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Dr S J Coker
Department of Pharmacology
University of Liverpool
P.O. Box 147
LIVERPOOL L69 3BX

1P THE PHARMACOLOGICAL CHARACTERISATION OF A STABLY TRANSFECTED CELL LINE EXPRESSING HUMAN NMDAR1a/NMDAR2A RECEPTORS, USING RADIOLIGAND BINDING TECHNIQUES

S. Grimwood, C.L. Barton, P.H. Hutson, J. Myers, B. Le Bourdèlles and P.J. Whiting. Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR.

Co-expression of the NMDAR1 (NR1) and NR2 subunits in *Xenopus* oocytes results in a receptor-channel complex with electrophysiological and pharmacological properties characteristic of the NMDA receptor (Monyer *et al.*, 1992). We have developed a stable cell line expressing human NR1a/NR2A receptors (BA7). NR1a and NR2A human cDNAs were stably co-transfected into L(tk)$^-$ cells under the control of a dexamethasone inducible promoter. Induced cells were grown in the presence of 0.5 mM ketamine for 3 days prior to harvesting and freezing. On the day of the experiment cell membranes were prepared and assays for the glutamate, glycine and ion-channel binding sites of the NMDA receptor complex performed as described by Grimwood *et al.* (in press).

Table 1. Saturation analyses for radioligand binding to NR1a/NR2A cell membranes.

	K_d (nM)	B_{max} (pmol/mg)
[^{3}H]L-689,560	3.70 (3.04, 4.50)	4.83 ± 0.96
[^{3}H]Dizocilpine*	7.05 (5.89, 8.43)	2.52 ± 0.36
[^{3}H]CGP 39653	3.28 (2.84, 3.80)	0.69 ± 0.15

K_d and B_{max} values are the geometric mean (-s.e.mean, +s.e.mean) and mean ± s.e.mean, respectively, for ≥ 6 experiments. *[^{3}H]Dizocilpine binding was not saturable (up to 100 nM) and data shown is for the high-affinity component.

Saturation experiments revealed high affinity binding for [^{3}H] L-689,560 (glycine site), [^{3}H] dizocilpine (ion-channel) and [^{3}H]CGP 39653 (glutamate site; Table 1). The ratio of B_{max} values obtained for these radioligands, respectively, was 7 : 3.6 : 1 for NR1a/NR2A receptors (Table 1), compared to a ratio of 1.7 : 1.4 : 1 for rat brain (cortex/hippocampus) membranes. Affinity values for glycine site agonists inhibiting [^{3}H]L-689,560 binding were >10-fold lower (eg glycine: K_i = 3.63 (2.29, 5.75) μM) and antagonist values similar (eg 7-chlorokynurenic acid: K_i = 0.618 (0.478, 0.798) μM), to those obtained using rat brain membranes, and very similar to values previously obtained with transiently transfected homomeric NR1a receptors (Grimwood *et al.*, in press). Glutamate site agonists were of similar affinity (eg NMDA: K_i = 5.67 (3.59, 8.97) μM) and antagonists approx. 3-fold more potent (eg R-CPP: K_i = 0.020 (0.015, 0.026) μM), at inhibiting [^{3}H]CGP 39653 binding to NR1a/NR2A cell membranes compared to rat brain membranes.

These results show that stably transfected NR1a/NR2A heteromeric receptors possess glutamate, glycine and ion-channel binding sites. Homomeric NR1a receptors also possess a high affinity binding site for [^{3}H]L-689,560 (but not for [^{3}H]CGP 39653 or [^{3}H]dizocilpine; Grimwood *et al.*, in press). The high ratio of [^{3}H]L-689,560 binding sites in the NR1a/NR2A cell line indicates that homomeric NR1a receptors may also be present and may obscure radioligand binding data obtained with glycine site ligands.

Grimwood, S., Le Bourdellès, B. & Whiting, P.J. (in press) *J. Neurochem.*
Monyer, H., Sprengel, R., Schoepfer, R. *et al.* (1992) *Science* 256, pp 1217-1221.

2P PHARMACOLOGICAL PROFILE OF RECOMBINANT HUMAN NR1a+NR2A AND NATIVE RAT CEREBELLAR GRANULE CELL NMDA RECEPTORS

P. Danks, B. Le Bourdelles, J. Myers, P. Whiting, JA Kemp & T Priestley. Merck Sharp & Dohme Research laboratories, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR.

The NMDA receptor is a multimeric assembly of subunits comprising of a common NR1 together with one or more distinct NR2 subunits. The precise subunit composition of natively expressed NMDA receptors is currently unknown, but those expressed by rat cultured cerebellar granule cells may have a predominantly NR1+NR2A composition (see Priestley and Kemp, 1994). The NMDA receptor is also unique in that activation requires the binding of two co-agonists, glutamate and glycine, therefore, we have compared glutamate and glycine site pharmacology of native NMDA receptors on rat cultured cerebellar granule cells with that of recombinant human NMDA receptors.

All experiments were performed on whole-cell, voltage-clamped (holding potential = -60mV) cells. Primary cultures of rat granule cells were prepared as described previously (Priestley & Kemp, 1994) and used for electrophysiological experiments between 8 and 21 days *in vitro*. A stable cell line (clone BA7) expressing recombinant human NR1a+NR2A heteromeric receptors was generated by co-transfecting L(tk)- cells with NR1a and NR2A subunit cDNAs which were engineered into a eukaryotic expression vector under the control of a dexamethasone inducible promotor. Experiments were performed 1-3 days after induction of receptor expression. The compositions of extracellular and pipette salt solutions were as previously described (Priestley & Kemp, 1994).

Concentration-response curves to glutamate, NMDA, and quinolinic acid revealed small, but statistically significant, higher affinities at recombinant NR1a+NR2A receptors compared to granule cell receptors (Table 1). The low affinity glutamate agonist, cis-2,3-piperidine dicarboxylic acid (cis-2,3-PDA) behaved as a partial agonist at the recombinant receptor (~ 3% efficacy of glutamate) but as an antagonist at the granule cell receptor. The competitive glutamate site antagonists, CGS19755, D-AP5, R-CPP and D-AP7 all failed to discriminate between recombinant and granule cell receptors. Glycine had a similar affinity at both granule cell and recombinant NR1a+NR2A receptors as had several glycine site antagonists: 7-Chlorokynurenic acid, L-689,560 and L-701,324. Interestingly, however, the glycine site partial agonists, L-687,414 and ACBC (aminocyclo-butanecarboxylic acid), showed a significantly lower affinity (Table 1) and higher efficacy at the recombinant receptor.

Table 1. Dissociation constants (K_d, μM) for NMDA receptor ligands

Ligand	Granule cells	L(tk)- cells	P (<)*
Glutamate	1.2 (1.1, 1.4)	0.8 (0.7, 0.8)	0.005
NMDA	21 (17, 25)	11 (8, 14)	0.05
Quinolinate	690 (590, 810)	400 (300, 500)	0.05
ACBC	34 (28, 43)	100 (81, 123)	0.005
L-687,414	1.7 (1.4, 2.2)	6.9 (6.0, 7.9)	0.0005

Data are geometric means (-sem, +sem), * = *t* test, 1 tailed

These experiments suggest that certain properties of the natively expressed rat granule cell NMDA receptor are shared with those of the recombinant human NR1a/NR2A receptor. However, there are also subtle differences, revealed particularly by partial agonists, which suggest the possibility of species differences and/or additional complexity associated with the native receptor.

Priestley, T & Kemp, JA (1994). *Mol. Pharmacol.* **44**, 1252-1257

3P CHARACTERISATION OF THE POLYAMINE MODULATORY SITE ON RECOMBINANT NR1/NR2A RECEPTORS

P.L. Chazot, M. Cik, S. Coleman & F.A. Stephenson (Introduced by M. A. Simmonds)
School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

N-Methyl-D-aspartate (NMDA) receptors are fast-acting glutamate-gated cation channels. Several cDNAs encoding NMDA receptor subunits (NR1, NR2A-NR2D) have been identified (Nakanishi, 1992). Combinations of these are thought to coassemble, probably as pentamers to form functional receptors with different pharmacological properties. As an approach to determining which subunits coexist in the brain, we have expressed transiently in human embryonic kidney (HEK) 293 cells, the major NR1 splice variant, NR1a, together with NR2A and NR2C in double and triple combinations. The cloned receptors were characterised with respect to their pharmacological, biochemical and single channel properties and compared to those found in situ in the brain (Chazot et al., 1994; Cik et al., 1993; Williams, 1994). We have previously reported a pharmacological profile by competition studies using [^{3}H]MK801 radioligand binding assays for a series of six disparate compounds active at NMDA receptors. Correlation coefficients of r = 0.98 and r = 0.992 were found between NR1/NR2A and native mouse forebrain and NR1/NR2A/NR2C mouse cerebellar NMDA receptors respectively. The latter was substantiated by immunoprecipitation studies from the triply transfected cells providing evidence that at least in this brain region, three subunit types coassemble (Chazot et al., 1994). The coexistence of three subunits has also been inferred in the forebrain by (Sheng et al., 1994). Electrophysiological studies by Williams (1994) have suggested that the polyamine class of compounds may be a useful tool to exploit in the dissection of NMDA receptor subunit complements. Therefore we have extended the characterisation of NR1/NR2A to include the effects of spermidine and ifenprodil on [^{3}H]MK801 binding activity. Spermidine showed no significant stimulation but inhibition of binding with IC_{50} = 2.8 ± 0.1 mM (n=3). Ifenprodil inhibited binding with IC_{50} = 151 ± 12μM (n=3). In native NMDA receptors of forebrain assayed under identical conditions, spermidine enhances binding (EC_{50} = 9 ± 3 μM, n=3) but inhibits binding to a low affinity site, IC_{50} = 1.8 ± 0.2 mM, n=3). Ifenprodil inhibits binding to native receptors with a biphasic displacement curve with IC_{50}(high) = 0.87 ± 0.6 μM (n=2) and IC_{50}(low) = 159 ± 17 μM (n=2). These results show that with respect to the polyamine site, the NR1/NR2A cloned receptors are not representative of the total forebrain receptor population. This is in contrast to the pharmacological profile of compounds active at the NMDA receptor channel domain. It is in agreement with the functional studies of Williams (1994) where it was shown that the high affinity polyamine modulatory site was endowed by the NR2B subunit.

Chazot PL, Coleman SK, Cik, M & Stephenson FA (1994) *J. Biol. Chem.* **39**, 24403-24409
Cik M, Chazot PL & Stephenson FA (1993) *Biochem. J.* **296**, 877-883.
Nakanishi, S (1992) *Science* **258**, 597-603.
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Stern P, Cik M, Colquhoun D & Stephenson FA (1994) *J. Physiol.* **476.3**, 391-397.
Williams K (1993) *Mol. Pharmacol.* **44**, 851-859.

4P ANTICONVULSANT ACTIVITY OF A NOVEL COMPETITIVE NMDA ANTAGONIST, THE S-ENANTIOMER SDZ EAB 515

A.G. Chapman, M.H. Millan, C. Kingsley, & B.S. Meldrum, Department of Neurology, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF, UK

A stereoisomer pair of competitive NMDA antagonists has recently been synthesized, where the S-enantiomer (L-form), SDZ EAB 515 (L-SDZ), has much higher affinity for the NMDA-receptor (Müller et al., 1992), than the R-enantiomer, SDZ 214 514 (D-SDZ). This contrasts with previously known competitive NMDA antagonists, including CPPene, where the affinity for the NMDA receptor and the anticonvulsant activity reside with the D-forms.
We have previously shown that D-CPPene is 10 fold more potent than L-CPPene as an anticonvulsant in DBA/2 mice, but that L-CPPene is 10 fold more potent than the D-form in inhibiting *in vivo* KCl-evoked hippocampal glutamate (GLU) release (Millan et al., 1994).
The aim of the present study is to determine if the novel NMDA antagonist pair exhibit stereo-specific anticonvulsant activity, and which, if any, of the enantiomers are capable of inhibiting evoked *in vivo* GLU release.
Anticonvulsant efficacy was assessed in DBA/2 mice following intraperitoneal administration (-1 h) of L-SDZ and D-SDZ. Both compounds provided dose-dependent protection against sound-induced clonic seizures, with the L-form being 20 fold more potent than the D-form, in agreement with their relative affinities for the NMDA receptor. The ED_{50} values were 13.9 (10.7-17.9) μmol/kg for L-SDZ, and 275 (200-378) μmol/kg for D-SDZ.
Microdialysis was performed in freely moving Sprague-Dawley rats with probes in dorsal hippocampus, perfused with artificial CSF (2μl/min; 10 min samples). Release was evoked with a 10 min 100 mM KCl stimulus, with or without L- and D-SDZ (0.25-1 mM via probe).
L-SDZ had no effect on GLU release at doses tested, while D-SDZ caused a reduction ($p < 0.05$) in evoked GLU release: from a 344% increase (controls; n=6) to a 193% increase (+1 mM D-SDZ; n=6). Thus, L- and D-SDZ exhibit a reversal of the pattern previously observed with L- and D-CPPene: L-SDZ has anticonvulsant activity and D-SDZ inhibits *in vivo* hippocampal GLU release; the SDZ pair is 10-40 times less potent than the CPPene pair on both accounts.

Millan, M.H., Chapman, A.G., and Meldrum, B.S. (1994) *J.Neurochem.* **62**, 217-222
Müller, W., Lowe, D.A., Neijt, H. et al. (1992) *Helv.Chim.Acta* **72**, 855-864

5P GENERALISED SEIZURES INHIBIT THE RELEASE OF GABA BUT AUGMENT THE RELEASE OF GLUTAMATE FROM RAT HIPPOCAMPUS *IN VIVO*

H. L. Rowley, K. F. Martin[1] and C. A. Marsden. Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH and [1]Boots Pharmaceuticals Research Department, Nottingham, NG2 3AA.

Although it is believed that an impairment of the amino acid neurotransmitters γ-aminobutyric acid (GABA) and glutamate (GLU) is involved in the neurological disorder epilepsy, there are few studies which have attempted to verify this *in vivo*. In this study we have used microdialysis to determine the effects of maximal electroshock (MES), used as a model of generalised tonic-clonic seizures, on extracellular levels of GABA and GLU.

Male, Lister hooded rats (250-350g; Olac) were anaesthetised with equithesin (4mg/kg i.p.) and a concentric microdialysis probe (4mm membrane) stereotaxically implanted into the ventral hippocampus. Probes were continuously perfused with artificial cerebrospinal fluid (aCSF). Animals were returned to their home cages and allowed at least 20 hours to recover from surgery with food and water available *ad libitum*. During the experiment, probes were perfused at a flow rate of 2.0μl/min and 20 min samples collected. Three or four basal samples were collected at the start of the experiment. All animals receiving MES were briefly anaesthetised with halothane (1.5-4.0%) in O_2 prior to stimulation via ear-clip electrodes. Stimulus parameters were 200V for 2 seconds which induced tonic hind-limb extensions. The concentration of amino acids in the dialysate samples was determined by high-performance liquid chromatography with electrochemical detection (Rowley *et al.*,1994).

In control animals, which were briefly anaesthetised and ear-clipped, basal GABA (0.20 ± 0.01pmol/20μl; mean ± S.E.M.) and GLU (0.61 ± 0.01pmol/20μl; n=7) levels remained stable for the duration of the experiment (240 min). In animals which received a single MES, basal GABA levels were significantly ($p<0.001$; Student's t-test) reduced by 46 ± 5% (n=7) in the 20 min immediately after the MES. The levels remained depressed until 60 min after the MES before gradually returning to basal. GLU levels were significantly ($p<0.05$) decreased by 11 ± 2% (n=7) in the 20 min immediately after the seizures were induced. In the next 40 min GLU levels significantly ($p<0.001$) increased by 44 ± 3% before gradually returning to basal 100 min after the MES.

Removal of calcium from the aCSF resulted in a gradual reduction of basal GABA levels which reached a plateau after 100 min (0.12 ± 0.04pmol/20μl; n=6). There were no significant differences between controls and those given MES. GLU levels in control animals were reduced to a minimum value of 0.40 ± 0.01pmol/20μl (n=6) following 120 min perfusion with calcium-free aCSF. However, MES again significantly increased GLU levels compared to the control group but without the initial reduction. 40 min after the MES, levels were increased by 20 ± 7% ($p<0.01$); 60 min by 42 ± 8% ($p<0.001$); 80 min by 19 ± 5% ($p<0.01$) and 100 min by 11 ± 3% ($p<0.05$).

Perfusion with aCSF containing 1mM Ni^{2+} throughout the experiment (240 min) to block voltage-dependent calcium channels had no effect on basal GABA (0.20 ± 0.01pmol/20μl; n=6) or GLU (0.59 ± 0.02pmol/20μl; n=6) levels but prevented the MES-induced changes in both amino acids.

These data demonstrate that induction of a generalised tonic-clonic seizure results in specific changes in the extracellular levels of GABA and GLU. Removal of calcium from the perfusing fluid prevented these changes in GABA, which suggests that it is neuronally-released GABA which is affected by the MES. Although the MES-induced increase in GLU was not markedly attenuated by removal of calcium, perfusion with 1mM Ni^{2+} prevented the changes in both GABA and GLU suggesting that MES-induced increases in GLU are due to increased neuronal release. The interaction between GABA and GLU following a generalised seizure is under further investigation.

Helen Rowley is a SERC CASE student in conjunction with Boots Pharmaceuticals.

Rowley, H. L., Martin, K. F. and Marsden, C. A. (1994) *Br. J. Pharmacol.* 111, 203P

6P GLUCOSE DEPRIVATION POTENTIATES GLUTAMATERGIC RECEPTOR-MEDIATED ARACHIDONIC ACID RELEASE FROM CULTURED STRIATAL NEURONES

R.J. Williams, J. Glowinski & J. Prémont (introduced by E. Kelly), INSERM U-114, Collège de France, Paris 75231, France.

L-glutamate stimulates the liberation of arachidonic acid (AA) from mouse striatal neurones via the activation of NMDA receptors and by the joint stimulation of AMPA and metabotropic receptors (Dumuis et al., 1990). In this study we investigated whether glucose deprivation would modify glutamatergic receptor-mediated AA release from these cells.

Cultured mouse striatal neurones were prepared as previously described (El-Etr *et al.*, 1989). Neurones were labelled overnight with 1μCi/ml [^{3}H]-AA and release experiments performed essentially as described by Tencé *et al.* (1994).

Preincubating neurones without glucose for 30 min strongly potentiated the maximum response to NMDA (100μM), compared to that observed under control conditions in the presence of 5mM glucose (Release of AA as a % of Control basal taken as 100%; Control NMDA, 181 +/- 17%; -Glucose basal, 127 +/- 6%; -Glucose NMDA, 345 +/- 53%; mean +/- s.e.mean, n=12), without significantly altering the potency (EC_{50} for NMDA with glucose 21 +/- 5μM; without glucose 20.5 +/- 4μM; mean +/- s.e.mean, n=3). Enhanced NMDA responses were observed when the concentration of added glucose was less than 0.5mM, increased with the time (up to 30 min) of incubation in glucose free medium, and were reproduced in the presence of 5mM glucose with the co-addition of 2-deoxyglucose. The enhanced NMDA response appeared to result from a release of glutamate and a subsequent additional liberation of AA due to the activation of AMPA and metabotropic receptors. Indeed the increased NMDA response was completely reversed when extracellular glutamate was enzymatically removed. Furthermore, glucose deprivation also potentiated the combined AMPA-metabotropic receptor-mediated release of AA, even in the absence of glutamate.

Thus, it appears that glucose deprivation in mouse striatal neurones induces an NMDA receptor mediated release of glutamate, which enhances AA release by activating AMPA/metabotropic receptors, which are themselves potentiated.under these conditions..

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El-Etr, M., Cordier, J., Glowinski, J. *et al.* (1989) *J. Neurosci.* 9, 1473-1480.
Tencé, M., Cordier, J., Prémont, J. *et al.* (1994) *J. Pharmacol. Exp. Ther.* 269, 646-653.

7P CHANGES IN EXTRACELLULAR GLUTAMATE IN THE NUCLEUS ACCUMBENS DURING A CONDITIONED EMOTIONAL RESPONSE

N.Saulskaya & C.A. Marsden, Department of Physiology & Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

A conditioned emotional response (CER) is a form of association learning caused by giving a rat a conditioned stimulus paired with footshock. Using the contextual cue (environment) as the stimulus we have shown that the CER is associated with increased extracellular dopamine in the medial nucleus accumbens which has an NMDA receptor-dependent (initial) and an NMDA-dependent (delayed) phase (Saulskaya & Marsden, 1994). The present study investigates the effects of a CER, using a contextual cue, on extracellular levels of glutamate in the nucleus accumbens.

Lister hooded male rats were prepared for *in vivo* microdialysis of the medial nucleus accumbens 24 h before the start of the experiment. Glutamate was measured in the dialysate samples using HPLC with electrochemical detection following pre-column derivatisation with orthophthalaldehyde (Rowley *et al.*, 1994). For the experiment rats were either placed in a test chamber for 10 min and exposed to inescapable footshock (0.5 mA for 1 sec x 10, 1 min apart), paired with tone, or exposed to the chamber alone and then returned to their home cage. 220 min later, both groups of rats were returned to the test chamber for 10 min but given neither shock nor tone (CER). Microdialysis samples were collected and analysed every 5 min throughout the experiment until 20 min after CER, after which 20 min samples were collected.

Footshock paired with tone caused a significant decrease (max 77 ± 6%, n=6) in glutamate during the period of footshock followed by an increase (145 ± 9%) when the rats returned ot their home cage. In contrast, rats exposed to the test chamber for the first time, but not given either shock or tone, showed an increase in glutamate which returned to basal shortly after the return to the home cage. When the rats given footshock on the first exposure were returned to the test chamber there was no change in glutamate while they were in the chamber, but an immediate increase on their return to the home cage. Rats not given shock on their first exposure to the box did, however, show a small and short lasting increase in glutamate when they were placed back in the box (Fig 1).

The results show that both footshock and CER are associated with marked, but short lasting, increases in extracellular glutamate in the nucleus accumbens. These increases, however, occur after the aversive stimuli, ie when the rats are returned to their home cage, are probably associated with relief from, rather than response to, danger; a conclusion supported by the decrease in glutamate during footshock.

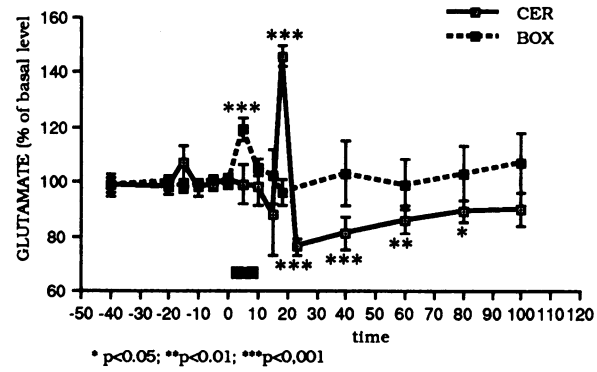


Fig 1: Changes in extracellular glutamate in the nucleus accumbens following CER (-□-) or second exposure to the test box alone (-■-) ■ = test period. **p<0.01, ***p<0.001 compared to pre-exposure value, Student's t-test n=6 for each group.

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Saulskaya, N. & Marsden, C.A. (1994). Br.J.Pharmacol., **112**, 297P.
Rowley, H., Martin, K. & Marsden, C.A. (1994). J.Neurosci. Methods (in press).

8P EFFECTS OF IONOTROPIC GLUTAMATE RECEPTOR AGONISTS ON THE ACTIVATION OF PHOSPHOINOSITIDE C BY METABOTROPIC GLUTAMATE RECEPTORS IN RAT CEREBELLAR GRANULE CELLS

D.W. Gray, R.A.J. Challiss and S.R. Nahorski, Dept. of Cell Physiology & Pharmacology, University of Leicester, Leicester.

Glutamate is the major excitatory neurotransmitter within the mammalian brain and has been implicated in a number of important neuronal processes e.g. memory, learning and excitotoxicity. The complexity of these glutamate responses does not appear to lie in the activation of a single receptor subtype, but rather are the result of integration of the responses of both ionotropic (iGluR) and metabotropic (mGluR) glutamate receptor activation. In the present study the effects of iGluR agonists on the mGluR activation of phosphoinositidase C (PIC) in rat cerebellar granule cells was studied.

Primary cultures of rat cerebellar granule cells were prepared as described previously (Whitham *et al.*, 1991) in 24 well multidishes and used between 4 and 6 days in culture. The cellular inositol pool was labelled to equilibrium with *myo*-[^{3}H]inositol for 48h. For total inositol phosphate ($InsP_X$) assay, incubations were carried out in the presence of LiCl (10mM). Where the individual inositol (poly)phosphates were investigated by HPLC (Batty *et al.*, 1989) LiCl was omitted.

The selective mGluR agonist, 1-aminocyclopentane-1S,3R-dicarboxylic acid (300μM; ACPD) and the mixed mGluR/α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) agonist, quisqualate (Quis; 100μM) elicited increases in $InsP_X$ (basal 3330 ± 150; ACPD 4962 ± 299; Quis 8525 ± 437 dpm/well at the 20min time-point; n=4 experiments performed in triplicate). Neither addition of AMPA (100μM) simultaneously with ACPD, nor the AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100μM) with Quis significantly altered the respective accumulations in $InsP_X$. N-methyl-D-aspartate (NMDA) at concentrations up to 100μM did not significantly stimulate $InsP_X$ accumulation. However, simultaneous addition of NMDA (100μM) and Quis (100μM) potentiated the accumulation of $InsP_X$ elicited by Quis (100μM) (Quis 8525 ± 437; Quis + NMDA 18515 ± 622 dpm/well). HPLC separation of inositol (poly)phosphates demonstrated the following isomeric changes at the 60sec time-point (in dpm).

	Cont	Quis	Quis + NMDA
$Ins(1,4,5)P_3$	601 ± 57	1029 ± 63	1989 ± 181
$Ins(1,4)P_2$	800 ± 118	2126 ± 191	4128 ± 228
Ins(4)P	491 ± 14	4564 ± 420	11293 ± 359
$InsP_4$	139 ± 30	285 ± 100	646 ± 63
$Ins(1,3,4)P_3$	70 ± 32	79 ± 10	931 ± 27
$Ins(3,4)P_2$	52 ± 22	87 ± 17	271 ± 22

These results indicate that, under the culture conditions used, rat cerebellar granule cells express mGluRs (linked to activation of PIC) and NMDA receptors. However, AMPA receptor agonists did not alter the PIC stimulation elicited by mGluR activation, possibly indicating that AMPA receptors, if present, rapidly desensitise. Thus Quis is a functionally selective mGluR agonist in this preparation.

Activation of NMDA receptors affects the phosphoinositide signal evoked by mGluR activation both by significantly potentiating the production of $Ins(1,4,5)P_3$, indicating a Ca^{2+}-modulation of PIC, and diverting the metabolism of $Ins(1,4,5)P_3$ through the 3-kinase route towards the production of the postulated second messenger, $Ins(1,3,4,5)P_4$. Such alterations in the level of PIC activity and phosphoinositide profile may significantly affect the level and temporal characteristics of Ca^{2+} release from intracellular stores.

Batty I.H. *et al.*, (1989) *Biochem. J.*, **258**, 23-32
Whitham, E.M *et al.*, (1991) *Eur. J. Pharmacol.*, **206**, 181-189

9P ANTAGONISM OF L-AP4- AND (1S,3S)-ACPD-INDUCED DEPRESSION OF DORSAL ROOT-EVOKED MONOSYNAPTIC EXCITATION OF NEONATAL MOTONEURONES BY THE NOVEL ANTAGONISTS MSPG AND MPPG

N.K. Thomas, D.E. Jane, K. Pittaway*, D.C. Sunter* and J. C. Watkins, Department of Pharmacology, University of Bristol, Bristol, BS8 1TD and *Tocris Cookson Ltd., Churchill Building, Langford House, Langford, Bristol, BS18 7DY.

L-2-amino-4-phosphonobutyrate (L-AP4) and (1S,3S)-1-aminocyclopentane-1,3-dicarboxylate (ACPD) are agonists at different presynaptic metabotropic glutamate receptors (mGluRs) on primary afferent terminals in neonatal rat spinal cord (Jane *et al.*, 1994). Their actions result in the depression of the dorsal root-evoked monosynaptic excitation of neonatal rat motoneurones. Both the L-AP4 and (1S,3S)-ACPD sensitive mGluR presynaptic sites can be effectively antagonised by (+)-α-methyl-4-carboxyphenylglycine (MCPG), with apparent K_D values of 227μM and 479μM respectively (Kemp *et al.*, 1994). We report here the actions of two novel antagonists synthesized in our laboratories, (RS)-α-methyl-4-phosphonophenylglycine (MPPG), and (RS)-α-methyl-4-sulphonophenylglycine (MSPG), on the agonist induced depressions of the monosynaptic response of neonatal motoneurones.

All experiments were performed on isolated hemisected spinal cords of 1-5 day old rats (Evans *et al.*, 1982). Recordings were made from the ventral root of motoneurones evoked by stimulation of the corresponding dorsal root. In order to isolate the non-NMDA receptor mediated components of the response, dorsal root-evoked ventral root potentials (DR-VRPs) were recorded from ventral roots in the presence of 2mM Mg^{2+} and 50μM D-2-amino-5-phosphonopentanoate (D-AP5).

Presynaptic depressions of DR-VRPs induced by L-AP4 were competitively antagonised by MPPG (50, 100 and 200μM) with a K_D of 9.2 ± 0.3μM (n = 10). The antagonism by MPPG at this L-AP4-sensitive mGluR was more than 10 times more potent than at the (1S,3S)-ACPD-sensitive mGluR with an apparent K_D of 113 ± 13μM (n = 3) (MPPG at 200μM). MSPG (200μM) was shown to be less potent and less selective than MPPG at both the L-AP4 and the (1S,3S)-ACPD receptors, with apparent K_D values of 157 ± 19μM (n = 3) and 221 ± 28μM (n = 3), respectively.

In conclusion, for both L-AP4 and (1S,3S)-ACPD sensitive presynaptic receptors in the neonatal rat spinal cord, the order of antagonist potency is MPPG > MSPG > MCPG. A phosphonic acid substituent in the 4-position of the phenyl ring thus not only enhances potency, but also selectivity for the L-AP4 sensitive receptor. MPPG is more than twice as potent as a previously reported selective L-AP4 receptor antagonist (S)-α-methyl-L-AP4 (MAP4) (Jane *et al.*, 1994), with a further increase in potency likely when the active stereoisomer of MPPG has been isolated.

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10P IDENTIFICATION OF THE STEREOISOMERS OF 3-(4-CHLOROPHENYL)-GLUTAMATE THAT POTENTIATE L-HOMOCYSTEIC ACID-EVOKED DEPOLARISATIONS IN NEONATAL RAT MOTONEURONES

G.A. Thompson, D.J. Chalmers, D.E. Jane, D.C. Sunter, P.M. Udvarhelyi, I.C. Kilpatrick & J.C. Watkins. Department of Pharmacology, University of Bristol, BS8 1TD.

The racemate of 3-(4-chlorophenyl)-glutamate ((±)-chlorpheg), has been shown to selectively potentiate responses to the putative excitatory transmitter, L-homocysteic acid (L-HCA; Davies *et al.*, 1985; Ito *et al.*, 1991) by a mechanism which may involve inhibition of L-HCA uptake. Recently in our laboratory, the four diastereomers of (±)-chlorpheg have been stereoselectively synthesised and each stereoisomer examined for its ability to influence excitatory responses to L-HCA.

Ventral roots of hemisected spinal cords from neonatal rats (1-5 days) were used to record amino acid-induced motoneurone depolarisations in a medium containing tetrodotoxin (10^{-7} M). Responses (0.25-0.75 mV) to L-HCA (10μM) or L-glutamate (50μM) were recorded in control conditions and in the presence of each chlorpheg isomer (100μM, Figure 1). Both the (2*S*,3*R*)- and (2*S*,3*S*)- isomers significantly potentiated L-HCA-evoked responses (% control ± s.e.mean: 114.5 ± 2.4, *n*=11 and 130.4 ± 3.6, *n*=20, respectively) whilst the (2*R*,3*S*)- isomer significantly reduced L-HCA response amplitude (94.2 ± 1.4, *n*=9) and the (2*R*,3*R*)- isomer was inactive. Responses to L-glutamate were unaffected by any isomer. The inhibition by (2*R*,3*S*)-chlorpheg was probably due to *N*-methyl-D-aspartate (NMDA) receptor antagonist properties since a sustained depolarisation induced by the continuous application of 5μM NMDA could be reversed by 100μM (2*R*,3*S*)-chlorpheg. Preliminary data indicate that this isomer is some 300 times weaker as an NMDA antagonist than D-2-amino-5-phosphonopentanoate. A complete investigation of the antagonism exhibited by (2*R*,3*S*)-chlorpheg was not possible due to its lack of availability. The other isomers (100μM each) did not produce measurable antagonism against depolarisations evoked either by NMDA (5μM) or (*S*)-2-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; 0.4μM).

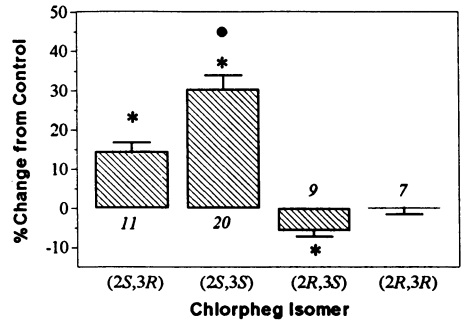


Figure 1 Effects of chlorpheg isomers (100μM each) upon 10μM L-HCA-induced depolarisations. Bars represent mean ± s.e.mean of the number of indicated tests. The (2*S*,3*R*)- and (2*S*,3*S*)- isomers caused potentiations that were significantly different from each other (•, p<0.005, Mann-Whitney U-Test). Only the (2*R*,3*R*)- isomer produced a non-significant change from control (Wilcoxon signed ranked test; *, p<0.01 for the other isomers).

In conclusion, the ability of (±)-chlorpheg to potentiate L-HCA- but not L-glutamate-evoked responses resides mainly with the (2*S*,3*S*)- and to a lesser extent, the (2*S*,3*R*)- isomer. In view of the weak NMDA antagonist properties of the (2*R*,3*S*)- isomer, future studies using the chlorpheg racemate may be compromised by contamination with this isomer.

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11P PHARMACOLOGY AND REGIONAL DISTRIBUTION OF (S)-[3H]5-FLUOROWILLARDIINE BINDING IN RAT BRAIN, A NEW RADIOLIGAND FOR THE STUDY OF AMPA RECEPTORS

L.M. Hawkins[1], P.J. Roberts[1], K.M. Beaver[1,2], D.E. Jane[1], P.M. Taylor[1] and D.C. Sunter[1,2] [1]Department of Pharmacology, University of Bristol, Bristol, BS8 1TD and [2]Tocris Cookson Ltd., Churchill Building, Langford House, Langford, Bristol, BS18 7DY.

The amino acid willardiine, 1-(2-amino-2-carboxyethyl)pyrimidine-2,4-dione, occurs naturally in the seeds of the species of *Acacia*. Evans *et al.*, (1980) were the first to describe that (±)willardiine was a non-NMDA receptor agonist in the neonatal rat spinal cord. Subsequently a series of structural analogues based on willardiine have been synthesised and their pharmacology has been well described (Patneau *et al.*, 1992 and Wong *et al.*, 1994). The most potent of the willardiine analogues identified to date is (S)-5-fluorowillardiine. This study describes the characterisation of the radiolabelled form of this compound, (S)-[^{3}H]5-fluorowillardiine.

Radioligand binding studies were performed on rat whole brain (minus cerebellum) synaptic membranes under non-chaotropic conditions, as described by Hawkins *et al.*, (1994). For the autoradiographic studies both transverse and saggital sections (10μm) were cut throughout male Wistar rat brains and thaw-mounted onto gelatin-coated slides. All sections were preincubated for 30 min at 4°C in 50mM Tris/HCl 100mM KCl (pH 7.4 at 4°C). This was followed by a 40 min incubation at 4°C in the Tris/KCl buffer with various competitors in the presence of 10nM (S)-[^{3}H]5-fluorowillardiine. Non-specific binding was defined in the presence of 1mM L-glutamate.

Saturation binding data for (S)-[^{3}H]5-fluorowillardiine, to rat brain synaptic membranes, can be best resolved by a two-site model into a high affinity (K_D 22.56 ± 4.33nM, B_{max} 1.45 ± 0.40 pmol/mg) and low affinity (K_D 1.59 ± 0.29μM, B_{max} 10.86 ± 2.52 pmol/mg) binding site. At 10nM specific (S)-[^{3}H]5-fluorowillardiine binding represents greater than 70% of the total binding. The excitatory amino acid receptor agonists (S)-AMPA, L-glutamate and kainate, with IC_{50} values of 0.63, 3.15 and 78.40μM respectively, displace (S)-[^{3}H]5-fluorowillardiine binding, whilst NMDA and (1S,3R)- ACPD were inactive.

Using quantitative autoradiography the regional distribution of the (S)-[^{3}H]5-fluorowillardiine binding sites were assessed. The highest levels of binding were in the hippocampus (CA1) and the dentate gyrus. Layers I-VI of the somatosensory cortex, cingulate cortex, striatum, hippocampus (CA2 and CA3), olfactory bulb and the molecular layer of the cerebellum displayed lower levels of binding. (S)-[^{3}H]5-fluorowillardiine binding in the cortex, striatum, dentate gyrus and hippocampus (CA1 and CA3) was completely displaced by excitatory amino acid receptor agonists with the following rank order of potency CNQX > (S)-AMPA > kainate.

The parallels in the pharmacology and regional distribution of (S)-[^{3}H]5-fluorowillardiine binding compared to that of (R,S)-[^{3}H]AMPA, the radioligand commonly used to identify the AMPA receptor, suggests (S)-[^{3}H]5-fluorowillardiine recognises the AMPA receptor. The advantages of high percentage specific binding and recognition of the two affinity binding sites, in the absence of KSCN, suggests that (S)-[^{3}H]5-fluorowillardine could be a better radioligand than (R,S)-[^{3}H]AMPA for the study of the AMPA receptor.

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12P PENTYLENETETRAZOLE KINDLING IN THE RAT IMPAIRS LONG-TERM HABITUATION OF EXPLORATORY RESPONSES

Sandra E. File & A. Becker Psychopharmacology Research Unit, UMDS Div. Pharmacology, Guy's Hospital, London SE1 9RT

Both electrial and chemical kindling have been reported to result in deficits in learning and memory (Lopes da Silva *et al.*, 1986; Voigt & Morgenstern, 1990; Becker *et al.*, 1992). So far, only negatively reinforced tasks have been studied following kindling with pentylenetetrazole (PTZ) (Voigt & Morgenstern, 1990; Becker *et al.*, 1992). The purpose of the present experiment was to determine whether deficits would be found in other tasks. The tasks selected were long-term habituation of exploratory head-dipping in the holeboard following 3 daily 10 min trials (with no objects) and response to stimulus change (introduction of 2 objects on day 4). Male Wistar rats received 13 injections of PTZ (30 mg/kg, 3 times weekly), at which point the mean (±sem) seizure stage was 4.0±0.15 on a modified RACINE scale (Becker *et al.*, 1992). The control group received vehicle injections. Behavioural testing (0800-1300h) commenced 96h after the last injection. The groups did not differ in their initial level of exploration on day 1, but habituation (shown by decreased head-dipping over days 1-3) was significantly impaired in the kindled rats (kindling x day interaction $F(2,48) = 7.6$, $p<0.001$). There was no impairment in response to stimulus change on day 4, both groups showing a significant increase in head dipping ($F(1,24) = 42.1$, $p<0.00001$). This pattern of results suggests a deficit in response inhibition.

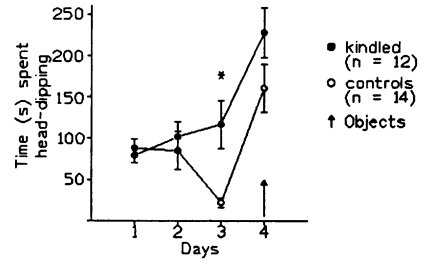


Figure 1 Mean (±sem) time (s) spent head-dipping in a holeboard

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13P EFFECT OF CALCIUM CHANNEL BLOCKERS ON EPILEPTIFORM ACTIVITY ON SLICES OF RAT NEOCORTEX

Julia Smith & David Lodge, Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey GU20 6PH.

Reduced extracelullar magnesium in the artificial cerebrospinal fluid (aCSF) superfusing slices of rat neocortex leads to bursts of epileptiform activity. Inhibition of this activity by competitive and non-competitive antagonists of N-methyl-D-aspartate (NMDA) correlates well with their potency as NMDA antagonists (Aram et al., 1989) and suggests that synaptically released glutamate plays an important role in the generation of such activity. Agents known to block various classes of voltage-dependent calcium channels (VDCCs), thought to be involved in neurotransmitter release, were therefore tested.

Wedge-shaped 450um slices of rat neocortex were placed in a two compartment bath so that a grease seal separated grey from white matter. The grey matter was superfused with aCSF with no added magnesium but containing 50uM picrotoxin. DC potentials were recorded between the two halves of the bath. About two hours later when epilpeptiform activity had stabilised, control values were obtained before superfusing the tissue for 40 min with the VDCC antagonists. Epileptiform activity was assessed as the frequency of bursts and the number of afterpotentials per burst (AP/B). At least 6 slices were used for each test and results expressed as % of control value (mean $\pm$ S.E.M.).

Neither the L channel antagonists, nifedipine 1uM and verapamil 1uM, nor the T channel antagonists, sFTX 1mM and amiloride 250uM, nor the N channel antagonist, ω-conotoxin GVIA 300nM, nor the P channel antagonist, ω-agatoxin IVA 50nM, had any significant effect on epileptiform burst frequency or duration. The Q channel blocker, ω-conotoxin MVIIC at 300nM, reduced AP/B by 40$\pm$7%. At 1uM both MVIIC and AgaIVA, which might be expected to block both Q and P type channels, reduced AP/B by about 70$\pm$9% and 73$\pm$14% and burst frequency by about 41$\pm$7% and 51$\pm$5% respectively.

Our results implicate a major contribution of the Q-type VDCCs in the release of glutamate that mediates epileptiform activity in rat neocortical slices.

We acknowledge Pfizer Inc for supplies of ω-agatoxin IVA.

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14P ENHANCEMENT OF SPINAL EXCITATORY POSTSYNAPTIC CURRENTS (EPSCs) BY α-METHYL-(2S,3S,4S)-α-CARBOXYCYCLOPROPYLGLYCINE (MCCG) *IN VITRO*

C.Q.Cao, D.E.Jane, H-W.Tse, P.M.Headley* & R.H.Evans, Departments of Pharmacology and *Physiology, School of Medical Sciences, University Walk, Bristol BS8 1TD.

1S3S-ACPD is a glutamate agonist selective for a subtype of metabotropic receptor which has a potent depressant action on spinal ventral root responses evoked by electrical stimulation of dorsal roots in vitro. MCCG has been shown to reverse this depressant action (Jane et al 1994). We have reported recently that, in addition to its synaptic depressant action, 1S3S-ACPD has an excitatory action (Cao et al 1994). In the present experiments we have examined interactions between MCCG and 1S3S-ACPD and between MCCG and L-α-amino-4-phosphonobutanoate (L-AP4). L-AP4 has a depressant action which is analogous to that of 1S3S-ACPD.

Whole cell excitatory postsynaptic currents (EPSCs) were recorded from lumbar motoneurones following electrical stimulation of dorsal roots in 5 to 8 day old rat spinal cord preparations maintained at room temperature (22 to 24 ^{o}C) and bathed in medium of the following composition (mM):- NaCl 118, KCl 3, $CaCl_2$ 1.5, $MgSO_4$ 0.75, $NaHCO_3$ 24, dextrose 12 gassed with 95%/5% O_2/CO_2. The patch pipettes (resistance 3 to 6 megohms) contained (mM):- Kgluconate 130, KCl 10, EGTA 11, HEPES 10, $CaCl_2$ 1, $MgCl_2$ 2, pH 7.4. Motoneurones were identified from their action currents evoked by electrical stimulation of ventral roots. EPSCs evoked from dorsal root stimulation had a mean peak amplitude (± s.e. mean) of 887 ± 77 pA (n= 8). Application of MCCG (200 μM) caused a mean increase of 14.2 ±6.3% in the amplitude of EPSCs (n= 6). In the presence of MCCG the mean EC_{50} (13.2 ± 12, n=3) for depression of EPSCs by 1S3S-ACPD was not significantly different to the value previously reported in the absence of MCCG (Cao et al, 1994). In two out of five cells 1S3S-ACPD failed to induce inward current in the presence of MCCG. The mean EC_{50} for the depressant effect of L-AP4 (4.8 ±0.3 μM, n=4) on EPSCs in the presence of MCCG (200 μM) was higher than but not significantly different from the mean value of 2.7 μM reported previously (Cao et al, 1994) in the absence of any antagonist.

Thus the present experiments show the antagonist potency of MCCG (200 μM) to be feeble. However, the enhancement of EPSCs by MCCG may reflect antagonism of endogenous L-glutamate at presynaptic glutamate receptors which may include those activated by L-AP4.

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15P MODULATION OF ACETYLCHOLINE RELEASE BY GLUTAMATE IN DIFFERENT BRAIN REGIONS

M.G. Giovannini, A. Mundula, and G. Pepeu. Department of Preclinical and Clinical Pharmacology, University of Florence, Viale Morgagni 65, 50134 Florence, Italy.

The glutamatergic regulation of the cortical and striatal cholinergic systems was studied by means of transversal microdialysis technique in freely moving adult Wistar rats (Wu et al., 1988). A microdialysis membrane was placed in the frontal cortex or dorsal striatum; 24 h after surgery it was perfused with Ringer solution containing 7 μM physostigmine sulphate at the flow rate of 2 μl/min. Samples were collected every 20 min. ACh levels in the dialysate were measured by an HPLC method with electrochemical detector. Drugs were administered either via the dialysis membrane or i.c.v. The mean ACh levels in the dialysates from the cortex and striata were 3.10 ± 0.18 (n=12) and 17.08± 1.30 (n=33) pmol/20 min, respectively, and in control experiments remained stable for 4 h and more. I.c.v. administration of the competitive NMDA antagonist CPP (5 nmol/5 μl saline) was followed by a long lasting 100 % increase in cortical ACh output (n=5). CPP (100 μM) perfused directly into the cortex through the dialysis membrane did not affect cortical ACh output. I.c.v injections of the non-NMDA receptor antagonist DNQX (0.5 and 20 nmol) had no effect on ACh release. These results indicate that CPP did not affect the cortical cholinergic nerve endings but neuronal bodies located in the forebrain cholinergic nuclei. Investigations are in progress in order to ascertain whether the increase in cortical ACh release results from an indirect effect mediated by GABAergic neurons activated by NMDA receptors, as shown in the septo-hippocampal pathway (Giovannini et al., 1994).
In the striatum CPP (25 and 100 μM) perfused through the dialysis membrane brought about a dose-dependent, reversible decrease in ACh output which was partly antagonised by 100 μM NMDA. This finding confirms that a direct glutamatergic input stimulates the striatal cholinergic neurons via NMDA receptors (Scatton & Lehman, 1982). Conversely, perfusion with the non-NMDA receptor antagonist NBQX (12.8 μM) resulted in a 55% increase in ACh (n=5). Perfusion with the non-NMDA receptor agonist AMPA (200 μM) was followed by a 50% decrease in ACh output and this effect was reversed by the $GABA_A$ receptor antagonist bicuculline (50 μM). These findings demonstrate that glutamate modulates the striatal cholinergic neurons directly through NMDA receptors and indirectly through non-NMDA receptors

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16P IS CLASS-III ANTIARRHYTHMIC ACTION OF TEDISAMIL DUE TO OPEN CHANNEL BLOCK OF I_{to} IN HUMAN VENTRICULAR MYOCYTES?

U. Ravens, H.M. Himmel, Qi Li, G.J. Amos, E. Wettwer & H. Posival[1], Institut für Pharmakologie, Universität Essen, D-45122 Essen, and [1]Herzzentrum Bad Oeynhausen, Germany.

The *in vivo* cardiac actions of tedisamil as shown in a variety of animals as well as in human subjects (Mitrovic *et al.*, 1992) include bradycardia, prolonged duration of both action potential and Q-T interval, and increased refractoriness. These effects are consistent with a class-III antiarrhythmic action. This action was suggested to be due to blockade of both transient outward K^+ current I_{to} in rat and delayed rectifier K^+ current I_K in guinea-pig ventricular myocytes (Dukes *et al.*, 1990). The effects of tedisamil in isolated myocytes of human cardiac origin, however, are completely unknown to date.

In human papillary muscle strips, action potential duration (35°C, 0.5 Hz, conventional microelectrodes) is prolonged by 3μM of tedisamil. Underlying membrane current changes were studied in voltage-clamped ventricular myocytes (stimulation rate 0.2 Hz) isolated from explanted failing hearts (Wettwer *et al.*, 1994). I_{to} was elicited by 300ms-clamp steps in the voltage range of -30 to +60 mV; Na^+ current was prepulse-inactivated (from holding potential -80 mV to -40 mV), Ca^{2+} current was blocked by Cd^{2+} (100μM). Current measurements were performed at room temperature.

I_{to} inactivated incompletely; at +60 mV, the ratio between peak- and late-current (300 ms) was 5.1±0.4 (n=28). Tedisamil (1-10 μM) concentration-dependently reduced peak-I_{to} (control: 9.98±2.56, tedisamil (10 μM): 6.71±1.51 pA/pF), but did not affect the current at the end of the 300ms clamp step (late-I_{to}: 2.42±0.48 and 2.50±0.69 pA/pF). The time constant τ of apparent inactivation of I_{to} at +60 mV decreased from 65.5±4.5 ms to 20.3±3.7 ms (tedisamil 10μM); this concentration-dependent effect was half-maximal at 3.3μM. Within the initial 60 ms of the clamp step, the fractional block of I_{to} increased monoexponentially from 0 at the beginning of the clamp step to a maximum of 61±5%; the respective time constant was 5.6± 0.9 ms. These findings are consistent with an open channel block by tedisamil, which is further supported by preliminary experiments demonstrating a use-dependent block of I_{to} by tedisamil (5μM).

In conclusion, I_{to} in human ventricular myocytes is a target for the class-III antiarrhythmic drug tedisamil. The block of I_{to} occurs in therapeutically relevant concentrations (approx. 4.4μM; Mitrovic *et al.*, 1992), and as its mechanism we suggest an open channel block.

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17P CONTROL OF RELAXATION IN CARDIAC MYOCYTES ISOLATED FROM THE VENTRICLES OF NORADRENALINE-TREATED GUINEA-PIGS AND PATIENTS WITH HEART FAILURE

K. Davia, D.W. Wynne & S.E Harding, Department of Cardiac Medicine, National Heart and Lung Institute, Dovehouse St., London SW3 6LY.

Contraction and relaxation are slowed in myocytes from failing human ventricle, and this appears to be related to depressed uptake of Ca^{2+} by the sarcoplasmic reticulum (SR). The low basal cyclic AMP levels present in failing human myocardium, (Danielsen *et al.*, 1989) which are a consequence of prolonged catecholamine exposure, may contribute to this. We have compared the contractile responses of cardiac myocytes isolated from failing human ventricle with those from the noradrenaline-treated (NA) guinea-pig. In this animal model β-adrenoceptor responses and basal cyclic AMP levels are depressed to a similar extent as those in human heart failure. Contraction amplitude (% change in cell length with each beat) was measured at 32°C, in Krebs-Henseleit buffer containing 4-6mM Ca^{2+} using a video/edge-detection system. SR function was assessed from the decline in contraction amplitude following a 3 min rest period, or by comparison of contraction before and after a 20 min exposure to 1μM of the SR Ca^{2+}-ATPase inhibitor thapsigargin.

Myocytes from NA guinea-pigs did not differ from sham-operated controls (SHAM) in contraction amplitude (7.1 ± 0.6%, NA v 7.8 ± 0.6%, SHAM), time-to-peak contraction (TTP) (0.22 ± 0.01s, NA v 0.24 ± 0.03s, SHAM) or time-to-50% relaxation (R50) (0.12 ± 0.01s, NA v 0.12 ± 0.01s, SHAM) (mean ± sem, n=9 animals SHAM, 16 NA). This contrasts with failing human heart where there was a prolongation of TTP (0.44 ± 0.02s, n=54 patients, failing v 0.31 ± 0.02s, n=16 subjects, non-failing, $P<0.001$) and R50 (0.26 ± 0.02s, failing v 0.19 ± 0.02s, non-failing, $P<0.02$) at frequencies where contraction amplitude was unchanged.

Myocytes from NA guinea-pigs were more sensitive to thapsigargin than those from controls. Exposure to 1μM thapsigargin lengthened R50 in cells from SHAM animals (0.10 ± 0.01s) but not those from NA (0.16 ± 0.03s, $P<0.05$ v untreated NA). However, myocytes from failing human hearts were less sensitive to thapsigargin than those from non-failing. Thapsigargin increased TTP by 42 ± 12% ($P<0.02$, n=8) in non-failing compared with 17 ± 8% (NS, n=9) in failing. R50 was lengthened by thapsigargin treatment in non-failing (0.20 ± 0.04s v 0.47 ± 0.07s, $P<0.01$) but not significantly increased in cells from failing hearts.

The first beat following a 3min rest was smaller than the steady state stimulation at 0.5Hz (guinea-pig) or 0.2Hz (human) when control myocytes were used. This post-rest decay was unchanged in myocytes from NA guinea-pigs, but considerably attenuated in cells from failing human heart. The first post-rest beat was 26.4 ± 7.2% of steady state in myocytes from 8 non-failing hearts compared with 113 ± 19% in cells from 19 failing ($P<0.001$). Thapsigargin abolished differences between the two, indicating that these were SR-dependent phenomena. We conclude that reduction of basal cyclic AMP levels by NA infusion in the guinea-pig does not mimic the SR-related changes seen in failing human heart.

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18P THE SEQUENTIAL GENERATION OF C5a AND IL-8 IN ISCHAEMIC/REPERFUSED RABBIT MYOCARDIUM

C.L. Ivey, P.D. Collins, P.J. Jose, T.J. Williams & F.M. Williams, Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London. SW3 6LY.

Acute myocardial infarction is associated with neutrophil accumulation in the infarcted region (Mallory *et al*, 1939); the mediators responsible are not clearly defined. We have detected C5a and interleukin-8 (IL-8) the sequential generation of C5a and IL-8 in ischaemic/reperfused rabbit myocardium, two potent neutrophil chemoattractants (Snyderman *et al*, 1970, Yoshimura *et al*, 1987), in reperfused rabbit myocardium.

New Zealand white rabbits were anaesthetised with sodium pentobarbitone (60mg/kg) and ventilated with room air. Arterial blood pressure and a lead I ECG were continuously monitored. The circumflex artery was occluded for 45 min followed by differing reperfusion times: 5min, 1.5, 3 and 4.5 h. Area at risk (AR) and normal zone (NZ) samples were subsequently subjected to homogenisation, centrifugation, dialysis and HPLC separation.

Elevated concentrations of C5a and IL-8 were detected by RIA in post-ischaemic AR, although the time course of generation differed. Data are presented in table 1 as mean ± s.e.mean *$p<0.05$, **$p<0.01$ (AR vs NZ). Neutrophil accumulation, measured using a myeloperoxidase (MPO) assay, also increased in the AR with reperfusion time: 1.5h, 1.12±0.5 U/g AR; 3h, 3.71±0.4 U/g AR; 4.5h, 4.23±1.6 U/g AR.

Four pooled AR samples (4.5h reperfusion) were further purified by cation-exchange and reversed-phase HPLC, resulting in a clear separation of the C5a (retention time 33.5-34 min) and IL-8 (retention time 42-42.5 min) immunoreactivity. The immunoreactive fractions were shown to have neutrophil-activating properties in a homotypic neutrophil aggregation assay, inducing 13.8±1.73 and 11.25±3.73 % maximal aggregation respectively (n=6). This study shows that C5a and IL-8 are produced in infarcted rabbit myocardium, but with differing time-courses; C5a appearing immediately and IL-8 after a delay.

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Table 1

Duration of reperfusion	Immunoreactive C5a (nmol/g tissue) NZ	AR	Immunoreactive IL-8 (nmol/g tissue) NZ	AR
5 min (n=5)	0.06±0.02	0.17±0.05*	0.01±0.02	0.01±0.02
1.5 h (n=5)	0.03±0.01	0.39±0.09**	0.01±0.01	0.03±0.01*
3 h (n=5)	0.05±0.02	0.62±0.15**	0.01±0.01	0.07±0.03**
4.5 h (n=6)	0.04±0.01	0.61±0.05**	0.02±0.01	0.23±0.05**

19P EFFECTS OF 1,3-DIPROPYL-8-SULFOPHENYLXANTHINE (DPSPX)-INDUCED HYPERTENSION ON CARDIAC FUNCTION

A. Rubino & G. Burnstock, Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT.

A role for purines in the development of essential hypertension has been suggested (Burnstock, 1993). In support of this hypothesis, long-term treatment with the adenosine antagonist 1,3-dipropyl-8-sulfophenylxanthine (DPSPX) induced systemic hypertension, associated with morphological changes in the arterial vascular wall (Matias *et al.*, 1991). However, no information is available on cardiovascular function during DPSPX-induced hypertension. This work aimed therefore to investigate what functional changes occur to cardiac sympathetic neurotransmission and the pharmacological regulation of cardiac contractility during DPSPX-induced hypertension.

Wistar rats (250–300 g) received constant i.p. infusion of DPSPX (30 μg/kg/h) or saline for 7 days. Systolic blood pressure (SBP) was measured daily with the tail-cuff technique. Sympathetic neurotransmission was evaluated in isolated atria driven at 4 Hz, by applying trains of field pulses (2–16). The pharmacology of α- and β-adrenoceptors was evaluated in strips of right ventricle driven at 2 Hz (8–10 V, 0.5 ms).

Following 7 days DPSPX treatment, SBP was 164.0±2.9 (n=5) mm Hg compared to 140.0±2.8 (P<0.05; n=7) in control rats. Electrical field stimulation of atrial tissue elicited contractile responses which were significantly reduced in DPSPX-treated preparations. Similarly, inotropic responses to increasing concentrations of noradrenaline were significantly reduced in DPSPX-treated atria, the maximal contractility being 391.7±50.7 (n=7) and 166.6±22.0 (P<0.01; n=5) mg in control and treated atria, respectively. Basal contractility of atrial preparations did not significantly differ between control and DPSPX-treated rats, being 213.0±28.5 (n=7) and 157.4±22.8 (n=5) mg, respectively. Isoprenaline (1nM–1μM) elicited inotropic responses in both control and treated ventricles and EC_{50} values did not significantly differ, being 21.4±6.4 (n=7) and 13.7±1.6 (n=5) nM in saline- and DPSPX-treated tissue, respectively. In the presence of propranolol 1 μM, phenylephrine (0.1–30 μM) increased cardiac contractility with EC_{50} values of 2.5±0.5 (n=4) and 3.1±0.5 (n=4) μM in control and DPSPX-treated ventricles, respectively. However, the positive inotropic effect of both isoprenaline and phenylephrine was less effective in hypertensive than in control rats. Maximal contractile responses to β-adrenoceptor stimulation were 716.0±111.4 (n=7) and 362.3±56.3 (P<0.01; n=5) mg in control and DPSPX-treated rats, respectively. Similarly, contractile responses to 30 μM phenylephrine were significantly less in DPSPX-treated than in control tissue. In both, atrial and ventricular preparations, contractile responses to increased concentrations of $CaCl_2$ did not differ between DPSPX- and saline-treated tissue.

In summary, this study demonstrates that DPSPX-induced hypertension affects cardiac sympathetic neurotransmission at postjunctional level causing reduction in responses to NA mediated by both α- and β- cardiac adrenoceptors. Since similar changes in the pharmacological regulation of cardiac performance have been reported in animal models of genetic hypertension (Head, 1989), this data further supports the hypothesis that purines play a role in essential hypertension.

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20P EFFECT OF PROTEIN KINASE C INHIBITION DURING ISCHAEMIC PRECONDITIONING ON THE SECOND WINDOW OF PROTECTION IN THE RABBIT

G. F. Baxter, F. M. Goma & D. M. Yellon, Hatter Institute for Cardiovascular Studies, Department of Academic & Clinical Cardiology, University College London Hospital & Medical School, London WC1E 6DB.

Ischaemic preconditioning (PC) of myocardium by brief periods of ischaemia is known to elicit a *biphasic* protection against infarction caused by a subsequent period of ischaemia (Baxter & Yellon, 1994). With regard to the late phase of protection, we have described enhanced resilience to infarction 24 h after PC in rabbit myocardium (Marber *et al.*, 1993). Although we have observed that this second window of protection may be triggered by adenosine receptor activation during PC (Baxter *et al.*, 1994), the intracellular signalling cascade is not known. In the present study we have examined the possibility that protein kinase C (PKC) activation may be a pivotal step in the development of the delayed protection by using the selective PKC inhibitor, chelerythrine, during PC.

Male New Zealand White rabbits (2.0-3.0 kg) were assigned to either PC or sham-operated groups. Each rabbit underwent a midline sternotomy, the pericardium was opened and a branch of the left coronary artery was identified. The branch was ensnared with a silk suture so that it could be reversibly occluded. The PC protocol consisted of four 5 min occlusions each separated by 10 min reperfusion. Five minutes before the PC or the sham period, animals received either chelerythrine chloride (chel; 5 mg/kg) or vehicle (water with 7% ethanol). Animals were allowed to recover for 24 h with antibiotic and analgesic cover. They were then re-anaesthetised and subjected to a 30 min coronary artery occlusion followed by 120 min reperfusion. The ischaemic risk zone (R) was determined *ex vivo* with fluorescent microspheres and the infarcted zone (I) was determined with triphenyltetrazolium staining. Computerised planimetry was used to determine the volumes of these zones and the ratio I/R was calculated.

Table 1. (Data are presented as mean ± s.e.m.)

	I (cm^3)	R (cm^3)	I/R (%)
Sham n = 6	0.48±0.11	0.92±0.15	48.6±5.4*
PC n = 6	0.25±0.05	0.80±0.13	31.3±4.1
Sham + chel n = 4	0.41±0.04	0.72±0.10	58.1±4.4*
PC + chel n = 6	0.48±0.11	0.79±0.11	55.4±8.4*

* P < 0.05 compared with PC (ANOVA)

Despite similar myocardial risk volumes in all groups, the reduction in I/R shows that there was enhanced resistance to infarction 24 h after PC. This delayed protection was completely abolished by chelerythrine treatment during the PC stimulus. We conclude that the myocardial adaptation underlying this delayed protection many hours after PC in the rabbit may involve the early activation of chelerythrine-sensitive PKC subtypes.

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21P ICI-118,551 IS AN INVERSE AGONIST IN ATRIA OF TRANSGENIC MICE WITH CARDIAC β_2-ADRENOCEPTOR OVEREXPRESSION

R.A. Bond and R.J. Lefkowitz [1], Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX 77204-5515, U.S.A. and [1]Howard Hughes Medical Institute and Department of Medicine, Duke University Medical College, Durham, NC 27710, U.S.A. (introduced by P. Leff).

An allosteric ternary complex model has been proposed to describe the ligand-receptor interaction at G protein coupled receptors (Samama et al., 1994). In this model, receptors exist in an inactive, R, and a constitutively active, R*. The model predicts that classical agonists promote the formation of R*, and that inverse agonists promote the formation of R. In this study we investigate the negative inotropic effect of ICI-118,551 (Samama et al., 1994) in the isolated left atria of transgenic mice with cardiac specific overexpression of the β_2-adrenoceptor.

Isolated left atria were suspended for isometric tension recordings in organ baths containing oxygenated Krebs-bicarbonate solution. Atria were paced at 3.5 Hz, with a 5ms pulse duration and voltage set at threshold plus 20%. Two lines of transgenic mice and age-matched controls were used. The TG4 line had ß-adrenoceptor densities 200 times above control levels and the TG33 line 90 times above control (Milano et al., 1994).

In the atria of TG4, but not control mice, ICI-118,551 ($1x10^{-9}$M to $3x10^{-6}$M) produced concentration-dependent inhibition of baseline left atrial tension. The maximal reduction in baseline was 76±6%. These responses were unaffected by reserpine treatment resulting in depletion of over 97% of cardiac catecholamine content. Alprenolol ($1x10^{-7}$M), produced a 15±6% (n=6) inhibition of baseline in TG4 atria, but produced a parallel, 33-fold rightward shift of the EC_{50} for ICI-118,551 (n=4). This shift is consistent with an interaction of the compounds at the β_2-adrenoceptor. Experiments with the ß-adrenoceptor alkylating agent, pindobind, revealed marked differences in the responses of inverse agonists compared to classical agonists. Pindobind ($1x\ 10^{-7}$M for 15 min followed by washout), produced a leftward shift of the CRC. The maximal response was reduced, but the EC_{20} was shifted to the left by 18-fold (n=4). Thus, ICI-118,551 produced greater responses for a given concentration after receptor alkylation. Similar results were observed comparing the ICI-118,551 CRC in TG33 versus TG4. In the line with lower ß-adrenoceptor densities (TG33), the CRC to ICI-118,551 was shifted to the left ($p<0.05$).

The data suggests that ICI-118,551 functions as an inverse agonist in the atria of transgenic mice with myocardial overexpression of β_2-adrenoceptors. The response of the inverse agonist is not only qualitatively opposite to that of classical agonists, but also, after receptor alkylation, the curves move in opposite directions.

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22P AN ATYPICAL β-ADRENOCEPTOR MEDIATES RELAXATION OF THE RAT ISOLATED MESENTERIC ARTERY

S. Sooch & I. Marshall, Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

Recent studies have suggested that not all β-adrenoceptor mediated responses can be classified as either β_1 or β_2, since an additional β-adrenoceptor subtype has been identified, referred to as atypical or β_3. In addition to adipocytes, atypical β-adrenoceptors have been identified in a number of gastrointestinal smooth muscle preparations, e.g., guinea pig ileum and rat distal colon (see Arch & Kaumann, 1993). The present study provides evidence for atypical β-adrenoceptors in the rat mesenteric artery.

Rat mesenteric arterial rings were suspended under 0.5g tension in Krebs solution at 37^0C and oxygenated with 95%O_2/5%CO_2 for isometric tension recording. Arteries were preconstricted with phenylephrine (10^{-6}M) and cumulative concentration-relaxation curves to agonists were constructed. Tissues were pretreated with propranolol, ICI 118551 or atenolol for 30 min before a second dose-response curve was obtained. Results are expressed as mean±s.e.mean.

Isoprenaline (10^{-9}M-10^{-4}M) produced a concentration-dependent relaxation of phenylephrine-induced tone in mesenteric arterial rings (EC_{50}:3.1±1.1x10^{-6}M; max. relax.= 98±1%). Propranolol produced dose-ratios of 7.2±1.1 (n=4), 5.8±2.0 (n=4) and 5.5±3.3 (n=4) at 10^{-7}M, 10^{-6}M and $3x10^{-6}$M, respectively with no depression of the maximum response. Isoprenaline-induced relaxations were not altered by the selective β_1-adrenoceptor antagonist, atenolol (10^{-7}M-$3x10^{-6}$M), but the selective β_2-adrenoceptor antagonist, ICI 118551, produced dose-ratios of 10.7±3.9 (n=4) and 6.5±1.7 (n=4) at 10^{-7}M and $3x10^{-6}$M, respectively. ZD 7114 (10^{-8}M-10^{-4}M), a β_3-adrenoceptor agonist (Holloway *et al.*, 1991; Growcott *et al.*, 1993) relaxed rat mesenteric arterial rings (EC_{50}:5.3±0.8x10^{-6}M; max. relax.=101±2%). ICI 215001 (10^{-8}M-10^{-4}M; Tesfamariam & Allen, 1994) and ZD 2079 (10^{-8}M-10^{-4}M; Grant et al., 1994) were less potent at relaxing rat mesenteric arterial rings (relaxations of 78±2% and 52±4% at 10^{-4}M, respectively). Propranolol (10^{-6}M) was without effect on the relaxant responses to ZD 7114, ICI 215001 and ZD 2079.

In conclusion, isoprenaline-induced relaxations were found to comprise a propranolol-sensitive component and a component resistant to blockade with increasing concentrations of this antagonist. The former component may be mediated by β_2-adrenoceptors because of the partial antagonism of isoprenaline relaxations by ICI 118551. Based on the relaxant responses to novel synthetic β_3-adrenoceptor agonists, i.e., ZD 7114, ICI 215001 and ZD 2079, all of which were resistant to blockade with propranolol, the additional receptor mediating vasorelaxation to isoprenaline appears likely to be an atypical β-adrenoceptor.

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23P ANTAGONISM OF α_1-ADRENOCEPTOR-MEDIATED CONTRACTIONS OF VASCULAR SMOOTH MUSCLE BY 5-METHYLURAPIDIL IN PACING-INDUCED HEART FAILURE

C. Forster, Department of Pharmacology, Medical Sciences Building, University of Toronto, Toronto M5S 1A8, Canada.

Alpha$_1$-adrenoceptor reactivity in the dorsal pedal artery (DPA) and saphenous vein (SV) is enhanced in dogs which had congestive heart failure (HF; end-stage HF occurring in 4 wk) induced by rapid ventricular pacing (250 bpm, Forster *et al.*, 1992). It was proposed that this was due to the activation of different α_1-adrenoceptor subtypes. In support of this, chloroethylclonidine (α_{1B}-adrenoceptor antagonist) was more effective against noradrenaline (NA) and phenylephrine (PE)-induced contractions of canine SV at end-stage HF compared to control (Forster & Albernaz, 1993). In this study, the effect of the α_{1A}-adrenoceptor antagonist, 5-methylurapidil (5MU), was examined in DPA and SV at control and end-stage HF.

Male mongrel dogs (N = 6 - 8, approx. 25kg) were used and sections of DPA and SV were dissected in the region of the hind paw at the time of pacemaker implantation (Forster *et al.*, 1989). One wk later, pacemakers were programmed to deliver 250 bpm and animals were paced to end-stage HF, at which time the contralateral vessels were removed. All blood vessels were treated in an identical manner and had their endothelium removed. Each vessel was cut into 5mm segments, mounted in organ baths and allowed to equilibrate for at least 1 hour. Propranolol and desipramine were present throughout. Concentration effect curves were constructed to either NA or PE in the absence and presence of increasing concentrations of 5MU (0.01, 0.1 and 1.0 μM). The results are shown below for IC_{50} [μM] of 5MU (geometric mean ± 95% confidence limits), ^ indicates $P<0.05$ vs control and no antag = no antagonism.

Table 1 IC_{50} values for 5MU

	Dorsal pedal artery		Saphenous vein	
	Control	HF	Control	HF
NA	0.9(0.7-1.1)	7.9(4.4-11.3)^	5.1(2.0-8.8)	22.0(9.9=56.6)^
PE	0.9(0.6-1.2)	no antag	7.3(1.0-11.2)	no antag

At control, 5MU inhibited contractions to NA and PE. Interestingly the degree of antagonism was greater in the artery compared to the vein. At end-stage HF, 5MU remained an effective antagonist against NA, albeit less potent than at control, but produced absolutely no antagonism (in the concentrations used) against PE.

These results indicate that under normal conditions i.e., in the absence of profound HF, contractions of the DPA and SV to NA and PE involve an α_{1A}-adrenoceptor component. However, at end-stage HF, this element is activated to a lesser degree by NA and is absent in the response produced by PE. Furthermore, the artery is much more sensitive to antagonism by 5MU indicating a greater α_{1A}-adrenoceptor constituent in response to α-adrenoceptor stimulation. Thus the enhanced vascular reactivity seen in HF may be due to activation of different adrenoceptor subtypes and could offer a new therapeutic approach to the management of HF.

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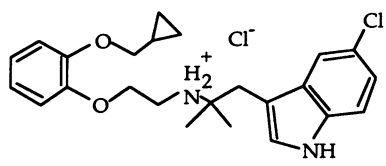
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24P DO α_{1A}(α_{1c})-ADRENOCEPTORS (AR) MEDIATE PROSTATIC SMOOTH MUSCLE CONTRACTION IN MAN? STUDIES WITH A NOVEL, SELECTIVE α_{1A}-AR ANTAGONIST, RS 17053

A.P.D.W. Ford, N. F. Arredondo, D.R. Blue, D.W. Bonhaus, M.S. Kava, T.J. Williams, R.L. Vimont, Q.-M. Zhu, J.R. Pfister & D.E. Clarke. Institute of Pharmacology, Syntex Discovery Research, Palo Alto, CA 94303, USA.

Current opinion evinces that α_{1A}-ARs (aka α_{1C}-ARs; Ford *et al.*, 1994), as distinct from α_{1B}- and α_{1D}-ARs, mediate sympathetically induced contractions of prostatic smooth muscle in man (Smith *et al.*, 1993; Forray *et al.*, 1994). Thus, a selective α_{1A}-AR antagonist may be of value in the therapy of urinary tract outlet dysfunction (e.g., benign prostatic hypertrophy). We report here the *in vitro* pharmacology of a novel, selective α_{1A}-AR antagonist, RS 17053 (Figure 1).

Figure 1. Structure of RS 17053



In radioligand binding studies, using [^{3}H]prazosin (0.1 nM) as ligand, RS 17053 displayed high affinity for α_{1A}-AR (rat submaxillary gland pK_i: 9.14±0.13; bovine clone α_{1C}-AR: 9.54±0.06) over the α_{1B}-AR (rat liver: 7.79±0.09; hamster clone: 7.84±0.06) and the α_{1D}-AR (rat clone: 7.76±0.05). In prostatic membranes from man, RS 17053 produced a biphasic displacement curve, with a pK_{iHIGH} of 9.90±0.24 (47% of total) and a pK_{iLOW} of 7.22±0.29 (n=3). (Data are mean ± s.e. mean; n = number of animals or patients.)

α_{1A}-AR selectivity was found in functional studies conducted *in vitro*. RS 17053 displaced with high affinity concentration-effect (E/[A]) curves to noradrenaline (NA) in isolated perfused kidney (estimated pA_2 =10.08±0.05), compared with aortic rings (pA_2 = 7.25±0.12) of rat. Such selectivity was observed in the same tissues from rabbit, dog, micropig and monkey.

In isolated smooth muscle preparations of tissues from lower urinary tract of man, RS 17053 shifted E/[A] curves to NA only at high concentrations. Estimates of affinity (pA_2) at α_1-ARs mediating NA-induced contractions were 7.52±0.17 (n=4) in prostatic periurethral longitudinal smooth muscle (prazosin = 8.61±0.07, n=6), 6.89±0.21 (n=4) in anterior fibromuscular stroma (prazosin = 8.94±0.33, n=5) and 7.10±0.29 (n=3) in bladder neck (prazosin = 8.54±0.14, n=7).

The data indicate that RS 17053 is 30-50 fold selective for the α_{1A}-AR, over the α_{1B}-AR and α_{1D}-AR. The high affinity binding of RS 17053 in prostate of man is consistent with the presence of an α_{1A}-AR in this tissue (Faure *et al.*, 1994). However, RS 17053 displayed only low affinity antagonism toward NA-induced contraction of prostate (and bladder neck). This finding suggests that the α_{1A}-AR may not mediate contractile responses to NA, a suggestion that contrasts with the conclusions of others (Forray *et al.*, 1994; Smith *et al.*, 1993). It is possible that multiple forms of the α_{1A}-AR may exist in prostate of man which are discriminated by RS 17053. Alternatively, another pharmacologically distinct α_1-AR may mediate the NA-induced contraction of prostatic smooth muscle (e.g. the α_{1L}-AR), as postulated by Muramatsu *et al.* (1993).

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25P CHARACTERIZATION OF A FUNCTIONAL α_{1D} ADRENOCEPTOR ON RAT AORTA

B. A. Kenny, D. H. Chalmers & A. M. Naylor. Department of Discovery Biology, Pfizer Central Research, Sandwich, Kent.

Two α_1 adrenoceptor subtypes, α_{1A} and α_{1B}, have been characterised pharmacologically in a number of tissues and a third subtype (α_{1D}) has been identified by molecular cloning (Bylund *et al*, 1994). However, the α_1 adrenoceptor mediating the contractile response to noradrenaline (NA) in rat aorta cannot be classified as either an α_{1A} or α_{1B} subtype (Oriowo & Ruffulo, 1992). In this study we have therefore characterised the pharmacological properties of rat aorta in comparison to cloned human α_{1D} adrenoceptors.

Determination of binding affinities for compounds at cloned α_1 adrenoceptors was carried out as previously described (Kenny *et al.*, 1994). Isometric contractions to noradrenaline were obtained using ring preparations of thoracic aorta from rats mounted in organ baths under a tension of 1g in Krebs Ringer bicarbonate containing cocaine (10 μM), corticosterone (10 μM) propranolol (1μM) and yohimbine (0.5 μM). BMY-7378, a selective α_{1D} antagonist (Saussy *et al.*, 1994) displayed high affinity for cloned α_{1D} adrenoceptors (pKi = 8.2 ± 0.1) with selectivity over both α_{1A} (pK_B = 6.2 ± 0.1) and α_{1B} subtypes (pKi 6.1 ± 0.1). In rat aorta, BMY-7378 potently inhibited NA mediated contractions (pA_2 = 8.3 ± 0.1). The α_{1A} selective compounds SNAP 1069 (α_{1A} pKi = 7.8 ± 0.2), 5-methyl-urapidil (α_{1A} pKi = 8.4 ± 0.10) and indoramin (α_{1A} pKi = 8.3 ± 0.03) also inhibited NA contractions with affinities consistent with α_{1D} binding potencies (Table 1). Regression analysis of these data (r = 0.98) suggests common identity between the cloned α_{1D} subtype and the functional α_1 adrenoceptor on rat aorta. In comparison, functional affinities correlated poorly with binding affinites at cloned α_{1A} (r = 0.44) and α_{1B} adrenoceptors (r = 0.59). Taken together, these data confirm the preliminary report of Saussy et al. (1994) and suggest that contractions of the rat aorta are mediated by an α_{1D} subtype.

Table 1. Functional potencies on rat aorta (pA_2/pK_B) and binding affinities (pKi) to cloned α_{1D} adrenoceptors.

COMPOUND	Rat aorta	α_{1D} binding affinity
Prazosin	9.8 ± 0.20	9.5 ± 0.10
BMY-7378	8.3 ± 0.10	8.2 ± 0.10
Benoxathian	8.5 ± 0.06	8.6 ± 0.12
Phentolamine	7.5 ± 0.04	7.8 ± 0.03
Indoramin	7.0 ± 0.10	7.2 ± 0.15
Spiperone	8.3 ± 0.06	8.1 ± 0.03
WB-4101	8.9 ± 0.08	9.2 ± 0.06
5-methyl-urapidil	7.8 ± 0.19	7.8 ± 0.03
SNAP-1069	6.8 ± 0.12	6.8 ± 0.12

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26P THE AFFINITY OF TAMSULOSIN (YM617) FOR FUNCTIONAL α_1-ADRENOCEPTOR SUBTYPES

A.J. Noble, D.J. Rosario*, C. Korstanje#, C.R. Chapple* & R. Chess-Williams. Departments of Biomedical Science, University of Sheffield and Urology*, Royal Hallamshire Hospital, Sheffield, U.K. and Yamanouchi# Europe B.V., Leiderdorp, The Netherlands.

Tamsulosin (YM617) is an α_1-adrenoceptor antagonist which, in radioligand binding studies, has a high affinity for the α_1-adrenoceptors of the human prostate (Yamada et al., 1991). The present study examines the effects of tamsulosin on the α_1-adrenoceptor mediated responses of the rat spleen which possesses a homogeneous population of α_{1B}-adrenoceptors (Han et al., 1987) and the responses of the human prostate, a tissue which expresses predominantly α_{1C}-adrenoceptors (Price et al., 1993).

Tissues were set up under 1g tension in Krebs solution at 37°C. Concentration-response curves to noradrenaline and phenylephrine were obtained in the absence or presence of tamsulosin. Experiments were performed in the presence of cocaine (10μM), corticosterone (10μM) and propranolol (1μM).

On the human prostate, tamsulosin (1-30nM) incubated with the tissue for 30min caused rightward shifts of noradrenaline concentration-response curves yielding a mean (±SEM) pA_2 value of 9.86 ± 0.12. Schild plots however had a slope significantly ($P<0.05$) greater than unity (1.5 ± 0.22) and maximum responses to noradrenaline were reduced by 49 ± 7% with the 10nM, and 46 ± 6% by the 30nM concentration of tamsulosin.

When the incubation period with the antagonist was increased to 60 min, tamsulosin competitively antagonised responses to noradrenaline without reducing maximum responses, yielding a mean pK_B value of 9.9 ± 0.4 (n=22). The Schild plot had a slope similar to unity (1.17 ± 0.18). Using phenylephrine as agonist and a 60 min incubation period tamsulosin again had a high affinity, with a mean pK_B of 10.0 ± 0.4 (n=30). Maximum responses were not altered significantly and the Schild plot had a slope of 1.0 ± 0.26.

On the rat spleen, tamsulosin was less potent and although concentrations of 3 and 10nM produced shifts of noradrenaline concentration-response curves (pA_2 = 8.6), 30nM failed to give any further shift. When phenylephrine was the agonist, tamsulosin antagonized responses competitively, yielding a pK_B value of 8.9 ± 0.1. The Schild plot was linear (slope = 1.0) and maximum responses of the spleen to phenylephrine were not altered by tamsulosin.

These results indicate that (i) tamsulosin has a greater affinity for prostatic α_1-adrenoceptors than for splenic α_{1B}-adrenoceptors (ii) At least 60 min is required for tamsulosin to equilibrate with prostatic α_1-adrenoceptor and (iii) Splenic responses to noradrenaline may be mediated by more than one receptor.

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27P AMINOGUANIDINE DOES NOT DIMINISH LIPOPOLYSACCHARIDE-INDUCED VASCULAR HYPERRESPONSIVENESS TO ACETYLCHOLINE OR METHOXAMINE IN CONSCIOUS RATS

J. Waller, S.M. Gardiner & T. Bennett. Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham. NG7 2UH.

Aminoguanidine (AG) is reported to be a selective inhibitor of inducible nitric oxide synthase (Corbett *et al.*, 1992). We have previously described the effect of AG on resting regional haemodynamics during low dose infusion of lipopolysaccharide (LPS) in conscious rats (Waller *et al.*, 1994). The effects of AG were particularly evident after 6h of LPS infusion, with the development of a hypertension and vasoconstriction in all the vascular beds monitored. As a continuation of this work we now report on the effect of AG on responses to acetylcholine (ACh) and methoxamine (ME) after 6h of LPS infusion.

Male Long Evans rats (350-450g) were chronically implanted with pulsed Doppler probes (renal, mesenteric and hindquarters) and intravascular catheters (under sodium methohexitone anaesthesia, 40-60mg kg^{-1} i.p.). Catheters were implanted 7-14 days after flow probe implantation and at least 24h before experiments were begun. Cardiovascular variables were measured during 3min infusions of ACh (22 nmol min^{-1}) and ME (120 nmol min^{-1}). The rats were then randomised into 3 groups (n=8 in each). Two groups received AG (400μmol kg^{-1}, 400μmol kg^{-1} h^{-1}) starting 1h before a continuous co-infusion of LPS (150 μg kg^{-1} h^{-1}; *E. coli* serotype 0127:B8; Sigma) or saline (0.3 ml h^{-1}). The third group received saline (0.1ml, 0.3ml h^{-1}) starting 1h before co-infusion of LPS (150μg kg^{-1} h^{-1}). Two, 6 and 24h after the start of the co-infusion of AG and saline, saline and LPS, or AG and LPS, all rats were re-challenged with ACh and ME.

ACh infusion caused a depressor response associated mainly with a renal vasodilatation. After 6h of saline infusion in the AG-treated group, responses to ACh were not different from control responses, whereas in both groups receiving LPS, the renal vasodilator response to ACh was significantly attenuated (Table 1). ME infusion caused a pressor response, associated with renal, mesenteric and hindquarters vasoconstrictions. After 6h of saline infusion in the AG-treated group, there were no changes in any of the haemodynamic responses to ME, whereas in both groups receiving LPS there was a marked attenuation of the pressor and hindquarters vasoconstrictor responses (Table 1). In addition, there was a reduced mesenteric vasoconstrictor response to ME in the AG-treated group receiving LPS, possibly because resting mesenteric vascular conductance was already reduced (Waller *et al.*, 1994).

Table 1 Responses to ACh and ME (areas under or over curves AUC, $AUC_{0\text{-}3min}$) before and 6h after the onset of saline (SAL) or LPS infusion after 1h pretreatment and continuous infusion of SAL or AG. Values are mean ± s.e. mean. * $P < 0.05$ versus control (Friedman's test). Units for renal (R), mesenteric (M) and hindquarters (H) vascular conductance (VC) are [kHz mm Hg^{-1}] 10^3 min.

		Acetylcholine		Methoxamine	
		Control	6h	Control	6h
ΔHR (beats)	AG/SAL	197 ± 17	191 ± 23	-149 ± 20	-139 ± 15
	SAL/LPS	167 ± 26	74 ± 11*	-149 ± 7	-90 ± 13*
	AG/LPS	168 ± 36	108 ± 18	-163 ± 23	-65 ± 10*
ΔMAP (mmHg min)	AG/SAL	-26 ± 4	-25 ± 4	88 ± 8	100 ± 6
	SAL/LPS	-16 ± 3	-12 ± 3	84 ± 6	33 ± 5*
	AG/LPS	-20 ± 3	-13 ± 5	78 ± 6	44 ± 4*
ΔRVC (units)	AG/SAL	74 ± 18	64 ± 11	-53 ± 11	-71 ± 11
	SAL/LPS	62 ± 12	33 ± 5*	-51 ± 6	-56 ± 11
	AG/LPS	56 ± 6	17 ± 5*	-34 ± 4	-37 ± 4
ΔMVC (units)	AG/SAL	16 ± 7	9 ± 5	-100 ± 8	-95 ± 6
	SAL/LPS	11 ± 2	25 ± 4*	-85 ± 6	-74 ± 9
	AG/LPS	9 ± 3	2 ± 1	-69 ± 6	-12 ± 2*
ΔHVC (units)	AG/SAL	15 ± 5	12 ± 3	-53 ± 5	-47 ± 6
	SAL/LPS	8 ± 4	6 ± 2	-60 ± 9	-11 ± 3*
	AG/LPS	12 ± 5	9 ± 1	-57 ± 5	-12 ± 3*

The significant attenuation of the renal vasodilator response to ACh after 6h of LPS infusion in both the saline- and AG-treated groups is unlikely to have been dependent upon resting renal vascular conductance, as the latter is increased by LPS only in saline-treated rats (Waller *et al.*, 1994). The inability of AG to prevent hyporesponsiveness to ME is in contrast to the study of Wu *et al.*, (1994) in which AG attenuated LPS-induced inhibition of the pressor response to noradrenaline in anaesthetised rats.

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28P VASODILATATION IN HUMAN ARTERIES *IN VITRO* INDUCED BY PDE III INHIBITORS

M.B. Vroom, M. Pfaffendorf[1], H.B. van Wezel, E.J.F. Timmenga[2], C.M.A.M. van der Horst[2] & P.A. van Zwieten[1], Dept. of Anesthesiology and [1]Dept. Pharmacotherapy, Dept. Plast. Reconstr. Surgery, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

The use of the currently available posphodiesterase (PDE)-III-inhibitors, such as amrinone, milrinone and enoximone in acute heart failure is associated with marked vasodilation at higher doses. The exact mechanism of these vasodilating properties remains to be elucidated in detail. In the present study, we investigated on human vessels *in vitro* whether the vasorelaxing effects of PDE III inhibitors used in clinical practice could be influenced by a nitric oxide (NO) synthase blocker, N^G-nitro-L-arginine methyl ester (L-NAME), or by glibenclamide (Glib), a blocker of ATP-sensitive potassium (K_{ATP}) channels.

Small arteries were obtained from 11 healthy female patients (age 18 to 49 years) undergoing reconstructive breast surgery under general anesthesia. Immediately after removal of a skin segment arteries with a diameter of 415 μm ± 20 (mean ± SEM, n=116) were dissected over a length of 1.8 ± 0.03 mm (n=116). The experiments were performed with an isometric myograph according to Mulvany and Halpern (1976). A priming procedure consisting of three subsequent 5 min. exposures to an isotonic, high potassium solution (120 mM) and one to 10 μM phenylephrine. The priming procedure was followed by an equilibration period of 30 min. In the case of pretreatment the drugs, L-NAME (100 μM) or Glib (1 μM), were administered at the beginning of this 30 min. period and remained present throughout the experiment. Subsequently, 1 μM U 46619, a vasoconstricting thromboxane A_2 mimetic agent, was administered. When the contraction has reached an equilibrium, cumulative concentration-response curves were constructed for the selective PDE III inhibitors amrinone, milrinone and enoximone as well as for theophylline and dipyridamol. A log concentration-response curve was fitted to the data of each experiment (n=5-7) by nonlinear regression analysis. The resulting EC_{50} values, the concentrations required for a 50% relaxation, are enumerated in Table 1.

No significant differences in diameter, length or active force development to 1 μM U46619 (5.27 ± 0.31 N/m) between the groups was observed.

Since the effect of none of the vasodilators investigated was modulated by glibenclamide we conclude that K_{ATP}-channels are not influenced by these compounds. L-NAME (100μM) shifted the concentration-response curve of milrinone significantly to the right, indicating that endothelium-dependent NO-release may contribute to the vasorelaxing properties of milrinone.

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Table 1. pEC_{50} values of phosphodiesterase inhibitor-induced vasodilation in precontracted small human arteries. Values given as mean ± s.e.m. (n).

	Amrinone (n)	Milrinone (n)	Enoximone	Theophylline	Dipyridamole
control	3.28 ± 0.10 (6)	3.49 ± 0.17 (7)	3.29 ± 0.08 (6)	2.72 ± 0.07 (5)	4.67 ± 0.22 (5)
+ L-NAME 100 μM	3.35 ± 0.09 (5)	2.77* ± 0.24 (5)	3.41 ± 0.10 (5)	2.69 ± 0.06 (5)	4.76 ± 0.09 (5)
+ Glibenclamide 1μM	3.25 ± 0.17 (6)	3.46 ± 0.15 (6)	3.49 ± 0.06 (6)	2.76 ± 0.19 (8)	4.53 ± 0.02 (5)

* denotes a significant difference when compared with the respective control group ($P < 0.05$)

29P INHIBITION OF CHEMOTACTIC PEPTIDE-INDUCED NEUTROPHIL SUPEROXIDE RELEASE BY THEOPHYLLINE, ROLIPRAM AND ISOPRENALINE IS REDUCED BY PROTEIN KINASE A ANTAGONIST H89

M Johnson, H.Sindi, A. Hussain, S. M. Banks, C. Page, J. Costello, S. A. Kilfeather*, Sackler Institute for Pulmonary Pharmacology, Department of Thoracic Medicine, Kings College School of Medicine and Dentistry, London SE5 9PJ.

The role of cyclic AMP in the actions of theophylline (Theo) on circulating cells at low therapeutic circulating concentrations (5-20mg/l) has not been established. We have investigated the effect of protein kinase A antagonist H89 on the inhibition of superoxide (SO) release by theophylline, rolipram and isoprenaline in neutrophils.

Peripheral blood neutrophils were isolated from healthy volunteers by density gradient centrifugation and dextran sedimentation of erythrocytes. SO production was monitored by reduction of cytochrome C in a 96-well plate at 37°C under conditions of high cell density (1.5 X $10^6/cm^2$) and adherence as previously described (Cerasoli et al., 1988) in PBS with BSA (0.1%) and adenosine deaminase (1unit/ml). H89 was preincubated with cells for 60 minutes before and throughout stimulation of superoxide production with the chemotactic peptide (Formyl-NLE-LEU-PHE-NLE-TYR-LYS, CHP, 10^{-7}M). Cells were preincubated with theophylline (30,100 x 10^{-6}M, 15 min), rolipram (Roli, 3 x 10^{-6}M, 15 min), isoprenaline (ISO, 0.3 x 10^{-6}M, 5 min) before stimulation with CHP or phorbol 12-myristate 13-acetate (PMA, 0.001-1 x 10^{-6}M). Theophylline, rolipram and isoprenaline inhibited SO production (Table 1). H89 reduced inhibition in each case. Theophylline (100 x 10^{-6}M) did not inhibit responses to PMA , demonstrating specificity of action.

Table 1 Effect of protein kinase antagonism on inhibition of neutrophil SO production

		% maximal response ± SE			
Superoxide production	H89 (4μM)	Theo, 30μM	Theo, 100μM	Roli, 3μM	ISO, 0.3μM
Total,	-	69.3 ± 6.3*	68.8± 3.5*	43.5± 5.3*	57.6± 10.5*
mOD units/ 2 hours	+	88.2 ± 5.3	84.2± 6.3	81.0± 8.7	100.6± 12.7
Maximum rate,	-	73.1 ± 7.3*	59.8± 4.3	37.7± 9.5*	53.7± 8.2*
mOD units/ min	+	91.6 ± 6.9	74.3± 7.3	66.9± 7.4	98.1± 11.0

*, P<0.05 compared to H89, n = 8 for all groups

These findings indicate phosphodiesterase inhibition in the action of theophylline at therapeutic circulating concentrations. Selectivity of H89 for protein kinase A over protein kinase G is approximately 10-fold and therefore involvement of cyclic GMP in addition to cyclic AMP cannot be excluded in the actions of theophylline in neutrophils.

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30P LYMPHOCYTE ADAPTION TO THE ANTIMITOGENIC EFFECT OF TYPE IV PHOSPHODIESTERASE INHIBITION OCCURS WITHOUT ALTERATION IN $CD4^+$, $CD8^+$ AND CD^- DISTRIBUTION

S. Wahid[1], E. Shittu[2], C. Page[1], J. Costello[1], S. A. Kilfeather[1]*, [1]Sackler Institute for Pulmonary Pharmacology, Department of Thoracic Medicine and [2]The Clinical Age Research Unit, Department of Health Care of the Elderly, Kings College School of Medicine and Dentistry, London SE5 9PJ.

Type IV phosphodiesterase (PDE) inhibition provides a potential route for immunomodulation in treatment of asthma (Nicholson and Shahid, 1993). Cyclic AMP exerts an inhibitory effect within the T-lymphocyte division cycle (Hadden, 1988). We have investigated inhibition of human lymphocyte division by the type IV PDE inhibitor rolipram (R).

Peripheral blood mononuclear cells were isolated from healthy volunteers by density gradient centrifugation and incubated with a submaximal mitogenic stimulus (phytohemagglutinin, PHA, 1-2 μg/ml). Effects of rolipram (0.01-10^{-6}M) on mitogenesis at 2 and 5 days were examined by inclusion of rolipram throughout the incubation period. Mitogenesis was monitored by ^{3}H-thymidine uptake. Increase in cell population was monitored by protein estimation (Baumgarten, 1985). Lymphocyte subset distribution was monitored through FACscan analysis.

Rolipram caused a dose-dependent inhibition of mitogenesis at day 2 (Inhibition with R [10^{-6}M]± SE: 31.7± 1.7%), but not at day 5 (2.2 ± 1.3%) (day 2 vs 5, n=6, P<0.0001). Rolipram (10^{-6}M) did not affect the PHA-induced increase in cellular protein at 5 days (% increase in protein: PHA vs PHA + R, slope = 0.965, r^2 = 0.68, n= 9, P<0.005). Inhibition of mitogenesis by rolipram (10^{-6}M) at day 2 was inversely related to mitogenic response (r^2=0.917, P<0.0001, n=9). PHA changed the proportion of CD4+ (day 0: 42.3± 2.9; day 5: 56.8± 3.8), CD8+ cells (day 0: 27.3 ± 2.4; day 5: 21.8 ± 2.6) and $CD3^-$ (day 0: 28.5 ± 2.0; day 5: 13.2 ± 3.3). PHA-induced changes in $CD4^+$, $CD8^+$ and CD^- cells were not affected by inclusion of rolipram (10^{-6}M, n = 6) over 5 days.

These findings demonstrate dose-dependent inhibition of mixed human lymphocyte division by a type IV PDE inhibitor at 2 days and loss of inhibition at 5 days. Inhibition of division is inversely related to the magnitude of mitogenic response. Adaption at 5 days to inhibition of proliferation by rolipram is independent of changes in lymphocyte distribution.

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31P POTENT INHIBITION OF THE DELAYED RECTIFIER POTASSIUM CURRENT IN RAT SYMPATHETIC NEURONS BY EXTRACELLULAR AMITRIPTYLINE REQUIRES THE DRUG TO BE UNCHARGED

Julian R.A. Wooltorton & Alistair Mathie, Department of Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF.

We have shown that tricyclic compounds such as amitriptyline can inhibit delayed rectifier (K_V) and transient (K_A) outward potassium (K^+) currents in rat sympathetic neurons (Wooltorton & Mathie 1993). The results presented here provide more information as to the mechanism underlying this action of amitriptyline on K_V.

Sympathetic neurons were isolated from 7-8 day old Sprague Dawley rats and K^+ currents were recorded under whole-cell voltage-clamp. Following a prepulse to -120 mV for 500 ms, neurons were stepped to 0 mV for 400 ms. This depolarisation evoked a rapidly activating current (K_A) of 3230 ± 467 pA (n=18; mean ± s.e.mean) which rapidly inactivated to a sustained outward current (K_V) of 879 ± 94 pA.

Under control conditions (pH 7.4), amitriptyline (10 μM) inhibited K_V by 47.7 ± 5.0% (n=10). Amitriptyline is a weak base with pK_a of 9.4, therefore, changing pH from 7.4 to 9.4 will change the ratio of charged to uncharged amitriptyline from 99:1 to 50:50. At pH 9.4, amitriptyline (10 μM) was significantly more effective at blocking K_V, inhibiting by 79.7 ± 5.6% (n=10). The block at this pH took much longer to reverse by washing than that at pH 7.4. The concentration response curve for amitriptyline inhibition of K_V at both pHs could be fitted by a Hill equation. At pH 7.4, amitriptyline had a K_D of 11.9 ± 1.2 μM with maximum inhibition of 101.0 ± 3.3% and slope 0.90 ± 0.05. At pH 9.4 amitriptyline was about 25 times more potent with a K_D of 0.47 ± 0.18 μM, a maximum inhibition of 83.8 ± 8.8% and slope of 0.95 ± 0.25. The quaternary K^+ channel blocking drug tetraethylammonium ion (TEA), at a concentration of 5 mM, was equally effective at blocking K_V at pH 7.4 (58.5 ± 3.0%, n=5) and at pH 9.4 (57.1 ± 6.1%, n=6).

Thus amitriptyline is a more potent blocker of K_V at pH 9.4 than 7.4. This suggests that the uncharged form of amitriptyline is required extracellularly for block. To test this idea further, the permanently charged analogue of amitriptyline, N-methyl amitriptyline was used. In four cells, 10 μM amitriptyline inhibited K_V by 36.2 ± 2.1% while in the same cells 10 μM N-methyl amitriptyline inhibited K_V by only 2.0 ± 0.8%.

These results show that amitriptyline must be present in its uncharged form, extracellularly, to block K_V potently. This may be because the blocking site has a high affinity for the uncharged form relative to the charged form. Alternatively, it may be that the uncharged form is required for the compound to cross the neuronal membrane and act from the inside.

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Wooltorton, J.R.A. & Mathie, A. (1993) *Br. J. Pharmacol.* 110, 1126-1132.

32P THE GLIBENCLAMIDE-SENSITIVE K^+ CURRENT IN RABBIT AORTIC MYOCYTES IS ACTIVATED BY INTRACELLULAR GDP, BUT NOT BY THE ABSENCE OF ATP

F.C. Halliday, A.M. Gurney & P.I. Aaronson, Department of Pharmacology, United Medical and Dental Schools, Lambeth Palace Road, London SE1 7EH

It has recently been proposed (Beech *et al*, 1993) that the glibenclamide (GLIB)-sensitive K^+ (putatively K_{ATP}) channel in vascular smooth muscle may be regulated primarily by nucleotide diphosphates, rather than by ATP itself. We therefore utilized the whole-cell, patch-clamp technique to examine the effect of varying the nucleotide composition of the pipette solution on the glibenclamide- and levcromakalim (LEV)-sensitive K^+ current, in smooth muscle cells isolated from the rabbit thoracic aorta. In order to visualize clearly the K_{ATP} current over the membrane potential range close to the resting potential, extracellular K^+ was set to 130 mM and voltage ramps of 100 ms duration were applied between -100 and +50 mV at 5s intervals, first under control conditions, then in the presence of LEV (20 μM) and finally in the presence of GLIB (10 μM). Total $MgCl_2$ in the pipette was set to 1 mM. When nucleotides were omitted from the pipette solution there was very little GLIB-sensitive current, although LEV induced a current at -80 mV (156 ± 54 pA; mean ± SEM, n=6), which was then blocked by GLIB. Similar results were observed with GTP (0.5 or 1 mM) or ATP (1 mM) in the pipette. If, however, GDP (1 mM) was included in the pipette solution, a large GLIB-sensitive current (117 ± 24 pA, n=12) was observed and the amplitude of the LEV-induced current was enhanced to 271 ± 72 pA (n=8). These GLIB- and LEV-sensitive currents were then strongly suppressed to 34 ± 5 pA (n=5) and 72 ± 19 pA (n=6) respectively, if both GDP (1 mM) and ATP (1 mM) were present. Raising the total pipette $MgCl_2$ to 5 mM had little effect on the GLIB-sensitive current regardless of the nucleotide content of the pipette solution; the LEV-induced current appeared to be reduced in the presence of GDP or ATP, but not when GTP or no nucleotide was present. Exclusion of $MgCl_2$ from the pipette solution, in the presence of GDP (1 mM) or in the absence of intracellular nucleotides, abolished the GLIB-sensitive and LEV-induced currents.

Thus, the activation of the GLIB-sensitive K^+ current required the presence of GDP or LEV and an optimal concentration of intracellular Mg^{2+}. Once activated, the current was largely suppressed by 1 mM ATP. Raising $MgCl_2$ to 5 mM had variable effects upon the GLIB-sensitive and LEV-induced currents, depending on the nucleotides present.

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G. Edwards, A. Niederste-Hollenberg, J. Schneider, Th. Noack[1] & A. H. Weston, Division of Physiology, Pharmacology & Toxicology, School of Biological Sciences, University of Manchester, G38 Stopford Building, Oxford Rd., Manchester M13 9PT, UK and [1]Department of Physiology, Philipps University, Deutschhausstrasse 2, 35033 Marburg, Germany

Electrophysiological studies with BRL55834 have suggested that this agent may open not only the ATP-sensitive K-channel (K_{ATP}) but also a glibenclamide-insensitive K-channel (Ward et al., 1992; Niederste-Hollenberg et al., 1994), an action which may form the basis of its tissue selectivity (Bowring et al., 1993). The aim of the present study was to characterise further the effects of BRL55834.

When enzymatically-dispersed portal vein cells were held at -10mV (using Ca^{2+}-free whole-cell recording conditions: Edwards et al., 1994), exposure to 3μM BRL55834 induced a voltage- and time-independent current of 311.2 ± 27.6 pA (mean ± sem, n=5) on stepping to a +40mV test potential. However, on stepping to this potential from a holding potential of -90mV, the *total* outward current was unaltered by BRL55834. Thus, like levcromakalim, BRL55834 reduces $I_{K(V)}$ and increases a time-independent current. This stimulatory effect of BRL55834 was Ca^{2+}-independent. Inclusion of AMP-PCP (5mM) in the pipette solution reduced the magnitude of the time-independent current induced by 3μM BRL55834 (to 68.2 ± 28.3 pA at +40mV, n=5) but totally abolished the response to 10μM levcromakalim. The glibenclamide-resistant current (275.1 ± 53.5 pA) obtained on stepping from -10mV to +40mV in the presence of 3μM BRL55834 and 10μM glibenclamide was reduced to 66.7 ± 7.8 pA in the additional presence of 100μM phentolamine, the inhibitor of K_{ATP} (Ibbotson *et al.* 1993). This value was similar to that of the initial control current in these cells (74.5 ± 9.2 pA, n=5). In contrast, the current induced by 33μM NS1619 (an opener of large conductance calcium-sensitive K-channels: Edwards et al., 1994) was unaffected by 100μM phentolamine (holding potential -10 mV, test potential +50 mV; control, 445.2 ± 130.9 pA; NS1619, 1235.5 ± 190.6 pA; NS1619+phentolamine, 1241.2 ± 252.9 pA, n=5).

It is concluded that the effects of BRL55834 may result from the opening of a single K-channel type which is probably K_{ATP}. The glibenclamide-insensitivity of the current induced by BRL55834, in contrast to that induced by levcromakalim, may reflect different mechanisms of action of these two K-channel openers.

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A.H. Weston, G. Edwards, M Stocker[1] & O. Pongs[1], Division of Physiology, Pharmacology & Toxicology, School of Biological Sciences, University of Manchester, G.38 Stopford Building, Oxford Road, Manchester M13 9PT and [1]Zentrum für Molekulare Neurobiologie, Institut für Neurale Signalverarbeitung, Martinistraße 52, Haus 42, 2000 Hamburg 20, Germany

The assessment of novel potassium (K) channel modulators is usually complicated by the presence of multiple K-channel types in the test tissue. Using whole-cell recording techniques, the electrophysiological and pharmacological properties of the delayed rectifier K-current ($I_{K(V)}$) in rat freshly-isolated portal vein cells were compared with those of the current ($I_{Kv1.1}$) carried by cloned Kv1.1 channels (RCK1; Baumann *et al.*, 1988) stably expressed in human embryonic kidney (HEK) 293 cells. RCK1 is identical to RK1, transcripts of which are present in a rat aorta cDNA library (Roberds & Tamkun, 1991). One objective of this study was to assess whether the pharmacology of a native K-channel could be modelled in such a heterologous expression system.

The voltage dependence of activation of Kv1.1 was different from that of K_V. When a Boltzmann function was fitted to the calculated peak conductance values obtained at different test potentials (assuming a K-equilibrium potential of -85mV), the $V_{1/2}$ values were -23.0 ± 3.8mV and 10.7 ± 2.4mV ($P<0.05$) with slope factors (k) of 12.4 ± 3.2 mV and 15.5 ± 1.5 mV for Kv1.1 and K_V, respectively. Furthermore, the rate of current decay of $I_{K(V)}$ was faster (τ=64ms) than for $I_{Kv1.1}$ (τ=290ms). 4-aminopyridine (5mM) and ciclazindol (100 μM) each inhibited $I_{K(V)}$, and $I_{Kv1.1}$. Unlike $I_{K(V)}$, $I_{Kv1.1}$ was inhibited by margatoxin (100nM, Garcia-Calvo *et al.*, 1993) but unaffected by phentolamine (100 μM) or levcromakalim (10 μM), both inhibitors of $I_{K(V)}$ in rat portal vein (Edwards *et al.*, 1993; Ibbotson *et al.*, 1993). Calphostin C (500 nM), the inhibitor of protein kinase C, had no effect on either $I_{K(V)}$ or $I_{Kv1.1}$. However, $I_{K(V)}$, but not $I_{Kv1.1}$ was inhibited by PKI(6-22) amide (100 nM), the inhibitor of protein kinase A. Inclusion of alkaline phosphatase (up to 100 U ml^{-1}) in the pipette had little effect on $I_{Kv1.1}$ whereas the rate of inactivation of this current was markedly enhanced by bath application of this enzyme (20 U ml^{-1}).

These results show that the spectrum of agents capable of inhibiting K_V is different from that active on Kv1.1. This either suggests that the native K_V is not a homomultimer formed from Kv1.1 α-subunits or that the properties of these are influenced by the co-expression of β- or other types of modulatory subunit in the portal vein smooth muscle. Alternatively K_V could be a heteromultimeric assembly of different α-subunits.

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35P INFLUENCE OF THE HYPERTENSIVE STATE ON THE EFFECT OF POTASSIUM CHANNEL MODULATORS IN MESENTERIC ARTERIES

B.C.P. Hüsken, M. Pfaffendorf & P.A. van Zwieten, Department of Pharmacotherapy, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

In vascular smooth muscle the ionic gradients and electrogenic ion transports, both contributing to the membrane potential are known to differ in spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats (Hermsmeyer, 1976).
In the present study we investigated different compounds that interfere with intracellular potassium homeostasis in the mesenteric arteries of Wistar rats, WKY rats and SHR. We evaluated the influence of glibenclamide, an ATP-sensitive K^+-channel blocker on the methacholine- (which may release the endothelium-derived hyperpolarizing factor (EDHF)) and on the levcromakalim- (an ATP-sensitive K^+-channel opener) mediated responses.
The experiments were performed in rat isolated mesenteric arteries using an isometric wire myograph (according to Mulvany & Halpern, 1977). The mesenteric arteries were incubated for 1 hour with vehicle, 0.1, 1 and 3 μM glibenclamide, respectively. After precontraction with 1 μM phenylephrine cumulative concentration-response curves for the vasodilator effects of methacholine and levcromakalim were constructed (n=4-7).

In the mesenteric arteries of the Wistar rats the pD_2-value (means±s.e.mean 7.51 ± 0.12) and the maximal relaxation (Emax) (98.97 ± 0.58%) for methacholine were not influenced by glibenclamide. The pD_2-values for levcromakalim were significantly ($p<0.05$) and concentration-dependently decreased by glibenclamide (6.55 ± 0.14, 5.80 ± 0.19, 5.44 ± 0.19 and 5.33 ± 0.18 for vehicle, 0.1, 1 and 3 μM glibenclamide, respectively). The Emax-value for levcromakalim (99.12 ± 0.40%) was not influenced by glibenclamide.
In the WKY preparations the pD_2- (7.66 ± 0.08) and Emax-values (100.0 ± 0.0%) for methacholine did not differ from those obtained in SHR vessels (pD_2: 7.56 ± 0.04, Emax: 95.73 ± 2.59%).
The preparations obtained from the WKY (pD_2 of 6.35 ± 0.12) were more sensitive to levcromakalim than those taken from the SHR (pD_2 of 5.55 ± 0.18). In the mesenteric arteries from the WKY glibenclamide caused a significant and concentration-dependent decrease in pD_2 (6.35 ± 0.12, 5.90 ± 0.11, 5.27 ± 0.14 and 5.19 ± 0.06 for vehicle, 0.1, 1 and 3 μM glibenclamide, respectively) and in Emax (99.11 ± 0.39%, 95.87 ± 0.71% and 55.46 ± 6.83% for vehicle, 1 and 3 μM glibenclamide, respectively). Only a high concentration of 3 μM glibenclamide caused a decrease in Emax (83.16 ± 7.94% and 48.00 ± 5.00% for vehicle and 3 μM glibenclamide, respectively) in mesenteric arteries obtained from the SHR. The pD_2 for levcromakalim remained uninfluenced by glibenclamide.

In conclusion, methacholine-mediated responses are neither influenced by glibenclamide nor by the hypertensive state. However, elevated blood pressure of the donor animals decreases the sensitivity to ATP-sensitive K^+-channels openers in isolated resistance vessels.

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36P INVESTIGATION OF THE EFFECT OF NPPB ON MEMBRANE CURRENTS IN RAT PORTAL VEIN

A. J. Kirkup & A. H. Weston, Physiology, Pharmacology and Toxicology Division, School of Biological Sciences, University of Manchester, G38 Stopford Building, Oxford Road, Manchester M13 9PT.

The lack of selective chloride (Cl^-) channel inhibitors precludes the determination of the contribution of these channels to excitation-contraction coupling in smooth muscle. 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) is a potent inhibitor of epithelial Cl^- channels (Wangemann *et al.*, 1986) but little is known of its actions on these channels in smooth muscle. The purpose of the present study was to examine the effects of NPPB on membrane currents in smooth muscle in order to determine its usefulness in the investigation of the role of Cl^- channels in this tissue.

Rat portal vein cells were isolated by enzymatic dispersion and were then superfused with a quasi-physiological salt solution. Whole-cell currents were recorded with the 'perforated-patch' configuration of the patch-clamp technique, using amphotericin B (pipette concentration of 300 $\mu g.ml^{-1}$). After 5 min exposure to 10 μM NPPB in Cs^+-containing solutions and at a holding potential of -60 mV, the inward calcium (Ca^{2+})-activated Cl^- currents induced by noradrenaline (NA, 10 μM) or caffeine (10 mM) were reduced (NA:- control: -91 ± 8 pA; 10 μM NPPB: -4 ± 4 pA; caffeine:- control: -73 ± 33 pA; 10 μM NPPB: -25 ± 3 pA). These effects of NPPB were reversible on washing.

In potassium (K^+)-containing solutions and at a holding potential of -2 mV (the Cl^- equilibrium potential), application of NA, caffeine or acetylcholine (ACh, 10 μM) induced large (110-1000 pA), outward Ca^{2+}-activated K^+ currents. After 5 min exposure to 10 μM NPPB the magnitude of NA- and ACh-induced K^+ currents was significantly reduced (NA:- control: +425 ± 96 pA; 10 μM NPPB: +57 ± 24 pA**; ACh:- control +470 ± 98 pA; 10 μM NPPB: +51 ± 30 pA*), effects which were reversible on washout. In contrast, the magnitude of the caffeine-induced currents was unaffected by the presence of NPPB (caffeine:- control: +892 ± 81 pA; 10 μM NPPB: +879 ± 110 pA).

NPPB 10μM also induced a noisy outward current (control holding current at -2 mV: +15 ± 3 pA; 10 μM NPPB: +118 ± 15 pA) and attenuated spontaneous transient outward currents. These effects were also reversible on washout. Bath application of glibenclamide (10 μM) reversed the stimulating effect of NPPB on the holding current but did not antagonise the inhibitory effects of the agent on evoked or spontaneous currents.

All values represent mean ± s.e.mean, n=3-18 (Student's paired t-test * $P<0.05$; ** $P<0.01$).

These data indicate that NPPB inhibits Cl^- currents in rat portal vein. However, this agent also modulates K^+ currents and Ca^{2+} homeostasis in the same tissue. Experiments are in progress to determine whether these diverse effects are exerted directly on ion channels or *via* biochemical systems involved in channel gating mechanisms.

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37P ACTIONS OF RYANODINE ON ACTION POTENTIAL AFTER-DEPOLARISATIONS IN CULTURED RAT DORSAL ROOT GANGLION NEURONES

A. Ayar, J. Riley & R.H. Scott, Department of Physiology, St. George's Hospital Medical School, London SW17 ORE.

Calcium entry during single action potentials can evoke after-depolarisations in 40-50% of cultured DRG neurones from neonatal rats. The purpose of this project was to investigate whether ryanodine-sensitive calcium release from intracellular stores is involved in the activation of Ca^{2+}-activated Cl^- channels which underlie the after-depolarisation. The whole cell recording technique was used to load cells with KCl-based patch pipette solution. The mean amplitude of the after-depolarisation was 22±3 mV from -75mV and the event decayed by 63% in 600±350 ms (n=7). In some cells the after-depolarisation was preceded by an after-hyperpolarisation so that two distinct after potentials were observed. The reversal potential of the after-hyperpolarisations and after-depolarisations were -75±2 mV (n=9) and -20±3 mV (n=3) respectively. Extracellular application of ryanodine (10μM) evoked transient changes in the resting membrane potential similar to those seen with caffeine. Under voltage-clamp conditions ryanodine caused transient activation of a Ca^{2+}-activated Cl^- current (n=5), which in part accounts the effects on membrane potential. Ryanodine also significantly attenuated the amplitude of the action potential after-depolarization by 42±20 % (n=3) (Figure 1). Our data suggests that ryanodine can transiently increase intracellular calcium possibly by releasing it from internal stores. Additionally, ryanodine reduced after-depolarisations which may indicate that these events are in part activated by calcium released from a ryanodine-sensitive store. Release of calcium from stores may be reduced because of depletion after the initial transient release of calcium or blocked by a secondary action of ryanodine.

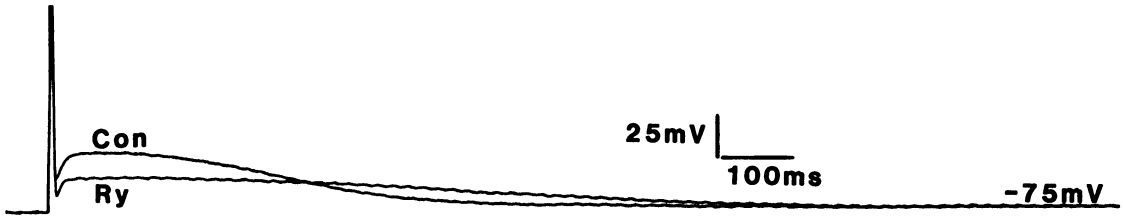


Figure 1. Ryanodine attenuates the amplitude of action potential after-depolarisations. Control action potential after-depolarisation (Con) and the action potential and after-depolarisation after 5 minutes application of 10μM ryanodine (Ry). No recovery was observed.

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38P CAPACITATIVE CALCIUM INFLUX IS STIMULATED IN CONTRACTION OF RAT SPLEEN TO PHENYLEPHRINE

Richard P. Burt[1], Christopher R. Chapple[2] & Ian Marshall[1]. [1]Department of Pharmacology, University College London, Gower Street, London WC1E 6BT and [2]Department of Urology, The Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF.

Contraction of rat spleen to phenylephrine is via α_{1B}-adrenoceptors which mediate their cell response through production of inositol triphosphate (IP_3) and diacylglycerol. IP_3 raises intracellular [Ca^{2+}] and this is often associated with a more sustained rise in intracellular [Ca^{2+}] due to influx of extracellular Ca^{2+}. The stimulus for this influx in some cells is the emptying of intracellular Ca^{2+} stores, called capacitative Ca^{2+} entry (Putney, 1986; Jacob, 1990). The aim of this investigation was to see if the contraction to phenylephrine in rat spleen was dependent upon capacitative Ca^{2+} entry.

Splenic strips from male Sprague Dawley rats (350-400g) were set up in Krebs solution at 37°C, bubbled with 95% O_2/5% CO_2, under 1.0g resting tension and equilibrated for 2 hours. A contraction to phenylephrine ($3x10^{-4}$M) which produced a maximal response was measured in each tissue to which all further responses were compared as a percentage. Results were plotted as the mean±s.e.mean of 4 experiments. Two hours after the initial contraction to phenylephrine another contraction to phenylephrine ($3x10^{-4}$M) was measured either in normal Krebs or in Ca^{2+} free Krebs solution (containing 1mM EGTA) in which the tissue was equilibrated for 30 minutes after a 90 minute recovery period in normal Krebs. The contraction to phenylephrine in normal Krebs consisted of an initial phasic contraction and a more slowly developing tonic contraction. The contraction in Ca^{2+} free Krebs was phasic (46±3% maximum response), returning to baseline in 5 minutes. To see if the influx of extracellular Ca^{2+} was stimulated by emptying of the intracellular Ca^{2+} stores, after the contraction in calcium free Krebs to phenylephrine the agonist was washed out for another 120 minutes in Ca^{2+} free Krebs so that the concentration of any second messengers generated by α_1-adrenoceptor stimulation were no longer raised. The intracellular Ca^{2+} stores should remain empty however as there had been no influx of Ca^{2+} to refill them. EGTA was removed from the Krebs for the last 30 minutes. Ca^{2+} (2.5mM) was then added to the Krebs which produced a slowly developing tonic contraction in each tissue, reaching 97±4% maximum response.

Cyclopiazonic acid (CPA), which releases Ca^{2+} from intracellular stores by inhibiting the endoplasmic reticulum Ca^{2+}-ATPase (Demaurex et al., 1992) was added to some tissues in normal Krebs. This produced a slowly developing tonic contraction (maximal effect of 202±8% at 10^{-5}M after 35 minutes). The effect of CPA (10^{-5}M) was then measured in Ca^{2+} free Krebs solution. This time the response was much smaller and not maintained, 30±4% maximum response, returning to baseline after 20 minutes. However if Ca^{2+} (2.5mM) was then added 1 hour later a tonic contraction was produced (263±12% maximum response). To see if the contractions to phenylephrine and CPA in Ca^{2+} free Krebs were due to release of intracellular Ca^{2+} from the same store a contraction to CPA (10^{-5}M) in Ca^{2+} free Krebs was followed 30 minutes later by a contraction to phenylephrine ($3x10^{-4}$M). The phenylephrine contraction was reduced to 9±2% maximum response. The effects of nifedipine (10^{-6}M) on the contractions induced by cumulative additions of phenylephrine (10^{-7}M-10^{-3}M) and to CPA (10^{-5}M) were then measured. It had no significant effect on either response.

The results are consistent with the response to phenylephrine in the rat spleen consisting of an initial phasic contraction due to release of intracellular Ca^{2+} and a tonic contraction due to influx of extracellular Ca^{2+} stimulated by the emptying of intracellular Ca^{2+} stores. CPA produces a tonic contraction by emptying the same intracellular Ca^{2+} stores causing influx of extracellular Ca^{2+} which for both phenylephrine and CPA is through a nifedipine insensitive pathway.

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39P TEMPERATURE MODULATES THE KINETICS OF INOSITOL 1,4,5-TRISPHOSPHATE-STIMULATED Ca^{2+} MOBILIZATION

Sandip Patel and Colin W. Taylor, Department of Pharmacology, Tennis Court Road, Cambridge CB2 1QJ.

Submaximal concentrations of inositol 1,4,5-trisphosphate ($InsP_3$) have previously been reported to rapidly mobilise only a fraction of the intracellular Ca^{2+} stores released by a maximal concentration of $InsP_3$. Since prolonged incubation with submaximal $InsP_3$ concentrations evokes no greater unidirectional Ca^{2+} efflux than that evoked within the first seconds of $InsP_3$ addition, the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilisation is independent of the time of exposure to $InsP_3$. These quantal responses to $InsP_3$ have been reported to be temperature-dependent: at 11°C, rather than 37°C, submaximal concentrations of $InsP_3$ evoked complete emptying of the $InsP_3$-sensitive stores albeit more slowly than maximal concentrations (Kindman and Meyer, 1993). However, since the sensitivity of intracellular stores to $InsP_3$ is itself temperature-sensitive (Nunn and Taylor, 1990), concentrations of $InsP_3$ that are submaximal at 37°C may become maximal at lower temperatures and thereby artefactually suggest that the quantal mechanism is abolished at low temperature. In the present study, we have examined the effects of temperature on the pattern of $InsP_3$-induced Ca^{2+} mobilization in saponin-permeabilized rat hepatocytes.

The intracellular stores of permeabilized hepatocytes were loaded to steady-state with $^{45}Ca^{2+}$ in cytosol-like medium with the free [Ca^{2+}] buffered at 200nM. The cells were then diluted 5-fold into a similar medium containing additional EGTA (10mM) and thapsigargin (1.25μM) at either 37°C or 2°C. This treatment allowed the incubation temperature to be rapidly changed simultaneous with the inhibition of further $^{45}Ca^{2+}$ uptake and reduction of the medium free [Ca^{2+}] to less than 5nM. After 30s, $InsP_3$ was added and at intervals thereafter the $^{45}Ca^{2+}$ contents of the intracellular stores were determined by rapid filtration. After a 30s incubation with $InsP_3$, the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization was 281± 37nM (n=4) at 37°C, and 68±3 nM (n=5) at 2°C, confirming that the intracellular stores are more sensitive to $InsP_3$ at the lower temperature.

At 37°C, unidirectional efflux of $^{45}Ca^{2+}$ from the preloaded stores occurred with a half-time ($t_{1/2}$) of 90 ± 12 s (n=3). The EC_{50}s for $InsP_3$-stimulated Ca^{2+} mobilization were similar after 30s (281± 37nM, n=4) and 150s (310± 48nM, n=4) exposures to $InsP_3$. These results indicate that $InsP_3$-stimulated Ca^{2+} mobilization is quantal at 37°C since submaximal concentrations of $InsP_3$ are equally effective after brief and sustained incubations. At 2°C, unidirectional efflux of $^{45}Ca^{2+}$ ($t_{1/2}$ = 372 ± 54 s, n=3) was slower than at 37°C. At this temperature, the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization progressively decreased as the incubations were prolonged: after 30s, the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization was 68±3 nM (n=5) and after 420s, the EC_{50} had decreased to 29±2 nM (n=5). These results indicate that at 2°C, submaximal concentrations of $InsP_3$ are capable of fully emptying the $InsP_3$-sensitive stores albeit at slower rates than maximal concentrations.

We conclude that the quantal pattern of $InsP_3$-stimulated Ca^{2+} mobilization observed at 37°C does not occur at 2°C

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40P HISTAMINE AND BRADYKININ STIMULATIONS OF CALCIUM TRANSIENTS ARE CONTROLLED BY DIFFERENT PROTEIN KINASE C ISOFORMS

C.M.Sena, L.M.Rosario, P.Parker[1], V.Patel[2] and M.R.Boarder[2], Department of Zoology, University of Coimbra, 3049 Coimbra CODEX, Portugal, [1]Imperial Cancer Research Fund, Lincoln's Inn Fields, London, and [2]Department of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, Leicester LE1 9HN, UK.

Both histamine and bradykinin stimulate inositol 1,4,5-trisphosphate (IP_3) levels, cytosolic Ca^{2+} and release of catecholamines from chromaffin cells; we have previously shown that activation of protein kinase C (PKC) attenuates the stimulation of IP_3 by both agonists (Boarder and Challiss, 1992). However nothing is known about the isoforms of PKC involved. Here we provide evidence that histamine and bradykinin receptors on cultured bovine adrenal chromaffin cells are controlled by different isoforms of PKC.

Chromaffin cells were prepared from fresh bovine adrenal glands and cultured as described earlier (Duarte et al, 1993). For the PKC isoform studies cells were cultured in a complete medium in 10 cm tissue culture dishes for 3-5 days; they were maintained serum free for 24h prior to use. For the Ca^{2+} studies cells were cutured in a complete medium in suspension for 2 days before use. PKC analysis was by Western blotting of extracts using antisera raised by P.Parker and specific for the isoforms of PKC, followed by scanning of autoradiographs. Cytosolic Ca^{2+} was monitored using Fura 2 loaded cells in suspension.

Western blotting of Triton (0.1%) soluble chromaffin cell extracts revealed the presence of α, ε and ζ isoforms of PKC. Treatment with tetradecanoyl phorbol acetate (TPA: 1μM) for 6 - 48h showed that PKCε was maximally down regulated by 12h (22.6 ± 4.2% of control), PKCα did not down regulate at 12h (101.9 ± 11.1% of control) but was substantially downregulated by 48h (28.1 ± 7.1% of control), while PKCζ did not down regulate at any time. Other studies showed that TPA stimulated translocation from cytosol to membrane was more rapid for PKCε than for PKCα. Measuring cytosolic Ca^{2+}, 10 min pretreatment with TPA abolished the histamine (100μM) stimulated Ca^{2+} transient and attenuated the bradykinin (1μM) response (6.37 ± 0.3% and 51.3 ± 1.9% of control respectively). However, 12h TPA followed by a 1h wash and further 10 min incubation with TPA resulted in a complete restoration of the bradykinin response but continuing loss of the histamine response (91.8 ± 2.3 % and 2.45 ± 0.7% respectively of that with no exposure to TPA).

These experiments show that under conditions of depletion of PKCε, but not of PKCα or PKCζ, TPA loses its ability to regulate bradykinin responses, but retains its ability to regulate responses to histamine. The results suggests that bradykinin responses are regulated by PKCε and that histamine responses are regulated by PKCα. This is the first report of receptor specific regulation by different PKC isoforms.

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A. Ahluwalia[1], S.Giuliani[2], & C.A.Maggi[2]. [1]Dept of Biochemical Pharmacology, The Med. College of St Bartholomew's Hospital, Charterhouse Sq, London EC1M 6BQ, [2]Dept of Pharmacology, A. Menarini Pharmaceuticals, Via Sette Santi, 3, Florence, Italy.

Substance P (SP) produces its effects via interaction with the tachykinin NK_1 receptor although it will also interact with other tachykinin receptors. There now exist several selective NK_1 agents such as the agonists, septide and Sar^9SP sulphone and antagonists such as RP 67, 580. Studies with these selective agents have however uncovered the possible existence of distinct subtype/binding sites of the NK_1 receptor. This site has been termed the 'septide-sensitive' receptor/binding site due to the apparent preferential binding of septide. We have now investigated whether there is any role for this site in tachykinin-induced rat skin inflammation.

Male Wistar rats (250-300 g) were anaesthetised using sodium pentobarbitone (45 mg/kg) and their backs shaved. Evans blue (10 mg/kg) was injected i.v. and 5 min later 6 different tachykinin solutions were injected i.d. into the dorsal skin in duplicate. After 30 min blood samples were taken via intracardiac puncture, the skin removed and the skin sites punched out using a 17 mm diameter hole punch. Samples were placed in 4 ml formamide for 24 h after which the absorbence was read at a wavelength of 620 nm. The Evans blue content in 1 μl of plasma was also determined and this value used to express oedema in the skin as μl of plasma.

Construction of dose-response curves to the natural tachykinins (0.001-1.0 nmol) showed equivalent potency for SP, NKA and NKB. Both septide and Sar^9SP sulphone were approximately 10-fold more potent than SP. Single doses of each agonist which gave responses equivalent to the SP EC_{50}=0.2 nmol were chosen for further studies. Neither the selective NK_2 or NK_3 agonists [βAla^8]NKA(4-10) and senktide respectively, (0.1-1nmol), had any significant oedema-inducing effects (n=6 for each dose). Likewise the NK_2 receptor antagonist, SR 48,968 (0.3 μmol/kg, i.v 5 min pretreatment), had no effect upon responses to the natural tachykinins, septide or Sar^9SP sulphone (n=4). The selective NK_1 receptor blocker, RP 67,580 (1 μmol/kg, i.v. 5 min pretreatment) significantly inhibited the response to only septide (0.002 nmol) giving 43.5 ± 19.5 % (n=6, $p<0.05$) of the control response (n=7). RP 67,580 (3 μmol/kg) significantly attenuated the responses to SP (0.2 nmol) to 44.7 ± 11.5 % Sar^9SP sulphone (0.002 nmol) to 45.9 ± 15.4 % and septide (0.002 nmol) to 29.2 ± 12.3 (n=5 in each case). The NO synthase inhibitor, L-NAME (0.1 μmol/site, co-injected with tachykinin), attenuated the response to septide (0.1 nmol) only giving 44.4 ± 6.8 % (n=6) of control while SP was unaffected (n=11) Compound, 48/80 (5 mg/kg for 3 consecutive days), a mast cell degranulator, significantly inhibited the response to only Sar^9SP sulphone (0.002 nmol), whereas equiactive doses of all other tachykinins tested were unaffected (n=6 in each case).

These results suggest different mechanisms of action for SP- and septide-induced oedema in rat skin. The response to septide is both NO-dependent and mast cell independent, whereas in contrast the SP response is only partially blocked by RP 67,580 and is NO-independent supporting the existence of a distinct 'septide-sensitive' receptor/binding site.

42P EFFECTS OF SR 142801, A POTENT NON-PEPTIDE TACHYKININ NK_3 RECEPTOR ANTAGONIST, ON CARDIOVASCULAR RESPONSES INDUCED BY SENKTIDE IN CONSCIOUS GUINEA-PIGS

D. Nisato, A. Roccon, D. Marchionni, X. Emonds-Alt, J.C. Brelière & G. Le Fur. Sanofi Recherche, 371, rue du Professeur Blayac, 34184 Montpellier cedex 04, France.

Several studies have documented a functional role for tachykinins in the peripheral control of the cardiovascular system (Couture *et al.*, 1989; Pompei *et al*, 1992). However, after injection of senktide (SK), a synthetic specific NK_3 receptor agonist, hypotension and no effect were described in anaesthetized and conscious rats, respectively. The purpose of the present study was twofold: firstly, to determine the effects of SK on mean arterial pressure (MAP) and heart rate (HR) after intravenous (i.v.) injection in conscious guinea-pigs, and secondly, to investigate the blockade of these SK effects using a new potent non-peptide NK_3 receptor antagonist, SR 142801 ((S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-N-methylacetamide; K_i = 0.11 ± 0.01 nM; [^{125}I]-iodohistidyl-[MePhe^7]NKB, guinea-pig brain cortex) (Emonds-Alt *et al.*, 1994) and its less active (R)-enantiomer, SR 142806 (K_i = 26 ± 3 nM) after i.v. or oral (p.o.) administration.

Male Dunkin-Hartley guinea pigs (250-350g) were anaesthetized (i.m.) with ketamine (80 mg/kg) and xylazin (10 mg/kg); the left carotid artery and the right jugular vein were cannulated for measurement of MAP and injection of drugs, respectively. Experiments were conducted at least 48h after surgery.

Cumulative injections of SK (1-1000 pmol/kg, n=7) elicited dose-dependent increases in MAP (28 ± 2 mmHg at the highest dose) accompanied by decreases in HR. Repeated injections at 15-min intervals of the highest doses of SK did not show any tachyphylaxis, and comparable responses were observed after 24 h.

The kinetics of the effects of SR 142801 and 142806 on the SK responses was studied by injecting a 300 pmol/kg dose of SK 15 min before and at 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 h after i.v. administration, and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 24 h after oral administration.

			Senktide responses				
			before treatment		after treatment (1)		
Route	Compound	Dose (mg/kg)	Δ MAP (mmHg)	Δ HR (beats/min)	ΔMAP (mmHg)	ΔHR (beats/min)	Duration (2) (h)
I.V.	Vehicle	-	23 ± 2	-66 ± 8	26 ± 1	-87 ± 19	-
	SR 142801	0.3	27 ± 1	-35 ± 16	23 ± 2	-23 ± 11	-
		1	24 ± 2	-43 ± 5	10 ± 3*	-5 ± 3*	0.5
		3	26 ± 3	-48 ± 9	6 ± 2*	-3 ± 3*	1
	SR 142806	3	24 ± 2	-52 ± 8	29 ± 2	-40 ± 5	-
P.O.	Vehicle	-	25 ± 2	-51 ± 9	29 ± 2	-35 ± 8	-
	SR 142801	3	24 ± 1	-41 ± 14	18 ± 1*	-15 ± 10*	3
		10	24 ± 2	-48 ± 9	12 ± 2*	-3 ± 3*	> 7; < 24
		30	25 ± 1	-42 ± 14	6 ± 1*	0*	> 7; < 24

Table 1: Values are mean ± s.e.mean n = 6 per group.
*, P < 0.05 versus corresponding vehicle-treated group (Dunnett's test following 2-way ANOVA)
(1), maximal effects
(2), times at which a significant difference of MAP response versus corresponding vehicle-treated group was observed.
Resting values ranged from 55 ± 2 to 68 ± 2 mmHg for MAP and from 347 ± 8 to 377 ± 10 beats/min for HR.

SR 142801 did not modify resting MAP; a minor bradycardia was observed only after i.v. administration at the highest dose. SR 142801 induced a dose-dependent inhibition of the cardiovascular responses to SK and displayed a long-lasting effect following oral administration (>7h). Maximal effects occurred 15 and 30 min after i.v. and oral administrations, respectively. This response was affected neither by SR 142806, the less active enantiomer, nor the vehicle.

In conclusion, this study showed, for the first time, a clear pressor effect of SK in conscious guinea pigs which was blunted by SR 142801, the first potent orally active non-peptide NK_3 receptor antagonist.

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43P OLVANIL INHIBITS SENSORY NEUROTRANSMISSION IN THE RABBIT IRIS SPHINCTER MUSCLE

A. D. Medhurst, K. A. Holland & S. J. Smith (introduced by A. A. Parsons), Department of Neurology Research, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AD.

Capsaicin contracts the rabbit iris sphincter muscle via release of tachykinins from sensory C-fibres and inhibits peptidergic contractile responses to electrical field stimulation (Ueda *et al.*, 1984). We have used the rabbit iris sphincter muscle to investigate the effects of olvanil, a capsaicin analogue, with antinociceptive activity (Dickenson *et al.*, 1990).

Strips of iris sphincter muscle were prepared for the measurement of isometric tension (resting tension 0.4g) in tissue baths containing Krebs-Henseleit solution (at 37°C), aerated with 95% O_2 and 5% CO_2. In some experiments carbachol (10μM) was added as a reference contractile agent. After incubation with 1μM atropine, (to abolish cholinergic contractile responses), three electrical stimulations were performed (S1, S2, S3) for 30s every 45min (2Hz; 0.3ms, 20V). Olvanil and capsaicin were added after S2 and remained throughout S3. Olvanil-induced responses were also investigated after 30min incubation with spantide (5μM) or capsazepine (30μM). The effect of 30μM olvanil on neurokinin A-induced concentration-effect curves was investigated in other tissues. All results are mean ± s.e.mean.

Olvanil (3, 30 and 300μM) induced slow onset contractile responses which were 41 ± 9, 61 ± 16 and 172 ± 70 % (n = 4-8) of contraction elicited by S2. Contractile responses to 1 and 3μM capsaicin were extremely rapid compared to olvanil and were 26 ± 4 and 234 ± 37% (n = 4) of contraction elicited by S2. Both olvanil- and capsaicin-induced contractile responses had returned to baseline before S3 was elicited. Olvanil and capsaicin also inhibited neurogenic contractile responses, with capsaicin being the more potent compound (Table 1).

OLVANIL μM	% inhibition	CAPSAICIN μM	% inhibition
3	27 ± 6	0.3	10 ± 3
30	47 ± 12	1	20 ± 6
300	90 ± 5	3	90 ± 5

Table 1. % inhibition of neurogenic contraction calculated from S3/S2 ratio (n = 4-8)

In separate experiments, olvanil (3 and 30μM) induced contractile responses which were 19 ± 3 and 30 ± 6 % of carbachol-induced contraction (n = 6 and 8). After treatment with spantide (5μM) responses to 3 and 30μM olvanil were reduced to 3 ± 1 and 13 ± 4 % of carbachol-induced contraction (n = 6 and 4), and after capsazepine (30μM) to 0 and 5 ± 3 % (n = 4) of the contraction induced by carbachol.

Olvanil (30μM) had no effect on concentration-effect curves to neurokinin A (n = 4).

We conclude that olvanil elicits contractile responses via release of tachykinins and inhibits electrically-induced contractions possibly due to depletion of neuronal tachykinin stores.

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44P BIBP 3226: A SELECTIVE AND HIGHLY POTENT NPY-Y_1 ANTAGONIST

H.N.Doods, K.-D. Willim, H.A. Wieland, M. Entzeroth, W. Wienen, W. Eberlein, W. Engel & K. Rudolf, Preclinical Research, Dr. Karl Thomae GmbH, P.O.Box 1755, 88397 Biberach.

Neuropeptide Y receptors can be classified into at least two subtypes namely Y_1 and Y_2 receptors. The pharmacological classification of these two receptor subtypes is primarily based on the affinity and relative activity of selective agonists such as [$Leu^{31}Pro^{34}$] NPY (Y_1-selective) and PYY(3-36) (Y_2-selective). Here we describe the pharmacological profile of BIBP 3226, (N^2-(diphenylacetyl)-N-[(4-hydroxyphenyl) methyl]D-arginin-amide), the first highly potent non-peptide Y_1 selective antagonist.

Since in the rat cortex Y_1 receptors predominate and in the rat hippocampus Y_2 receptors, we used membranes of these two tissues to perform receptor binding studies in order to determine the subtype selectivity of BIBP 3226. Binding studies were performed using a modified Tris/NaCl incubation buffer. [^{125}I]NPY (30 pM) was incubated with membrane homogenate and BIBP 3226 (10^{-10} - 10^{-4}) for 2 hours at room temperature. The incubation was stopped by centrifugation and the non-specific binding was determined in presence of 100 nM NPY. The in vitro antagonistic properties of the compound were investigated in the electrically stimulated isolated rat (Y_2 model) and rabbit vas deferens (Y_1 model) according to Doods and Krause (1991). Moreover, it was tested whether BIBP 3226 was able to antagonize the NPY (10 μg/kg/i.v.) induced increase in blood pressure in pithed rats.

BIBP 3226 inhibited dose dependently specifically bound [^{125}I]NPY from Y_1 sites in the rat cortex. The K_i value amounted to 7.7 ± 0.9 nM (mean ± sem, n=4). On the contrary, concentrations up to 10 μM were not able to displace [^{125}I]NPY from Y_2 sites in the rat hippocampus. Accordingly, BIBP 3226 possesses a Y_1 selectivity of at least 1000-fold. NPY inhibited the twitch response due to electrical stimulation of the rat and rabbit vas deferens in a concentration dependent manner with EC_{50} values of 12 nM and 1.5 nM, respectively. The addition of BIBP 3226 (1 μM) produced a rightward shift of the concentration-response curve to NPY in the rabbit vas deferens (prejunctional Y_1 receptors). A mean pK_b value of 7.31 ± 0.10 (n=4) was calculated. BIBP 3226 (10 μM) had no effect on Y_2 receptors in the isolated rat vas deferens preparation. NPY (10 μg/kg) given i.v. to pithed rats elicited an increase in blood pressure of approx. 55 mmHg. Pretreatment with BIBP 3226, administered i.v. 1 min before NPY was injected, caused a dose-dependent inhibition of the NPY-induced pressor response. The ID_{50} value was 0.11 ± 0.03 mg/kg, i.v. (n=8).

The present results show that BIBP 3226 is a potent and selective Y_1 receptor ligand. The functional studies demonstrate that BIBP 3226 is devoid of agonistic properties and is a competitive antagonist. The fact that BIBP 3226 attenuates the in vivo effects of NPY on blood pressure makes it a useful tool to investigate the role of NPY in the cardiovascular system.

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45P CHOLECYSTOKININ-OCTAPEPTIDE MEDIATES CONTRACTION OF RAT ISOLATED PROXIMAL COLON BY ACTIVATING CCK_B RECEPTORS

M. Patel[1]., S. Bowden, and C.F. Spraggs., Departments of Cellular Science[1] and Pharmacology 1, Glaxo Research and Development, Park Road, Ware, Herts., SG12 ODP UK.

Sulphated cholecystokinin-octapeptide (sCCK-8) mediates contraction of gastrointestinal smooth muscle by activation of both CCK_A and CCK_B receptors, where these receptors have been shown to coexist. (Patel and Spraggs, 1992; Boyle, *et al.*, 1993). The aim of the present study was to study the contractile effects of sCCK in the rat proximal colon (RPC) and to characterise the receptor(s) that mediate these effects.

The proximal colon (3-4 cm segment) from female AH/A rats (180 g) was removed and separated from mucosa by blunt dissection. Individual tissues were suspended under a resting tension of 0.6g in modified Krebs solution in 4ml organ baths and gassed with $95\%O_2/5\%CO_2$ at 37°C. Single agonist concentration-response curves (CRC) were constructed in a sequential manner, antagonists were allowed a 30 min equilibration period prior to constructing a CRC to sCCK-8. Data are shown as means of individual tissues.

sCCK-8 (0.3-1000 nM) produced concentration-dependent transient contractions of the PC under resting tension. The contractile effects of sCCK-8 were not inhibited by tetrodotoxin (300 nM). The rank orders of potency for CCK peptides were consistent with activation of CCK_B receptors (sCCK-8>G-17=pentagastrin=CCK-4; Table I).

Table I. Gastrin/CCK peptide potencies on proximal colon.

Agonist	EC_{50} (nM)	E_{max} (g)
sCCK-8	5.3(4.0-6.8)	0.84±0.06
Gastrin	25.3(13.2-48.4)	0.90±0.13
Pentagastrin	19.3(7.0-53.5)	0.71±0.07
CCK-4	19.8((7.0-55.9)	0.89±0.15

Values shown are mean with 95% confidence limits or SEM as appropriate ($n \geq 5$).

The CCK_B receptor-selective antagonist, L-365,260, blocked the contractile responses to sCCK-8 with a pA_2 value of 8.0 and a slope of 0.7 (significantly different from unity; n=14). In contrast, the CCK_A receptor-selective antagonist, devazepide (100 nM) was a less potent antagonist of the contractile responses to sCCK-8 (pK_B =6.4; n=11). These results are consistent with the view that sCCK-8 mediates contraction of the RPC by activation of CCK_B receptors.

In conclusion, sCCK-8 activates a homogeneous population of CCK_B receptors in the rat isolated proximal colon. This is the first description of a homogeneous population of CCK_B receptors in an isolated smooth muscle preparation.

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46P BRONCHOPULMONARY HYPERREACTIVITY IN IMMUNISED AND CHALLENGED "BP2" MICE IS NOT DEPENDENT UPON STRICT EOSINOPHIL RECRUITMENT TO THE AIRWAYS

S. Y. Eum, J. Lefort, S. Hailé[1], M. Huerre[1] & B.B. Vargaftig, Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur-INSERM n° 285 and [1]Unité d'Histopathologie, Paris, France.

Bronchopulmonary hyperreactivity (BHR) and eosinophilic bronchial inflammation are present in asthma. We recently showed that BHR is only induced if the eosinophils are activated in the airways of guinea-pigs made hyper-eosinophilic by antigen provocation (Pretolani et al.,1994). In Swiss, CBA and transgenic mice for IL-5 the recruitment of eosinophils to lungs and BAL is not accompanied by BHR (Lefort et al., submitted). We compared BHR and antigen-induced eosinophil recruitment to lungs and BAL in mice from a selection which produces large amounts of IgE ("Bons producteurs 2", "BP2", for High Line of Selection 2; Biozzi et al., 1979) to those of Balb/C. Airways resistances were evaluated with the Mumed system on urethane-anesthesised mice (1 mg/kg, i.p.) challenged i.v. with 5-HT. Mice were challenged by the intranasal (i.n.) instillation of 50 µg of OA on day 14 after immunisation with ovalbumin (OA; 2 x 100 µg in 1.6 mg of $Al(OH)_3$ s.c., at one week interval). No eosinophils were recruited in immunised mice after i.n. saline nor 6 h after antigen provocation. They were present after 24 and 96 h (21 % and 46% for "BP2" and 17% and 37% for Balb/C, respectively). The PD90 (amount of 5-HT in µg/kg required to increase resistance by 90%), did not change in both strains after the challenge, irrespective of the intensity of eosinophil recruitment. Nevertheless, when mice were challenged twice a day for 2 days with OA, and used the day after the 4th challenge, a marked BHR was detected on "BP2" (PD90 was reduced from 44 ± 4 to 24 ± 2 µg/kg, $p < 0.001$), but not in Balb/C mice, even though the eosinophil counts were the same as after the single challenge. BHR was also only noted when "BP2" mice were challenged once a day for 10 days and used on day 11, when 62 % and 53% of the BAL cells were eosinophils for "BP2" and paired Balb/C, respectively. Using whole body plethysmography (Buxco Electronics LS/20 lab system), BHR was confirmed in awake "BP2" mice exposed to inhaled methacholine (Mch). Histological studies suggest that eosinophils reach the respiratory epithelium after at least 2 OA challenges in "BP2" mice, but remain sequestered in the bronchial submucosa in Balb/C. Our results confirm that the presence of eosinophils alone does not support BHR, which is uncovered by 5-HT i.v. or inhaled Mch. The selective effect on BP2 mice may involve their increased ability to produce high levels of IgE (Biozzi et al., 1979 and Coyle, personal communication) and to trigger eosinophil migration to the epithelium followed by their *in situ* activation upon repeated provocations.

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M.M. Teixeira, T.J. Williams, P.G. Hellewell and [1]A.G. Rossi, Department Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY.

Eosinophils are thought to play an important functional role in the control of parasitic infection and in allergic diseases. Aggregation of eosinophils has been observed both *in vitro* (Koenderman *et al*, 1991) and *in vivo* (Meurer *et al*, 1993). In this study, we have investigated the mechanisms by which guinea pig eosinophils aggregate as determined by changes in light transmission.

Horse serum-elicited eosinophils were purified (> 96% purity) on a discontinuous Percoll gradient and resuspended at a final concentration of 10^7 eosinophils ml^{-1}. Aliquots (300 μl) were dispensed into a siliconized cuvette in a dual channel platelet aggregometer (Chronolog 440 VS), incubated for 5 min at 37°C with continuous stirring at 700 r.p.m. and then stimulated with various agonists. The reference cuvette contained buffer alone. Drug pretreatment was added 3 min prior to stimulation of the eosinophils. Responses were allowed to develop for at least 5 min and the results expressed as the percentage maximal aggregation induced by phorbol myristate acetate (PMA) 10^{-6} M. In some experiments glutaraldehyde (1.5% final concentration) was added to the cell suspension, an aliquot (20 μl) taken, added to Kimura's stain and visualized under a light microscope.

Eosinophils stimulated by platelet-activating factor (PAF, 10^{-10} to 10^{-6} M), leukotriene B_4 (LTB_4, 10^{-10} to 10^{-6} M) and human recombinant C5a (C5a, 10^{-9} to 10^{-6} M) underwent a rapid and partially reversible concentration-dependent aggregation as assessed by changes in light transmission (n= 4-5). PMA (10^{-10} to 10^{-6} M) also induced concentration-dependent aggregation which was slower in onset, took longer to reach maximum and was irreversible. Whereas PMA induced maximal aggregation (100%, EC_{50} 5 x 10^{-9} M), PAF, LTB_4 and C5a induced 35%, 44% and 50% of maximal aggregation, respectively. Light microscopy confirmed that changes in light transmission represented true aggregation. When PAF and LTB_4 were used as stimuli, there was homologous but little heterologous desensitization (n= 4). In the presence of the calcium chelator EGTA (10^{-3} M), aggregation induced by PAF (10^{-7} M) and PMA (10^{-6} M) was inhibited by 92% and 94%, respectively (n=4). In the absence of Mg^{2+}, aggregation induced by PAF and PMA was inhibited by 62% and 63%, respectively (n= 3). The $F(ab')_2$ fragment of the anti-CD18 monoclonal antibody 6.5E (150 $\mu g\ ml^{-1}$) significantly inhibited eosinophil aggregation induced by 10^{-7} M PAF, LTB_4, C5a and 10^{-6} M PMA by 52%, 56%, 68% and 69%, respectively (n= 4).

In summary, guinea pig eosinophils exposed to different agonists undergo homotypic aggregation dependent on the concentration, Ca^{2+}, Mg^{2+} and CD18. We suggest that eosinophil aggregation is a reliable marker of eosinophil activation and should prove a useful technique for mechanistic and pharmacological investigation of eosinophil responsiveness.

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[1]- present address: Syntex Research Centre, Edinburgh, Scotland.

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48P THE VASOACTIVE INTESTINAL PEPTIDE RECEPTOR IS FUNCTIONALLY COUPLED TO G_{i3} IN RAT LUNG MEMBRANES

S.M. Shreeve & N.L. Diehl, Department of Pharmacology, Vermont Center for Vascular Research, College of Medicine, Given Building, University of Vermont, Burlington, Vermont 05405, U.S.A.

Hormones, neurotransmitters and drugs can activate multiple receptor subtypes, G-proteins and effectors to generate an array of second messengers which are integrated by the cell to produce a physiological response. Vasoactive intestinal peptide (VIP) is a physiological agonist in the lung which elicits smooth muscle relaxation in the airways and blood vessels and also may modulate inflammatory cell function. The goal of this study was to test the hypothesis that VIP can activate multiple G-proteins in membranes derived from rat lung tissue.

Using a novel approach we have demonstrated directly that the VIP-receptor (M_r = 54,000) is coupled to G_s (Kermode *et al.*, 1992). The functionally interacting, physically associated VIP/receptor/G_s complex in rat (Sprague-Dawley) lung membranes was cross-linked by incubating with 5 mM ethylene glycol bis(succinimidyl succinate) (EGS) for 30 min at room temperature. Two high molecular weight protein bands were generated which were identified using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography and immunoblotting. The M_r = 114,000 band was found to be composed of VIP, its receptor and $G_s\alpha$ while the M_r = 184,000 complex comprised VIP, its receptor and $G_s\alpha\beta(\gamma?)$.

In the present study, lung membranes have been prelabelled with VIP, exposed to EGS and analyzed by SDS-PAGE and immunoblotting as before except that we have probed with an antiserum specific for $G_{i3}\alpha$ (Calbiochem). This antiserum detected the M_r = 184,000 band which co-migrated with a [^{125}I]VIP cross-linked protein visualized by autoradiography. Increasing the concentration of VIP enhanced the formation of the M_r = 184,000 VIP/receptor/G_{i3} immunoreactive complex in a dose dependent manner. From densitometric scans a dose response curve was constructed and the EC_{50} for VIP was calculated to be 45±7 pM (n=3). Formation of the M_r = 184,000 complex was inhibited 30±2 % by 1 mM GTP and 90±5 % by 1 mM GTPγS (n=4, $P<0.05$). Pretreatment of the membranes with pertussis holotoxin (Gibco) was found to reduce high affinity [^{125}I]VIP labelling to its receptor by 53±3 % (n=3, $P<0.01$) compared to control (pertussis toxin B-oligomer).

These data demonstrate that the VIP/receptor/G_{i3} complex in native membranes is functional prior to cross-linking. We conclude that VIP binding to its receptor(s?) activates G_s and G_{i3} in the rat lung.

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49P IDENTIFICATION AND CHARACTERIZATION OF SOMATOSTATIN RECEPTORS EXPRESSED IN RAT LUNG TISSUE

J. Schloos, F. Raulf, D. Hoyer, G. Engel, and Ch. Bruns
Sandoz Pharma Ltd., Preclinical Research, CH-4002 Basel, Switzerland

The hormone and neuropeptide somatostatin (SRIF) exerts its biological effects after binding to specific, high affinity receptors expressed in various tissues, including brain, pituitary, pancreas and the gastrointestinal tract. Until now, five different SRIF receptor subtypes (SSTR1-5) have been identified and characterized. Recently, the existence of mRNA of the SRIF receptor subtypes SSTR1, SSTR3, and SSTR4 in lung tissue has been demonstrated (for review see: Bruns et al., 1994).

The aim of the present study was to investigate the expression of SRIF receptor protein in rat lung tissue. For this purpose we performed radioligand binding studies as well as receptor autoradiography to identify and characterize the SRIF receptors with respect to the different subtypes in rat lung.

Radioligand binding studies using [^{125}I]-Leu^{8},D-Trp^{22},Tyr^{25} SRIF-28 ([^{125}I]-LTT-SRIF-28) as specific ligand demonstrated high affinity binding to membranes of rat lung which was time-dependent, saturable, and reversible. The time course of binding at room temperature is described by an association rate constant (k_1) of 3.7 x 10^{10} M^{-1} h^{-1} and a dissociation rate constant (k_{-1}) of 0.44 h^{-1} in the mean. Saturation binding experiments reveal an equilibrium dissociation constant (K_D) of 47 ± 6 pM and a capacity (B_{max}) of 83 ± 5 fmol/mg protein (mean ± s.e.mean, n=3). Different short chain SRIF analogues inhibited the binding of [^{125}I]-LTT-SRIF-28 in a concentration-dependent manner demonstrating a rank order of potency of SRIF-28 > SRIF-14 > CGP 23996 >> RC 160 > BIM 23014 > SMS 201-995 ≥ BIM 23056 > MK 678. The binding affinities of these compounds were in close correlation to those determined using Chinese hamster ovary (CHO) cells stably expressing the SRIF receptor subtype SSTR1 (r = 0.92) and SSTR4 (r = 0.95), respectively. In accordance with these binding results a predominant occurence of mRNA for the SRIF receptor subtype SSTR4 as well as a very weak signal for the subtype SSTR1 were obtained by analysis of reverse transcriptase-polymerase chain reaction (RT-PCR). The evaluation of the distribution pattern of SRIF binding sites by receptor autoradiography showed a homogenous distribution of high affinity SRIF receptors in rat lung tissue.

In conclusion, the present study establishes the predominant expression of the SRIF receptor subtype SSTR4 in rat lung tissue. These results support the hypothesis for a role of SRIF in modulating lung cell function. SRIF has been reported to modulate cholinergic neurotransmission in lung (Sekizawa et al., 1989) and to inhibit secretion processes at birth (Perks et al., 1992). Whether SSTR4-selective agonists or antagonists could be useful for the treatment of airway diseases remains to be elucidated.

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Sekizawa, K. et al. (1989) *J. Appl. Physiol.* 67, 2397-2400

50P THE LEUKOTRIENE B_4 ANTAGOINST, U-75,302, INHIBITS ANTIGEN-INDUCED AIRWAY HYPERRESPONSIVENESS BUT NOT LEUCOCYTE ACCUMULATION IN SENSITIZED GUINEA-PIGS

A.A.Y. Milne* & P.J. Piper. Department of Pharmacology, The Royal College of Surgeons, Lincoln's Inn Fields, London. WC2A 3PN.

It is widely held that increased responsiveness of the airways in asthma is secondary to pulmonary inflammation (British Thoracic Society, 1993). However, this hypothesis has recently been contested (Chapman *et al.*, 1993). The present study aimed to investigate the effect of a leukotriene B_4 (LTB_4) antagonist, U-75,302, in a guinea-pig model of airway inflammation. Previous studies have shown that U-75,302 reduced eosinophil accumulation in guinea-pigs in response to antigen (Richards *et al.*, 1991), but effects on bronchial hyperresponsiveness were not investigated. The present study sought to examine the effects of U-75,302 on both airway hyperresponsiveness and eosinophilia following exposure of sensitized guinea-pigs to allergen.

Guinea-pigs were sensitized to ovalbumin (OA) over two weeks and challenged with an aerosol of OA on day 14. 24h later, animals were anaesthetized (midazolam 7.5 $mgkg^{-1}$, fentanyl 0.63 $mgkg^{-1}$, fluanisone 20 $mgkg^{-1}$) and airway resistance in response to acetylcholine (Ach, 10 μgkg^{-1} i.v.) was measured using a computerized pulmonary monitoring system (Mumed, London). Lungs were lavaged (4x10ml buffer) and total and differential counts made of leucocytes in bronchoalveolar lavage fluid (BALF). Eosinophil peroxidase (EPO) in BALF was assayed using a colorimetric assay (Strath *et al.*, 1985). U-75,302 or vehicle (5% ethanol, 1% Tween 80) was given (10 $mgkg^{-1}$, n=5) by gavage, 1h prior to and 4h following OA exposure. Sensitization and exposure to aerosolized OA induced bronchial hyperresponsiveness to Ach and an increase in total leucocytes, eosinophils and EPO in BALF 24h later (Milne & Piper, 1994). Treatment with U-75,302 resulted in a decreased hyperresponsiveness to Ach relative to vehicle treated animals (U-75,302, 204± 42; vehicle 472± 88 cmH_2O $l^{-1}s^{-1}$; mean±s.e.mean, $p<0.05$) without altering numbers of total leucocytes (U-75,302, 36.4± 5.3; vehicle, 33.8± 6.7 x10^6 cells), eosinophils (U-75,302, 12.9± 2.2; vehicle, 9.0± 2.6 x10^6 cells) or levels of EPO (U-75,302, 0.12 ± 0.02; vehicle, 0.09±0.03 units ml^{-1}) in BALF. These results, in contrast to those of Richards *et al.*, 1991, provide evidence that LTB_4 has a role in the development of bronchial hyperresponsiveness but not leucocyte accumulation in this model. Inhibition of bronchial hyperresponsiveness without attendant effects on inflammatory cell recruitment or activation suggests that these two events are not causally linked.

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*Present address: Syntex Research Centre, Edinburgh, EH14 4AP.

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51P LOW CONCENTRATIONS OF BQ123 POTENTIATE THE PULMONARY VASOCONSTRICTOR AND BRONCHOCONSTRICTOR ACTIONS OF ENDOTHELIN-1 IN THE RAT ISOLATED PERFUSED LUNG

H.Lal, B. Woodward & K.I. Williams, School of Pharmacy & Pharmacology, University of Bath, BA2 7AY

We have studied the effects of the selective ET_A receptor antagonist BQ123 (Ihara et al., 1992) on pulmonary vascular and bronchial actions of endothelin-1 (ET-1) and sarafotoxin 6c (SX6C) in a ventilated rat perfused lung model, which allows simultaneous measurement of pulmonary perfusion pressure (PPP), pulmonary inflation pressure (PIP) and lung weight (Lal et al., 1994a).

ET-1 produced dose-dependent increases in PPP (ED_{50} 130 ± 2 pmol; mean ± sem, n=10) with a maximum pressure increase at 800 pmol of 10.4 ± 1.6 mmHg (n=4). In the presence of 1 μM BQ123 PPP responses to low doses of ET-1 (50-100 pmol) were significantly inhibited ($p<0.05$) where as the response to the highest dose of ET-1 (800 pmol) was significantly augmented to 21 ± 3.6 mmHg, n=5 ($p<0.05$) when compared with the control. The slope of the dose-response curve (DRC) was also significantly steeper than the control ($p< 0.001$). In the presence of 2 μM BQ123 the DRC to ET-1 showed a rightward parallel shift with a significant increase in ED_{50} to 311 ± 3 pmol, n=4 ($p< 0.01$) compared with control.

We have previously speculated that changes in lung weight caused by ETs are probably associated with increased hydrostatic pressure within the pulmonary microvasculature (Lal et al., 1994b). However in the present experiments where 1 μM BQ123 significantly potentiated the PPP response to 800 pmol ET-1, it caused a significantly smaller increase in lung weight compared with control (increase in lung weight with 800 pmol decreased from 8.1 ± 0.1 g to 4.5 ± 0.8 g, n=4, $p < 0.001$). 2 μM BQ123 further attenuated the ET-1 (800 pmol) induced increase in lung weight (0.8 ± 0.2 g, n=4, $p <0.001$).

There was also disparity between the effects of the different concentrations of BQ123 on the PIP responses to ET-1. Surprisingly the control ED_{50} (216 ± 2 pmol, n=10) for ET-1 on PIP was reduced to 78 ± 12.3 pmol, n=5 $p< 0.01$) in the presence of 1 μM BQ123. In addition the DRC to ET-1 in the presence of 1 μM BQ123 was significantly steeper ($p< 0.001$). Furthermore in the presence of a higher concentration of BQ123 (2 μM) the ED_{50} value for ET-1 (107 ± 17 pmol, n=4) was still significantly lower than the control value ($p< 0.05$).

Neither concentration of BQ123 had any effect upon the pulmonary actions of the selective ET_B receptor agonist SX6C. The control ED_{50} values of SX6C on PPP (165 ± 2 pmol, n=6)and PIP (81 ± 6 pmol, n=6) were not altered in the presence of BQ123 (2 μM). Interestingly the slopes of the control DRCs for SX6C on PPP and PIP were similar to those for ET-1 in the presence of 1 μM BQ123. Indomethacin (10 μM) did not affect the actions of ET-1 or SX6C in this lung model.

These results suggest that low concentrations of BQ123 potentiate both the pulmonary vasoconstrictor and bronchoconstrictor effects of ET-1. The mechanisms underlying these changes merit further study.

H.Lal is an Indian government Scholar.

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52P INFLAMMATORY CELL INFILTRATION ASSOCIATED WITH AN EARLY AND LATE PHASE BRONCHOCONSTRICTION AFTER ALLERGEN CHALLENGE IN CONSCIOUS SENSITIZED GUINEA-PIGS

Christine A. Lewis & K. J. Broadley, Department of Pharmacology, Welsh School of Pharmacy, University of Wales, Cardiff CF1 3XF

Human bronchial asthma is a multicellular-dependent process involving chronic inflammatory changes in the lungs. The immediate bronchoconstriction is followed by a late-phase increase in airflow obstruction (Durham *et al*, 1984). Few studies have shown both phases in animal models, but Hutson *et al* (1988) obtained both phases of equal size in conscious guinea-pigs. This study determines a time-course for bronchoconstriction in conscious sensitized guinea-pigs after allergen challenge and leukocyte infiltration.

Guinea-pigs were sensitized with 10μg ovalbumin (OA) and 100mg $Al(OH)_3$ in normal saline (1ml). 14-21 days later they were exposed to a macroshock of OA (10mg/ml) by inhalation in a sealed box for 2 min, 30 min after mepyramine (30mg/kg i.p.), to protect against fatal anaphylaxis. Changes in specific airway conductance (sGaw) were determined by whole body plethysmography (Griffiths-Johnson *et al*, 1987) up to 72h after the OA exposure. Bronchoalveolar lavage (BAL) was also performed at 5 and 24h after OA challenge immediately after killing with an overdose of pentobarbitone (i.p.). The trachea was cannulated and approx. 6ml Hanks balanced salt solution (without calcium, magnesium or phenol red) was introduced into the lungs using a syringe. After 3 mins approx. half of the solution was withdrawn. This procedure was repeated. Total cell counts using a haemocytometer (Improved Neubauer) were performed on the BAL fluid and differential counts on cytocentrifuged preparations (Cytospin II; Shandon) stained with May-Grünwald-Giemsa. 500 cells were counted using standard morphologic criteria to classify the cells.

An early phase bronchoconstriction peaked at 15 min after OA exposure with a max. sGaw value of -70.8±3.8% ($P<0.05$, n=8) which resolved after 5h. A late phase peaked variably at between 17-24h with a max. sGaw value of -21.6±3.7% ($P<0.05$, n=8). A control exposure to saline showed no significant variation in sGaw over the predicted early and late phases. At 5 and 24h after the OA macroshock, significant increases in total cell numbers were seen (108.9 and $89.2x10^5$/ml respectively, $P<0.05$) compared with a saline challenge (6.3 and $14.1x10^5$/ml respectively). Differential cell counts showed a significant ($P<0.05$) increase in neutrophils (42.0±4.6%) and decrease in macrophages (59.4±2.3%) at 5h compared with saline controls (2.2±0.8% and 88.8±2.8% respectively). At 24h after an OA exposure there were significant ($P<0.05$) increases in neutrophils (15.0±3.0%) and eosinophils (25.6±3.0%) and decreases in macrophages (59.4±2.3%) compared with saline controls (2.0±0.3%, 11.0±3.4% and 86.9±3.3% respectively). Thus, a macroshock of OA causes an early and smaller late phase bronchoconstriction associated with significant increases in neutrophils at 5h and neutrophils and eosinophils at 24h, with a significant decrease in macrophages at 5 and 24h.

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S.P. Grix, P.J. Gardiner, J. Westwick[1] and C.T. Poll, Bayer plc, Stoke Court, Stoke Poges, SL2 4LY, and [1]Dept of Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY.

Despite strong evidence for a key inflammatory role for eosinophils, in the pathology of asthma, relatively little is known about their stimulus-response coupling mechanisms. In human eosinophils, C5a has been shown to cause elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), release of eosinophil peroxidase (Kernen et al., 1991) and secretion of eosinophil cationic protein (Takafuji et al., 1994). In this study, we have investigated the relative contribution of intracellular Ca^{2+} release and Ca^{2+} influx (using fura-2) to C5a-induced generation of O_2^- (cytochrome C reduction) and LTC_4 (radioimmunoassay) in human peripheral blood eosinophils, from normal healthy donors.

C5a (0.03-30nM) caused concentration-dependent increases in $[Ca^{2+}]_i$ and Mn^{2+} (a Ca^{2+} surrogate) influx, with mean EC_{50} values (n=4; 95% confidence limits) of 0.14 (0.06-0.3)nM and 0.09 (0.02-0.46)nM, respectively. The putative phospholipase C (PLC) inhibitor, U73122 (1μM), caused ≥85% (n=3-4) inhibition of C5a- (30nM) induced Ca^{2+} mobilisation, O_2^- and LTC_4 release, suggesting that these responses were mediated via a PLC-dependent pathway.

Chelation of extracellular Ca^{2+} using EGTA (4mM), caused only 13.2±6.3% (mean±s.e.m.; n=4) inhibition of the C5a-induced peak increase in $[Ca^{2+}]_i$, suggesting that this represented release of Ca^{2+} from intracellular stores. However, generation of O_2^- and LTC_4 was inhibited by 84.6±16.6% and 96.8±2.1% (n=4), indicating a requirement for extracellular Ca^{2+} for these responses.

The inorganic cation Ni^{2+} (3mM), which blocks both receptor mediated Ca^{2+} entry (RMCE) and voltage-operated Ca^{2+} channels (VOCs), caused only 28.5±8.2% (n=4) inhibition of the rise in $[Ca^{2+}]_i$ produced by C5a (30nM), whereas the Mn^{2+} influx response was markedly inhibited (73.6±2.2%; n=4). In contrast, the potent, selective VOC blocker, nifedipine (10μM), was inactive against either Ca^{2+} elevation or Mn^{2+} influx responses to C5a. The putative RMCE blocker, SK&F 96365, had a similar inhibitory profile to Ni^{2+}, with a mean IC_{50} value of 19.0 (13.7-26.4)μM against Mn^{2+} influx. Furthermore, we have previously shown that release of O_2^- and LTC_4 were inhibited by Ni^{2+} and SK&F 96365, but not by selective VOC-blocking concentrations of nifedipine (Grix et al., 1994).

These data provide further evidence that PLC activation and receptor mediated Ca^{2+} influx may be involved in C5a-induced human eosinophil activation.

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54P THE EFFECT OF ROLIPRAM ON ANTIGEN-INDUCED AIRWAY RESPONSES IN THE NEONATALLY IMMUNISED RABBIT

N.Gozzard, C.M.Herd[1], S.M.Blake, and C.P.Page[1]. Celltech Research Ltd., 216 Bath Road, Slough, SL1 4EN. and [1]Dept Pharmacology, Kings College, London, SW3 6LX.

The phosphodiesterase type IV (PDE IV) inhibitor rolipram is reported to exhibit anti-inflammatory and bronchorelaxant activity (Nicholson & Shahid, 1994). Litter-matched NZW rabbits immunised within 24h of birth and until 12 weeks of age with *Alternaria tenuis* antigen (Ag) in Al(OH)3 gel (*i.p.*) were pre-treated twice daily on days 1-3 with rolipram (1mg kg^{-1} *i.p*) or vehicle (ethanol:saline 1:5 v/v); (2ml kg^{-1}). On day 3, the concentration of inhaled aerosolised histamine required to provoke a 50% increase (PC_{50}) in total lung resistance (R_L) and 35% decrease (PC_{35}) in dynamic compliance (C_{dyn}) were determined and bronchoalveolar lavage (BAL) performed. On day 4, rabbits were given a final dose of rolipram or vehicle 1 h prior to Ag aerosol (20,000 PNU ml^{-1} in saline for 20 min). On Day 5, PC_{50} and PC_{35} values were determined and BAL performed as on day 3 (Herd *et al*,1994).

Ag-induced acute bronchoconstriction was unnaffected by rolipram pre-treatment (% increase in R_L 30.5±5.1 Vs vehicle 32.4±5.2; % decrease in C_{dyn} 23.3±3.1. Vs vehicle 31.0±3.0).

Table 1. Histamine PC_{50} and PC_{35} values pre and post antigen.

Treatment	PC_{50}(mg ml^{-1})		PC_{35}(mg ml^{-1})	
	Pre	Post	Pre	Post
vehicle n=9	16.6±1.4	4.8±1.4†	12.7±1.4	5.0±1.3†
rolipram n=9	17.4±1.3	11.0±1.2†#	11.1±1.2	10.6±1.3#

†P<0.05 Vs Pre (paired t-test); #P<0.05 Vs post vehicle (unpaired t-test)

In vehicle-treated rabbits, Ag-induced airway hyperresponsiveness was evidenced by reduced PC_{50} and PC_{35} values to inhaled histamine 24h post antigen. This hyperresponsiveness was significantly reduced by rolipram pre-treatment (Table 1).

Pre-treatment with rolipram did not alter basal lung function. (R_L 32.2±1.6 Vs.vehicle 32.0±1.7 cmH_2O^{-1} L^{-1} sec^{-1} and C_{dyn} 4.5± 0.2 Vs vehicle 4.3±0.1 ml cmH_2O).

Total leucocytes recovered in BAL 24h post Ag challenge were increased, but not significantly, in vehicle-treated rabbits only. Ag-induced increases in eosinophils were significantly reduced by rolipram (P=0.002). Neutrophil and mononuclear cells tended to be reduced in rolipram-treated rabbits before and after Ag challenge (Table 2).

Table 2. Total and differential cell counts in BAL (10^4 cells ml^{-1}) pre and post Ag.

Treatment	Total		Mono		Neut		Eosin	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Vehicle	28.2 ±2.6	36.1 ±6.3	27.0 ±2.5	26.5 ±6.8	1.2 ±0.5	7.9† ±2.2	0.0 ±0.0	1.7† ±0.4
Rolipram	21.0 ±2.6	19.2# ±2.1	20.7 ±2.8	15.1 ±2.3	0.3 ±0.1	3.9† ±1.3	0.01 ±.01	0.3†# ±0.1

†P<0.05 Vs Pre; #P<0.05 Vs post vehicle (Wilcoxon test), *n*=9 per group.

These data demonstrate that the PDE IV inhibitor rolipram attenuates Ag-induced airway hyperresponsiveness and pulmonary eosinophilia in the immunised rabbit.

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55P HISTAMINE FORMING CAPACITYAND ITS MODULATION BY THE H_3 RECEPTOR IN A MODEL OF ACUTE BRONCHIAL HYPERRESPONSIVENESS

M.C. Allen, Pamela Graham & G. Morris, (introduced by W. Dawson) Clinical Pharmacy Unit, University of Brighton, Moulsecoomb, Brighton BN2 4GJ

The role of histamine in asthma remains uncertain but it is generally accepted that asthmatic airways are hyper-responsive to this amine. Although the lungs have the ability to form histamine utilising the enzyme histidine decarboxylase, most work has concentrated on that released from mast cells rather than new formation. The present study was designed to investigate the histamine forming capacity (HFC) of acutely challenged airways from sensitised guinea pigs and at the same time to observe the reactivity to exogenous histamine and electrical stimulation of airway smooth muscle from the same animal.

Adult male guinea pigs, sensitised to bovine serum albumin (BSA) by i.p. injection were subsequently exposed to nebulised BSA, 4% in normal saline (ns), or to ns. Groups were sacrificed immediately or at varying times after exposure and lungs and trachea removed. HFC of lung tissue was measured by a modified radio isotope method (Keeling et al 1982). Concurrently, histamine dose response curves were constructed for parenchymal strips and tracheal muscle and the latter was subjected to electrical field stimulation to record neurogenic responses. Some animals were pretreated with either saline, α-methyl histamine (an H_3 receptor agonist), thioperamide (an H_3 receptor antagonist), or combinations of these to investigate the modulation of inducible HFC via H_3 receptors.

Acute exposure to BSA led to an increase in HFC above the saline controls for a period of 24 hours ($p< 0.001$ ANOVA). The peak at 2 hours was significantly different from its control (mean ± s.e.mean 13.46±3.2 and 5.23±0.77 fmol/mg protein/3h, respectively, n=6 $p<0.05$ t-test). Prior treatment with thioperamide significantly ($p<0.02$) augmented the HFC measured 2 hours post exposure (8.68±1.04 fmol/mg protein/3h compared with 5.0±0.55 for controls). This increase was inhibited by α-methyl histamine ($p<0.05$). Concurrent *in vitro* measurements showed a shift to the left of the histamine log concentration response curves for the parenchymal strip with an increased maximum response e.g. mean±s.e.mean EC_{50} 1.14μM± 0.28 and 3.41±1.3; maximum response 2.03±0.33g tension /g wet weight and 1.2±0.26 for BSA and control groups respectively. This change developed slowly and was first apparent 3 hours after exposure to BSA, remaining elevated at 24 hours. Similar changes did not occur with the tracheal preparation, indeed a reduction in responsiveness was observed at 24 hours in the BSA group (2.17±0.47g tension/g wet tissue compared with control 4.04±0.53,$p<0.05$). Field stimulation of trachea resulted in a contraction followed by a long lasting relaxation. BSA exposure did not significantly influence any of these neurogenic responses.

A possible relationship may exist between increased responsiveness of lower airways to exogenous histamine and a raised endogenous formation, regulated by the H_3 receptor, the site of which is speculative. Tracheal data from the same animals suggests that acute challenge of sensitized guinea pigs did not result in overall airway hyper-responsiveness.

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56P EVIDENCE THAT CENTRAL 5-HT_{1A} RECEPTORS MODULATE REFLEX BRONCHOCONSTRICTION CAUSED BY ACTIVATION OF PULMONARY VAGAL MOTONEURONES IN THE CAT AND GUINEA-PIG

D.J. Bootle, A.G. Ramage & J.J. Adcock[1], Departments of Pharmacology, Royal Free Hospital School of Medicine, Hampstead, London, NW3 2PF and [1]Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS

Preliminary studies have shown that drugs which block 5-HT_{1A}/5-HT_7 receptors can attenuate the reflex activation of pulmonary vagal motoneurones (PVM's) by inhaled capsaicin in anaesthetised cats (Bootle *et al.*, 1994). The present study was carried out to determine if this was due to a central action of these drugs, the relative roles of central 5-HT_{1A} receptors versus 5-HT_7 receptors in the modulation of this reflex, and to extend these observations to another species, guinea-pigs. Animals were anaesthetised with α-chloralose (60-80 mg kg^{-1}; i.v.) following induction with 5% halothane in O_2 and blood pressure and heart rate monitored. Cats and guinea-pigs were artificially ventilated after neuromuscular blockade with dimethyl-tubocurarine and vecuronium bromide respectively. In cats, dynamic lung compliance and lung resistance were monitored with an on-line digital data acquisition system (PO-NE-MAH), and also pressure in an air-filled balloon (P_{TB}; index of reflex bronchoconstriction), placed in the trachea above the endotracheal cannula was measured. In guinea-pigs tracheal pressure (P_T) was measured as an index of bronchoconstriction. Capsaicin (100 μg ml^{-1} in cats; 0.9 μg ml^{-1} in guinea-pigs; 6 breaths) aerosols were generated with a modified DeVilbiss ultrasonic nebuliser and administered to the lower airways. Saline and drug-evoked changes were compared by one-way ANOVA followed by Dunnett's Test. 8-OH-DPAT is a 5-HT_{1A} receptor agonist, all other test drugs were 5-HT_{1A} receptor antagonists. All drugs (except 8-OH-DPAT in guinea-pigs), when given intracisternally (i.c.), caused significant changes in either P_{TB} or P_T increases evoked by capsaicin. However, WAY-100635 (Fletcher *et al.*, 1994) caused no significant change when administered i.v., suggesting that these drugs are acting at a central site (see Table 1.). None of these drugs, with the exception of 8-OH-DPAT in cats which significantly increased dynamic lung compliance (12 ± 4 % from a value of 3.4 ± 0.5 ml cmH_2O^{-1}), affected baseline pulmonary variables. Further, the bradycardia and hypotension also evoked by capsaicin were unaffected by any of the drugs tested. As all the drugs have the common ability to act at 5-HT_{1A} receptors (Hoyer & Fozard, 1991; Fletcher *et al.*, 1994), and since pindolol does not act at the putative 5-HT_7 receptor (Lovenberg *et al.*, 1993), this data supports the view that 5-HT_{1A} receptors play an important role in modulating the excitability of PVM's.

WAY-100635 was a gift from Wyeth (U.K.). D.J.B. is an M.R.C. student.

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Table 1. Capsaicin-evoked increases in P_{TB} (cats) and P_T (guinea-pigs) before (control) and after drug or saline. Mean ± s.e.mean cmH_2O *$p<0.05$

Cats, *Drug, Dose, Route*	*n*	*Control*	*3 min*	*33 min*
Saline, 10 μl, i.c.	5	4.0 ± 1.5	3.6 ± 1.4	3.2 ± 1.4
WAY-100635, 100 μg kg^{-1}, i.c.	4	6.3 ± 1.1	3.1 ± 0.6*	2.7 ± 0.4*
WAY-100635, 100 μg kg^{-1}, i.v.	4	4.4 ± 2.0	4.5 ± 2.2	3.6 ± 1.7
Methiothepin, 200 μg kg^{-1}, i.c.	4	2.3 ± 0.4	1.4 ± 0.3*	1.0 ± 0.4*
(-)-Pindolol, 100 μg kg^{-1}, i.c.	4	7.9 ± 1.1	5.6 ± 0.9	4.1 ± 0.8*
8-OH-DPAT, 50 μg kg^{-1}, i.c.	5	3.9 ± 1.4	4.5 ± 1.3*	2.8 ± 0.8

Guinea-pigs, *Drug, Dose, Route*	*n*	*Control*	*3 min*
Saline, 10 μl, i.c.	5	3.7 ± 0.7	3.6 ± 0.6
WAY-100635, 100 μg kg^{-1}, i.c.	5	2.1 ± 0.6	1.0 ± 0.5*
WAY-100635, 100 μg kg^{-1}, i.v.	5	3.2 ± 1.2	3.0 ± 0.9
(-)-Pindolol, 100 μg kg^{-1}, i.c.	5	3.6 ± 0.3	0.8 ± 0.3*
8-OH-DPAT, 50 μg kg^{-1}, i.c.	5	2.3 ± 0.8	2.2 ± 0.7

57P THE MECHANISM BY WHICH THE ADENOSINE A_3 RECEPTOR AGONIST APNEA INDUCES BRONCHOSPASM

C.J. Meade, Department of Pharmacology, Boehringer Ingelheim KG, D-55216 Ingelheim, Germany

APNEA [N^6-2-(4-aminophenyl)ethyladenosine] is an adenosine A_3 receptor agonist (Van Galen et al, 1994). The A_3 receptor facilitates release of allergic mediators from mast cells (Ramkumar et al, 1993). A hypotensive effect of APNEA has been described by Fozard and Carruthers (1993). We here describe APNEA's effect on the pulmonary system.

We administered APNEA (0.01 - 1.0 μmol/kg) i.v. to spontaneously breathing, anaesthetised BDE rats. Within minutes there was a marked increase in total lung resistance (R_L). Tidal volume (TV) was decreased [ED_{50} 0.22 μmol/kg (95% confidence limits 0.14-0.41)]. As anticipated from the results of Fozard and Carruthers, mean arterial blood pressure fell (ED_{50} 0.019 μmol/kg, confidence limits 0.015-0.024). APNEA-induced bronchospasm and hypotension were substantially and significantly ($P<0.05$, Wilcoxon) reduced by the combined A_3/A_1 receptor antagonist BW-A 1433, 0.1 mg/kg, but not by A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) 0.5 mg/kg, although this DPCPX dose substantially and significantly reduced bronchospasm and hypotension induced by A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA). Rats were treated with compound 48/80 to deplete mast cells (daily dose gradually increased from 1 to 5 mg/kg over 15 days). APNEA (but not CCPA)-induced increase in R_L was significantly ($P<0.001$) and specifically (no inhibition of bronchospasm after subsequent methacholine challenge) reduced [median increase in R_L after 0.2μmol/kg APNEA: 48/80 treated group (n=9) 28%, vehicle group (n=9) 285%]. The anti-allergic and mast cell stabilising agent epinastine, 10 μg/kg i.v. 2 minutes before 0.1μmol/kg APNEA also significantly ($P<0.01$) reduced APNEA-induced increase in R_L (median values: epinastine (n=12) 30%, controls (n=25) 106%) without affecting CCPA-induced bronchospasm. Bilateral cervical vagotomy 40 minutes before, together with atropine 0.1 mg/kg 2 minutes before APNEA challenge, blocked APNEA-induced bronchospasm. NK_2 receptor antagonist L-659877 1.0 mg/kg i.v. 2 minutes prior to 0.1 μmol/kg APNEA also significantly ($P<0.05$) blocked APNEA-induced bronchospasm (increase in R_L, decrease in TV).

It is therefore concluded, in BDE rats A_3-receptor agonist induced bronchospasm is predominantly via a mast-cell and neurally (NK_2-receptor binding neuropeptide) mediated mechanism.

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58P THE AMYLIN BINDING SITE ON LUNG TISSUE IS DISTINCT FROM THE FUNCTIONAL AMYLIN RECEPTOR ON SKELETAL MUSCLE

R.M.Mayers, S.P.Quayle, S.Pariagh, T.L.Grant and B.R.Holloway, Cardiovascular and Metabolism Research Department, ZENECA Pharmaceuticals, Mereside, Alderley Park, Macclesfield Cheshire SK10 4TG.

Amylin is a 37-amino acid peptide cosecreted with insulin from pancreatic β-cells, which has been implicated in the etiology of insulin resistance. Amylin has been shown to inhibit insulin-stimulated glycogen synthesis in isolated rat soleus muscle (Leighton & Cooper, 1988). Low or negligible specific binding of ^{125}I-amylin has been detected in muscle or liver membranes, however, high-affinity amylin binding sites have been detected in the nucleus accumbens (Beaumont et al. 1993). In addition, Bhogal et al., (1992) reported specific binding to lung membranes. The objective of this study was to identify a membrane source expressing a high level of amylin binding, and to compare this binding site with the receptor associated with the action of amylin on isolated soleus muscle.

In membranes prepared from lung tissue from a range of species, the binding of 50pM ^{125}I-rat amylin was compared. Significantly higher levels of binding were found in lung membranes from cat (95.7±10.1fmols/mg protein), dog (87.4±12.2) and mouse (85.9± 16.8) compared to rat (20.8±6.8) and bovine lung (37.4±2.9). Cat lung membranes contained a single binding site: K_D 223.3pM (n=2), B_{max} 1967fmols/mg. Related ligands competed for the cat lung binding site with the following IC_{50}s (nM): rat amylin 0.32 (95% confidence limits 0.29-0.36), rat α-CGRP 45.4 (19.1-108.1), β-CGRP 1.65 (0.77-3.53), salmon calcitonin (sCT) 17.8 (11.5-27.5), rat adrenomedullin 0.46 (0.31-0.67) (all n=3). The CGRP antagonist hCGRP(8-37) displaced ^{125}I-amylin with IC_{50} 0.20nM (0.09-0.46), whereas the amylin antagonist AC187 (Young et al., 1994) was of low affinity, IC_{50} 118.0nM (97.1-143.6).

In isolated strips of rat soleus muscle, 100μU/ml insulin elicited a 2-3-fold increase in the rate of glucose incorporation into glycogen, which was inhibited by rat amylin in a concentration-related manner (IC_{50} 4.4nM (3.8-5.2, n=3). sCT was also capable of inhibiting the insulin-stimulated effect (IC_{50} ≤1nM), but 100nM rat adrenomedullin had no effect as a mimetic of the amylin response. The effect of 4nM amylin was completely reversed by 300nM AC187 ($p<0.001$) but 3μM CGRP(8-37) was required to cause a 50% reduction in the amylin response.

The difference in order of potency of these peptides as antagonists and agonists leads us to conclude that the amylin binding site detected on lung membranes is distinct from the receptor involved in the effect of amylin on insulin-mediated glucose metabolism in skeletal muscle.

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59P THE GLN-GLU27 β_2 ADRENOCEPTOR POLYMORPHISM PROTECTS AGAINST RECEPTOR DOWNREGULATION IN CULTURED HUMAN AIRWAY SMOOTH MUSCLE CELLS

I.P. Hall (introduced by P.C. Rubin). Department of Therapeutics, University Hospital of Nottingham, Nottingham NG7 2UH

Recently a number of polymorphisms have been identified in the human beta 2 adrenoceptor (Reishaus et al 1992). The physiological significance of these polymorphisms remains unclear. The aim of this study was to investigate the effect of 2 common polymorphisms upon beta 2 adrenoceptor coupling in human airway smooth muscle (HASM). Primary cultures of HASM were obtained as previously described (Hall et al 1992) and changes in receptor coupling were studied using isoprenaline induced cyclic AMP production as an end point. We have previously shown that these cells express beta 2 adrenoceptors, and that exposure to beta adrenoceptor agonists results in a marked reduction in respone to a subsequent challenge to isoprenaline (Hall et al 1993).

HASM cell cultures derived from 12 individuals were genotyped using allele specific oligonucleotide hybridisation and direct sequencing and 3 informative cell populations were used for further studies. These were homozygous for the wild type form of the receptor (WT), for the Arg-Gly16 form of the receptor, and for the Gln-Glu27 form of the receptor. [^{3}H]-cyclic AMP formation was measured in HASM labelled with [^{3}H]-adenine as previously described. Cyclic AMP responses to isoprenaline were not significantly different in the 3 cell populations (EC_{50} 2uM, fold stimulation to 1uM isoprenaline 9.5 cf basal (=1), Hall et al 1992). Following 18h exposure to isoprenaline (1nM-1uM), the WT cells demonstrated a markedly reduced cyclic AMP response to a subsequent challenge to agonist (reduction with 1uM isoprenaline= 84 $\pm$ 5%, IC_{50} for downregulation= 30 $\pm$ 5nM, n=4). HASM cells homozygous for the Arg-Gly polymorphism behaved similarly to WT cells. However, HASM cells homozygous for the Gln-Glu 27 polymorphism were markedly less sensitive to modulation following prior exposure to isoprenaline (reduction to 1uM isoprenaline =33 $\pm$ 7%, IC_{50} for downregulation 2 $\pm$ 0.5uM, n=4, $p<0.05$ cf WT cells).

These results demonstrate that the regulation of beta 2 adrenoceptor coupling in a nontransformed cell population derived from HASM is dependent upon beta 2 adrenoceptor genotype. Hence, the Gln-Glu27 polymorphism is likely to be of physiological significance.

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60P DETECTION OF INTRACELLULAR LIPOCORTIN 1 IN MOUSE PERIPHERAL BLOOD LEUCOCYTES BY FLOW CYTOMETRY

M Perretti, SK Wheller, F Mancuso, JG Harris, #NJ Goulding, JD Croxtall & RJ Flower.
Depts of Biochemical Pharmacology and #Rheumatology, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ, U.K.

Glucocorticoid hormones induce the anti-inflammatory protein lipocortin 1 (LC1) in human monocytes and macropahges (Flower & Rothwell, 1994) as detected by a semi-quantative western blot technique as well as an ELISA specific for human LC1 (Goulding et al., 1990). Here we describe a quantitative flow cytometry analysis for measuring LC1 in mouse blood leucocytes.

A mixed population of leucocytes was prepared by centrifugation of blood on a ficoll-metrizoate gradient (400 *g* x30 min) followed by two washes in posphate-buffered solution (PBS). 0.5-1.0 x10^6 leucocytes (approximately 70% lymphocytes, 10% monocytes and 20% neutrophils) were aliquoted in 96-well plates, fixed with 1% paraformaldehyde, and permeabilized with 0.015% saponin (which remained throughout the experiment). After blockade of non-specific sites with diluted (1:20) plasma, either a specific anti-LC1 or a non-immune sheep serum were added (1:50 dilution) and incubated for 1h at 4°C. Cells were then washed in PBS with 0.2% bovine albumin and 1.5 mM $CaCl_2$, and stained with a FITC-conjugated donkey anti-sheep IgG antibody for 30 min at 4°C. This was followed by three washes in the buffer. For storage purposes cells were fixed with an equal volume of 2% paraformaldehyde. Flow cytometry was performed using a FACScan II, and distinct cell populations were discriminated using forward and side scatter characteristics. In some cases animals were treated with dexamethasone (Dex; 5 μg i.v.) or RU486 (20 mgkg^{-1} p.o., -24h and -2h) prior to blood collection. In other cases cell were taken from an inflammatory site: neutrophils migrated into a 6-day-old air-pouch in response to cytokine administration (Perretti & Flower, 1993) and compared with those in the circulation. Data are mean ± s.e.mean of n experiments.

Circulating neutrophils were found to have 27,600±4,300 LC1 molecules per cell, monocytes 34,800±5,500 and lymphocytes 7,900±2,700 (n=10-14). Detection of endogenous LC1 was lower when buffer without calcium ions was used (-38% in neutrophils, and -100% in mononuclear leucocytes). Following treatment of mice with Dex, there was a significant increase in cell-associated LC1 at 30 min time point: 307±75% of basal for neutrophils, 209±80% for monocytes and 384±190% for lymphocytes (n=3, $p<0.05$). This increase persisted at the 2h time point (≈ 230% of basal) and then gradually disappeared within 20h. When animals were challenged with RU486 there was a marked reduction in cell-associated LC1, being only 46% of basal for neutrophils and 45% for monocytes (n=3). When levels of endogenous LC1 between migrated and circulating neutrophils were compared, it was found that cells taken from an inflammatory site had ≈40% less cell-associated LC1: 15,800±2,800 *vs.* 31,400±4,900 (n=3, $p< 0.05$).

In conclusion, we have described a quantitative FACS analysis for LC1 and observed a modulatory action of endogenous and exogenous glucocorticoid hormones on protein expression in circulating leucocytes. Moreover, it was also possible to monitor changes in LC1 related to the induction of an inflammatory process.

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61P TOPICAL GLUCOCORTICOIDS INHIBIT NEUROGENIC INFLAMMATION: INVOLVEMENT OF LIPOCORTIN 1

A. Ahluwalia, P. Newbold[1], & RJ Flower. Dept. of Biochem. Pharmacol., The Medical College of St Bartholomew's Hosp., London EC1M 6BQ, [1]Biomedical Sciences Division, Kings College, London, SW3 6LX

The topical glucocorticoids (GCs) represent first-line treatment for many types of inflammatory skin-disease, although their mechanism of action remains rather speculative. Our studies in the rat suggest that one possible site of action may be the sensory C-fibre (Ahluwalia & Flower, 1993). To further explore this possibility we have used a model of neurogenic inflammation i.e. electrical stimulation of the rat saphenous nerve and investigated the effect of GC treatment upon the ensuing oedema response. In other studies we have shown that topical GC treatment results in induction of the GC-inducible, anti-inflammatory protein lipocortin 1 (LC1) in rat skin and in light of this we have investigated whether LC1 may be involved in GC action in the described model.

Briefly male Wistar rats were anaesthetised using sodium pentobarbitone (45 mg/kg), the hair on both paws shaved and a depilatory cream applied to remove any remaining hair. After 1 h the left paw was treated with betamethasone-17-valerate (0.18 mg /cm^2) or with the acetone vehicle alone, the other paw remaining as an untreated control. After 3 h , a portion of the left saphenous nerve, in the thigh, was carefully dissected free, tied proximally, immersed in paraffin oil and placed on platinum bipolar electrodes. The right saphenous nerve was also dissected free but not tied and acted as sham control. After 15 mins ^{125}I-labelled human serum albumin (2 μCi/kg) in Evans blue (1 %) was injected i.v. via the tail vein. The left nerve was stimulated for 5 min (2 Hz, 0.1 ms and 15 V). After this time a blood sample was taken by intracardiac puncture and the animal killed. The skin of the left paw, which had turned blue, was removed and an equivalent area was taken from the right unstimulated paw. Oedema was expressed in μl of plasma per 100 mg of tissue following determination of the number of counts found in 1 μl of plasma. Values of oedema are shown as net values of oedema per mg of tissue where the extravasation measured in the control paw has been subtracted from that of the stimulated paw.

Topical GC treatment significantly ($p<0.05$) attenuated oedema formation in the paw, with a response of 12.5 ± 1.3 μl following vehicle-treated paws and 5.4 ± 2.3 μl/mg (n=6) in GC-treated paws. Pretreatment with sheep anti-LC1 (1 ml/kg, 24 h prior to use) caused partial reversal of the inhibitory effect of topical GC treatment i.e. 13.4 ± 2.7 μl for controls and 8.9 ± 1.3 μl (n=6) in topical GC treated rats. Pretreatment with control sheep serum had no significant inhibitory effect upon topical GC activity (n=6). The GC receptor antagonist RU38486 (20 mg/kg 2 and 20 h pretreatment o.p.) reversed the anti-inflammatory effect of the topical GC with a response of 11.32 ± 1.7μl, n=5 which was not significantly different from vehicle treated paws in animals also treated with RU 38486 (14.53 ± 2.3 μl, n=6)

Thus topical GC treatment of the rat paw results in inhibition of neurogenic inflammation induced by antidromic stimulation of the saphenous nerve. This inhibitory effect of the GC is via specific binding to GC receptors and partially dependent upon the GC-inducible anti-inflammatory protein LC1.

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62P SUPPRESSION OF EXPERIMENTAL ACUTE LUNG INJURY IN THE MOUSE BY DEXAMETHASONE AND THE ROLE OF LIPOCORTIN-1

JM Miotla[1], M Perretti[3], RJ Flower[3], PK Jeffery[2], PG Hellewell[1], Depts of [1]Applied Pharmacology and [2]Lung Pathology, NHLI, Dovehouse Street, London SW3 6LY and [3]Dept. Biochemical Pharmacology, WHRI, St. Bart's Hospital, Charterhouse Square, London EC1M 6BQ

Lipocortin-1 (LC-1) is known to mediate some of the anti-inflammatory effects of the glucocorticoid steroids (Flower, 1988). For example, anti-LC-1 antiserum has been shown to reverse the inhibitory effects of dexamethasone on IL-1-induced neutrophil accumulation in a mouse air pouch model (Perretti & Flower, 1993). In this study, we have therefore investigated the effect of dexamethasone on acute lung injury in the mouse as assessed by extravascular albumin accumulation (Miotla *et al*, 1994) and have assessed the involvement of LC-1 in mediating these effects.

BALB/c mice (18-20g) were anaesthetised (Hypnorm/Diazepam, 16mg/kg, i.p.) and injected with LPS from *Escherichia Coli* 0111:B4 (3mg/kg, i.v.) 2 hours prior to zymosan i.v. (10mg/kg) along with ^{125}I- human serum albumin (^{125}I-HSA, 0.25μCi). After 30 minutes, ^{131}I-HSA (0.5μCi) was injected i.v. 5 minutes before sacrifice, as a marker of pulmonary intravascular volume. The activities of ^{125}I-HSA and ^{131}I-HSA in whole lung tissue were measured in a gamma counter and compared with that in plasma. Albumin accumulation was expressed as μl of plasma retained extravascularly in whole lung. Dexamethasone (2.5mg/kg, i.v.) was injected 2 hours prior to combined LPS and zymosan, whereas anti-LC-1 antiserum (50μl s.c.) or normal control sheep serum (50μl s.c.) were injected 24 hours prior to subsequent treatment. The efficacy of the anti-LC-1 antiserum was tested against dexamethasone in a mouse model of glycogen-induced peritonitis. All lung tissue was processed for examination by light microscopy.

Glycogen-induced neutrophil accumulation in the peritoneal cavity ($1.8\pm0.6\times10^6$, n=3) was inhibited by 80% by dexamethasone. Anti-LC-1 antiserum reversed the inhibitory effect of dexamethasone ($1.6\pm0.1\times10^6$, n=3) and when administered without dexamethasone, enhanced the response to glycogen ($6.8\pm0.6\times10^6$). Combined LPS and zymosan treatment led to significant extravascular albumin accumulation in lung tissue (12.5±1.2μl, n=6; $p<0.01$) when compared with saline-treated controls (0.7±0.4μl, n=4) and induced the sequestration of neutrophils in pulmonary microvessels as seen by light microscopy. Pretreatment with dexamethasone partially inhibited extravascular albumin accumulation in lung tissue in response to LPS and zymosan treatment (6.7±0.9μl, n=7; $p<0.05$ compared with LPS+zymosan group). This was associated with a reduction in neutrophil sequestration as seen by light microscopy. However, the anti-LC-1 antiserum was ineffective in reversing the inhibitory effect of dexamethasone on LPS and zymosan-induced albumin accumulation (7.0±0.8μl, n=5) and neutrophil sequestration was still suppressed.

These observations demonstrate an inhibitory effect of dexamethasone on LPS and zymosan-induced acute lung injury in the mouse. However, unlike other anti-inflammatory effects of dexamethasone in this species, the effect in the lung is not mediated via lipocortin-1.

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63P CHANGES IN THE CELLULAR DISTRIBUTION OF LIPOCORTIN-1 (ANNEXIN-1) IN C6 GLIOMA CELLS AFTER EXPOSURE TO DEXAMETHASONE

J.D. McLeod, P. Jelic, A. Goodall & C. Bolton, Pharmacology Department, School of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY

Lipocortin-1 (LC-1) is a member of the Annexin family of calcium and phospholipid binding proteins and is a putative mediator of the anti-inflammatory action of glucocorticoid steroids (Flower, 1988). We have shown that the synthetic glucocorticoid, dexamethasone (Dex.), affects the cellular distribution of LC-1 in astroglia cells (McLeod & Bolton, 1994). In this study we investigated the effect of Dex. on LC-1 distribution in C6 glioma cells.

The effect of Dex. on three separate cell fractions; extracellular, intracellular and membrane, was investigated (Peers *et al.*, 1993). Densitometric quantitation of the staining intensity of LC-1 bands was undertaken and results expressed as % change (mean ± s.e.m.) from control levels. All experiments were done in steroid-depleted medium. Dex. (0.01μM and 0.1μM) was added for 2h, 6h or 16h. Glioma cells in the presence of Dex. (0.01μM) for 2h showed minimal change in LC-1 content in all fractions (-27 ± 16%, 11 ± 17% and -24 ± 4%; extracellular, intracellular and membrane fractions respectively; n=7). After 6h treatment with low dose Dex. increases in LC-1 were detected in extracellular and membrane fractions (60 ± 32% and 36 ± 23% respectively, n=3). The increased LC-1 present at the cell-surface remained high following incubation with Dex. for 16h (118 ± 62%, n=4). In the presence of the higher concentration of Dex. (0.1μM) for 2h C6 glioma cells responded by increasing LC-1 content associated with the membrane (25 ± 8% of control, n=3), with no change occurring in the remaining fractions (-20 ± 20% and -7 ± 3% ; extracellular and intracellular respectively, n=4). Following 6h glucocorticoid treatment a substantial rise in cell-surface LC-1 was apparent (368 ± 189% of control), the levels of which did not alter after further incubation (16h) (331 ± 179%, n=3).

Cycloheximide (5μg/ml), a de novo protein synthesis inhibitor, was added concurrently with Dex. (0.01μM and 0.1μM) for 2h, 6h and 16h and LC-1 content detected as before. The changes in LC-1 associated with all cell fractions in the presence of Dex. (0.01μM) were abolished at 2h (-15 ± 12%; mean LC-1 changes of all fractions), 6h (-49 ± 7%) and 16h (-52 ± 19%, n=6). Furthermore, in the presence of high-dose Dex. (0.1μM) cycloheximide reduced the LC-1 content below control levels in all cell fractions (-67 ± 7%, 2h; -35 ± 13%, 6h; -56 ± 7%, 16h; n=6).

These results indicate that Dex. affects the intracellular distribution of LC-1 in C6 glioma cells leading to externalisation of the protein. Since extracellular LC-1 has biological effects (Cirino & Flower, 1987) the influence of glucocorticoids on the translocation process may be part of their mechanism of action.

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64P MODULATION OF PROLACTIN RELEASE *IN VITRO* AND *IN VIVO* BY DEXAMETHASONE, LIPOCORTIN 1 (LC1) AND ANTI-LC1 ANTISERA

A.D.Taylor,[1]R.J. Flower & J.C.Buckingham. Dept. Pharmacology, Charing Cross & Westminster Medical School, London W6 and [1]Dept Biochemical Pharmacology, The William Harvey Research Institue, St. Bartholomew's Hospital Medical College, London EC1M

Glucocorticoids inhibit the release of a number of anterior pituitary hormones including corticotrophin (ACTH), prolactin (PRL) and thyrotrophin (TSH). Our previous studies suggest the second messenger protein lipocortin 1 (LC1) contributes to the steroid-induced inhibition of ACTH release (Taylor *et al.*, 1993) but its role in the regulatory actions of the glucocorticoids on PRL secretion remains to be determined. In the present study we have used *in vitro* (Hadley *et al.*, 1993) and *in vivo* models to examine the influence of dexamethasone (dex), anti-LC1 antisera (LC1-Ab) and LC1 on the release of PRL. Peptide release was measured by radioimmunoassay and the data were expressed as either ng/mg tissue and analyzed by Duncan's multiple range test *(in vitro)* or as ng/ml serum and analyzed by Schèffe's test *(in vivo)*. *In vitro* anterior pituitary segments (n=6/group), removed post mortem from adult male CFY rats (≈200g) (Taylor *et al.*, 1993), responded to vasoactive intestinal peptide (VIP, 0.1nM-1μM), thyrotrophin releasing hormone (TRH, 0.1nM-1μM), the adenylate cyclase activator forskolin (1μM-10mM) and the L-Ca^{2+}-channel agonist BAY K8644 (0.01-10μM) with significant ($P<0.05$ vs. basal which is ≈4ng/mg tissue±15%), concentration-dependent increases in ir-PRL release. Submaximal concentrations of VIP (10nM), TRH (10nM), forskolin (100μM) and BAY K8644 (10μM) which provoked the significant ($P<0.05$ vs. basal) increases in PRL release were used in subsequent experiments. Preincubation of the tissue with dex (0.1μM) significantly reduced($P<0.05$ vs. dex-free control), in a protein synthesis-dependent manner, the secretory reponses to VIP (6.2±1.9 vs. 11.7±2.2), TRH (7.5±2.9 vs. 16.1±4.1) and forskolin (2.9±0.9 vs. 7.4±2.9) but not to BAY K8644 (9.6±0.8 vs. 7.9±0.1). The attenuating effects of dexamethasone on the VIP and forskolin evoked release of PRL were reduced ($P<0.05$) by a monoclonal anti-LC1-Ab (Zymed; diluted 1:15000) but not by a correspondingly diluted control antibody (Sigma; anti-spectrin α + ß) while human recombinant LC1 (100fg-100ng/ml) and its stable N-terminal fragment (LC1$_{1-188}$; 10pg-10ng/ml) mimicked the actions of the steroid. By contrast neither the anti-LC1-Ab nor LC1$_{1-188}$ influenced the PRL responses to TRH in the presence or absence of dexamethasone.

For *in vivo* studies adult male CFY rats (≈200g) were injected subcutaneously on two successive days with either a purified polyclonal LC1-Ab or a corresponding volume (1ml/kg) of similarly purified non-immune sheep serum (NSS). On the following morning, rats from each group (n=12-16) were given either dexamethasone (100μg/kg, i.p.) or its saline vehicle (1ml/kg, i.p.) followed 1h 15 min later by laparotomy under ether anaesthesia (controls were left undisturbed in their cages). Laparotomy produced, within 1h, significant ($P<0.05$ vs. controls) rises in the serum ir-PRL concentration in NSS and anti-LC1-Ab treated animals (NSS treated 4.5±1.0 vs. 1.9±0.2 ng/ml: anti-LC1 Ab treated 3.9±0.5 vs 2.1±0.2 ng/ml). The rises in PRL release precipitated in NSS treated rats by the surgical trauma were abolished by pretreatment of with dex ($P<0.05$; 1.9±0.4 vs. 4.5±1.0ng/ml). By contrast, in animals treated with anti-LC1 Ab the inhibitory effects of the dex were not apparent (dex-free control; 4.1±1.9vs.3.9±0.4ng/ml, N.S.).

The results provide novel evidence to suggest that LC1 contributes to the mechanisms underlying the glucocorticoid-induced inhibition of PRL secretion in the rat.

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65P EVIDENCE FOR ARGININE VASOPRESSIN AS THE PRIMARY ACTIVATOR OF THE HYPOTHALAMO-PITUITARY ADRENAL AXIS DURING CHRONIC INFLAMMATORY STRESS

H.S. Chowdrey, P. J. Larsen[2], M. S. Harbuz[1], D.S. Jessop[1], & S. L. Lightman[1].
School of Biological & Chemical Sciences, University of Greenwich, London. [1]Department of Medicine, BRI, Bristol. [2]Institute of Medical Anatomy, University of Copenhagen, Denmark.

Adjuvant-induced arthritis (AA) is an immunologically mediated inflammatory disease that results in chronic activation of the hypothalamo-pituitary-adrenal (HPA) axis, as indicated by increased adrenal size, increased expression of anterior pituitary proopiomelanocortin mRNA, increased morning circulating corticotropin (ACTH) and corticosterone and loss of the normal diurnal rhythm of these hormones (Sarlis et al., 1992; Harbuz et al., 1992). We have further characterised the role of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) in the regulation of the HPA axis during development of AA in Piebald-Viral-Glaxo (PVG) and Sprague-Dawley (SD), the two susceptible rat strains. AA was associated with a significant increase in AVP mRNA levels in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN). In contrast, the expression of CRF mRNA in the same parvocellular division of the PVN was unchanged in the SD or decreased in the PVG rats. Coincident with the onset and the progression of inflammation, AVP levels in the portal plasma of PVG rats markedly increased from a control of 14 ± 2 fmol/ml to 42 ± 22 on day 7 and 101 ± 30 on day 11 following the injection of adjuvant. These AVP levels were further elevated by day 16 (217 ± 9) and were sustained on day 21 (224 ± 40), coincident with the period of maximal inflammation. The basal release, in vitro, of ACTH from pituitaries quarters of arthritic PVG rats was significantly greater than the respective controls (436 ± 91 pg/tube in AA v 167 ± 23; values are means ± s.e. mean; n=16). The ACTH release in response to CRF or the combination of CRF and AVP in the PVG arthritic rats was significantly greater compared with the controls although the ACTH response in the SD rats was unchanged. The data are consistent with the view that activation of the parvocellular vasopressinergic system has an important role in the adaptation of the HPA axis to chronic stress.

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66P EFFECTS OF MASTOPARAN UPON THE LATE STAGES OF THE ADRENOCORTICOTROPHIN (ACTH) SECRETORY PATHWAY OF AtT-20 CELLS

B.W. McFerran & S.B. Guild, Molecular Endocrinology Unit, School of Biological and Medical Sciences, University of St Andrews, St Andrews, UK.

Guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) stimulates adrenocorticotrophin (ACTH) secretion from permeabilised cells of the mouse AtT-20/D16-16 anterior pituitary cell line in the absence of calcium and without the activation of protein kinase C (PKC) suggesting that a guanosine 5'-triphosphate-binding protein (G-protein), termed G_E, is involved in mediating the effects of calcium upon secretion (McFerran & Guild, 1994). Mastoparan activates heterotrimeric G-proteins by a mechanism similar to that of agonist-occupied receptors (Higashijima et al., 1988) and was used to investigate the possibility that G_E in AtT-20 cells may be a heterotrimeric G-protein. The effects of mastoparan upon ACTH secretion from electrically permeabilised AtT-20 cells were studied as previously described (McFerran & Guild, 1994).

Mastoparan (10^{-8}-10^{-5}M) stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner with a threshold of 10^{-6}M and a 940 ± 88 % (n=3) increase over control at 10^{-5}M mastoparan, the highest concentration of this agent investigated. The stable guanosine 5'-diphosphate (GDP) analogue, guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) significantly reduced ACTH secretion in response to 10^{-5}M mastoparan from a 940 ± 88% (n=3) increase over control in the absence of GDP-β-S to a 585 ± 92 % (n=3) increase over control in the presence of GDP-β-S. ACTH secretion evoked by mastoparan (10^{-8}-10^{-5}M) was not significantly different in the presence or absence of chelerythrine chloride (10^{-5}M), a PKC inhibitor (Herbert *et al.*, 1990). Mastoparan (10^{-5}M) evoked a 738 ± 42% (n=3) increase in ACTH secretion over control in the absence of chelerythrine chloride and a 712 ± 82% (n=3) increase in ACTH secretion over control in the presence of chelerythrine chloride. ACTH secretion evoked by mastoparan (10^{-8}-10^{-5}M) was significantly inhibited by the omission of adenosine 5'-triphosphate (ATP, 5mM) from the permeabilisation medium. ACTH secretion in response to 10^{-5}M mastoparan was reduced from a 905 ± 83 % (n=3) increase over control to a 416 ± 34% (n=3) increase over control when ATP was omitted.

Mastoparan stimulates ACTH secretion from permeabilised AtT-20 cells in the absence of calcium and without activating PKC, characteristics consistent with those of GTP-γ-S in this system (McFerran & Guild, 1994). This suggests that mastoparan may act upon G_E and that this G-protein may be heterotrimeric. Mastoparan may, therefore, be a useful alternative to GTP-γ-S, which is a general activator of G-proteins, as a means of directly activating G_E.

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67P ACTIVATION OF HUMAN EOSINOPHILS BY C-C CHEMOKINES AND IL-5: A ROLE FOR PHOSPHOINOSITIDE 3-KINASE

A.D. Bourne, M.L. Watson & J. Westwick, Department of Pharmacology, University of Bath, Avon BA2 7AY

Members of the chemokine family and IL-5 have been proposed as the major eosinophil specific activators in allergic disease (Lindley *et al.*, 1993; Sanderson, 1992). We have compared the effect of the C-C chemokines and IL-5 on intracellular free calcium ($[Ca^{2+}]_i$) and hydrogen peroxide (H_2O_2) production in human eosinophils.

Human eosinophils (approximately 90% pure) were prepared from heparinised blood by negative selection with anti-CD16 particles using a protocol slightly modified from that of Hansell *et al.* (1991). $[Ca^{2+}]_i$ and H_2O_2 production were measured as previously described for guinea pig eosinophils (Bourne *et al.*, 1992).

All the C-C chemokines tested, except MIP-1 β (1-100nM), dose dependently elevated $[Ca^{2+}]_i$ above basal levels of 77.7±5.6nM (mean±s.e.mean). For example RANTES and MCP-3 at 100nM elevated $[Ca^{2+}]_i$ to 357.5±79.7nM and 232.3±24.2nM respectively. The rank order of potency was RANTES ≥MIP-1 α>MCP-3 >MCP-1. Neither MIP-1 β (100nM) or IL-5 (40nM) were able to elevate $[Ca^{2+}]_i$. Calcium desensitisation experiments indicated the presence of a RANTES specific receptor and other C-C chemokine receptors. RANTES, MCP-3 and IL-5 were all able to stimulate H_2O_2 production from eosinophils. Wortmannin, which at low nM concentrations is a selective phosphoinositide 3-kinase (PI3-kinase) inhibitor (Arcaro and Wymann, 1993), inhibited the H_2O_2 released from human eosinophils (table 1).

Table 1. Inhibition by wortmannin of H_2O_2 produced by human eosinophils in response to added agonist. Control H_2O_2 release (nmol/2x10^5cells over 30min, mean±s.e.mean , n=3-4) was 0.4±0.1; 1.1±0.3 and 1.4±0.4 for basal, RANTES (100nM) and IL-5 (40nM) treated cells respectively. Significant difference versus agonist alone: *, $p<0.05$; **, $p<0.01$ (Dunnetts t test).

Agonist	n	Wortmannin (nM); % Inhibition		
		3	10	30
Basal	4	46±22%	77±21%	78±22%
RANTES	4	46±6%*	76±13%**	87±13%**
IL-5	3	56±25%*	71±18%**	83±8%**

This data suggests that chemokines and IL-5 activate a common signalling pathway, PI3-kinase, for the generation of H_2O_2 production. Thus PI3-kinase could prove a useful target for the control of eosinophil function.

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68P THE ROLE OF CYTOKINES IN THE EFFECTS OF BACTERIAL LIPOPOLYSACCHARIDE (LPS) ON CYCLOOXYGENASE ACTIVITY IN CULTURED BOVINE AORTIC ENDOTHELIAL CELLS (BAEC)

P. Akarasereenont, Y.S. Bakhle, C. Thiemermann & J.R. Vane, *The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Sq., London EC1M 6BQ.*

Exposure of cultured BAEC to LPS over 24h increased cyclooxygenase (COX) activity and the amount of COX-2 protein in these cells (Akarasereenont et al., 1994). Many of the effects of LPS *in vivo* are mediated by cytokines (Mantovani et al., 1992). In order to assess the role of two cytokines as possible mediators of the induction of COX-2, we investigated the effects of specific antibodies to tumour necrosis factor-α (TNF-α) and epidermal growth factor (EGF) on the induction of COX activity afforded by LPS in BAEC. The effects of exogenous TNF-α and EGF on COX activity were also investigated.

The preparation and culture of the BAEC have been described (Akarasereenont et al., 1994); BAEC were incubated with LPS (1 μg/ml) and antibodies to cytokines for 24h. The major COX metabolite, prostacyclin, in the supernatant medium was measured by radioimmunoassay (as 6-oxo-PGF1α). BAEC were also incubated with exogenous cytokines (but without LPS) for 12h. Medium was then removed, the cells washed, incubated for 15 min with fresh medium containing arachidonic acid (30 μM) and the output of 6-oxo-PGF1α was measured. In other experiments, BAEC with LPS (1 μg/ml) or cytokines were washed, extracted and the extract examined by immunoblotting (Western blot) for COX-2 protein using a specific antibody (Akarasereenont et al., 1994). Results are given as means ± s.e.m. from 9 assays from 3 different cultures; $p<0.05$ (*t*-test for unpaired samples) for significant differences between means. The release of COX metabolites increased from 1.8±0.6 ng 6-oxo-PGF1α per ml in untreated cells to 56±7.2 ng/ml at 24h of treatment with LPS. Antibodies to TNF-α or EGF (0.1-10 μg/ml) caused a graded inhibition of this increase with a maximum inhibition of 70±3% for anti-TNF-α and 30±4% for anti-EGF (at 1 μg/ml). TNF-α and to a lesser degree EGF caused a significant increase in COX activity (TNF-α: 8-fold; EGF: 4-fold). Similarly, TNF-α (100 ng/ml) and to a lesser degree EGF (1 μg/ml) also caused the expression of COX-2 protein (Western blot analysis). Thus, the majority of the increase in COX activity and COX-2 protein induced by LPS in BAEC is mediated by TNF-α with a minor contribution from EGF.

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69P PAF AND LTB4 PRIME INTERLEUKIN-5-INDUCED EQUINE EOSINOPHIL ADHERENCE, BUT EOSINOPHILS FROM HORSES WITH ALLERGIC SKIN DISEASE DO NOT EXHIBIT ENHANCED ADHESIVE PROPERTIES

A.P. Foster, *P. McCabe, *S. Sanjar & F.M. Cunningham. Department of Veterinary Basic Sciences, The Royal Veterinary College, North Mymms, Herts., AL9 7TA and *Glaxo Group Research Limited, Ware, Herts.

Eosinophils isolated from horses with clinical signs of the allergic skin disease sweet itch show decreased migratory responses to LTB4 and PAF *in vitro* (Foster *et al.*, 1994a). One explanation for this finding could be that eosinophils from sweet itch horses have enhanced adhesive properties. Previous work has shown that human recombinant interleukin (hr IL)-5, but not PAF or LTB4, induces the adherence of eosinophils from normal horses to fibronectin-coated plastic (Foster *et al.*, 1994b). In the present study the priming effect of LTB4 and PAF on IL-5-induced eosinophil adherence has been examined in normal horses and the adhesive responses to these mediators of eosinophils isolated from sweet itch horses investigated.

Microtitre plates (96-well) were sequentially coated with human fibronectin (10 μg ml^{-1}) and blocked with bovine serum albumin (20 μg ml^{-1}, 2% BSA), each for 1 h at 37°C. Hr IL-5 (0.1-1000 ng ml^{-1}), LTB4 (10^{-10}-10^{-6}M), PAF (10^{-10}-10^{-6}M), or vehicle (PBS or 0.2% BSA) were added to test wells (n=6 for each concentration of agonist). Eosinophils (1 x 10^5 cells/well) purified from normal or sweet itch horses (Foster *et al.*, 1994a), with or without pre-treatment for 1 h at 37°C with LTB4 (10^{-6}M), PAF (10^{-6}M), lyso-PAF (10^{-6}M) or vehicle (0.2% BSA), were then added and incubated for 25 min at 37°C. Adherence was quantified as previously described (Foster *et al.*, 1994b). Dose-response curves to IL-5 were fitted using a non-linear curve modified Marquart procedure and maximum responses (EMax), EC_{50}s and Hill slopes determined.

Pre-treatment with LTB4 or PAF, but not lyso-PAF, significantly increased IL-5-induced maximum adherence of eosinophils isolated from normal horses.

Pre-treatment	Vehicle	Agonist
LTB4	2.3±0.6	3.6±0.7*
PAF	1.2±0.3	1.8±0.4*
lyso-PAF	1.6±0.4	1.7±0.4

Values are mean % maximum adherence (EMax) after subtraction of background levels ± s.e.mean; *$p<0.05$ compared to vehicle, paired Student's t test; n=4. Mean background adherence was in the range 0.2-0.4 %. The EC_{50}s and Hill slopes were no different after pre-treatment with LTB4, PAF or lyso-PAF. LTB4 and PAF did not cause adherence of eosinophils from sweet itch horses and there was no difference between IL-5-induced adherence in these and normal animals (n=4).

These findings show that LTB4 and PAF prime eosinophils of normal horses to the effects of IL-5. However, the decreased migratory responses of eosinophils from sweet itch horses observed previously is unlikely to result from increased adherence due to priming with LTB4 or PAF since adherence responses to IL-5 were no different in sweet itch and normal animals and neither LTB4 nor PAF caused adherence of these cells.

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70P ANTI-INFLAMMATORY EFFECTS OF THE PROTEIN KINASE C INHIBITOR, Ro 31-8220

S. Reynia, F.M. Williams, T.J. Williams, [1]A.G. Rossi. Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY.

Protein kinase C (PKC) activation plays an important role in signal transduction mechanisms in various isolated inflammatory cells (Nishizuka, 1986). PKC activation is necessary for increased endothelial cell permeability *in vitro* (Siflinger-Birnboim *et al*, 1992) and there is some evidence for the involvement of PKC in increased microvascular permeability to phorbol esters *in vivo* (Kuchera *et al*, 1993).

In this study we have investigated the effect of a specific PKC inhibitor, Ro 31-8220 (Ro 31), on inflammatory responses in rabbit skin. Rabbit peripheral neutrophils were purified and radiolabelled with $^{111}InCl_3$. ^{125}I-albumin, a marker of microvascular protein leakage and ^{111}In-neutrophils were injected i.v. into recipient rabbits. After 5 min inflammatory mediators were injected i.d ± Ro 31 at 10^{-8} and 10^{-9} mol/site. The vasodilator PGE_2 (3 x 10^{-10}mol/site) was co-administered to potentiate responses. The animals were killed after 1h. Microvascular permeability and neutrophil accumulation were expressed as μl of plasma and number of ^{111}In neutrophils/skin site, respectively. In other experiments rabbit neutrophils were isolated and the effect of Ro 31 on superoxide production and homotypic aggregation in response to agonists measured *in vitro*. All results are expressed as the mean ± s.e. mean.

Neutrophil accumulation induced by PMA (2 x 10^{-8} mol/site, 6352 ± 1976), FMLP (5 x 10^{-11} mol/site, 39946 ± 7982) and HrC5a (2.5 x 10^{-11}mol/site, 29616 ± 7249) was significantly inhibited by Ro 31 at 10^{-8} mol/site (3310 ± 1196, 25582 ± 4403, and 15906 ± 6618, respectively, n=5, $P<0.05$). In contrast, plasma protein extravasation induced by PMA (28.1 ± 2.8μl), FMLP (82.9 ± 20.2μl) and HrC5a (53.8 ± 15.2μl) were unaffected by 10^{-8} mol/site Ro 31 (34.6 ± 6.6μl, 67.8 ± 15.3μl, 56.5 ± 15.9μl, n=5). Similarly, responses to bradykinin (10^{-10} mol/site, 68.1 ± 11μl), a direct acting mediator of increased microvascular permeability were unaffected by 10^{-8}mol/site Ro 31 (59.6 ± 11.1μl, n=5). Ro 31 inhibited agonist-induced neutrophil aggregation and superoxide production (Table 1; *$p<0.05$, **$p<0.01$) in response to the PKC activator PMA (10^{-8}M), FMLP and HrC5a (both at 10^{-7}M). Neutrophil aggregation in response to 10^{-7}M A23187 was significantly inhibited, but superoxide production was unaffected.

These results suggest that PKC is important for the accumulation of neutrophils into inflammatory sites, without alterations in microvascular permeability. In the neutrophil, PKC can regulate secretion of endogenous mediators and alter aggregation properties of the cell in response to neutrophil activators.

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[1]Present address: Syntex Research Centre, Edinburgh, EH14 4AP.

Table 1. Effect of PKC inhibitor Ro 31-8220 on agonist-induced rabbit neutrophil superoxide production and aggregation

	Superoxide production (nmol $O_2^{\cdot-}$/ 5 x 10^5 cells)				Neutrophil aggregation (% from baseline)		
	PMA	FMLP	C5a	A23187	PMA	FMLP	A23187
Control	6.7 ± 0.8	3.1 ± 0.7	2.5 ± 0.5	1.0 ± 0.3	98 ± 5	44 ± 11	85 ± 18
+ Ro 31 10^{-6}M	0.8** ± 0.1	1.1* ± 0.3	2.0 ± 0.3	0.8 ± 0.1	56* ± 9.3	33* ± 4.1	66 ± 23
+ Ro 31 10^{-5}M	1.0** ± 0.3	0.7* ± 0.3	1.2* ± 0.5	0.7 ± 0.3	41**± 6.1	34* ± 3.4	56* ± 12

71P ELEVATION OF cGMP IN HUMAN ENDOTHELIAL CELLS BY FLURBIPROFEN-NITROXYBUTYLESTER, A NOVEL NONSTEROIDAL ANTI-INFLAMMATORY DRUG WITH REDUCED ULCEROGENIC ACTIONS *IN VIVO*

A.R. Baydoun, G. Cirino[+] & J.L. Wallace[++], Vascular Biology Research Centre, Biomedical Sciences Division, King's College, Campden Hill Road, London W8 7AH, U.K. [+]Department of Experimental Pharmacology, University of Naples, Via Domenico Montesano 49, 80131 Naples, Italy. [++]Department of Pharmacology and Therapeutics, 3330 Hospital Drive NW Calgary, Alberta, T2N 4N1 Canada.

Flurbiprofen-nitroxybutylester (FNBE) is one of a new class of compounds with potent anti-inflammatory activity but markedly reduced ulcerogenic effects in rats (Wallace *et al.*, 1994). Although the precise mechanism for the reduced ulcerogenic properties are as yet unclear it has been demonstrated that administration of FNBE *in vivo* results in a marked increase in plasma nitrite levels and reduced leukocyte adherence at sites of inflammation. These effects may be due to the generation of nitric oxide (NO), a potent vasodilator agent with anti-thrombotic properties (Moncada et al., 1991). We have now explored this hypothesis, examining whether FNBE is metabolised *in vitro* to liberate NO by measuring changes in cGMP levels in cultured human umbilical vein endothelial cells (HUVEC) following exposure to FNBE.

Monolayers of endothelial cells isolated from human umbilical veins by collagenase digestion were plated in 24 well trays and cultured to confluency in medium M199 supplemented with 10 % foetal and 10 % new born calf serum. Cells were preincubated with 0.5 mM isobutylmethylxanthine (IBMX) for 15 min followed by a 5 min incubation with FNBE either alone or in combination with other agents. cGMP levels in cell extracts was determined by standard radioimmunoassay.

Incubation of HUVEC with FNBE (0.1 - 100 μg/ml) resulted in a concentration-dependent increase in cGMP, with levels elevated from a basal value of 1.76 $\pm$ 0.25 pmol/10^6 cells to 3.99 $\pm$ 0.18 pmol/10^6 cells ($p < 0.05$; $n = 6$) at the maximum concentration (100 μg/ml) of FNBE used. The parent compound, flurbiprofen (0.1 - 100 μg/ml), caused no significant change in cGMP levels. In comparison, administration of a maximal concentration of histamine (10 μM), an agonist which elevates cGMP via activation of constitutive nitric oxide synthase (cNOS), resulted in a more dramatic increase in cGMP, elevating basal values to 21.3 $\pm$ 1.92 pmol/10^6 cells (n= 4). In contrast to histamine, increases in cGMP levels induced by FNBE were unaffected by pretreatment of cells for 15 min with either L-N^G-monomethyl-L-arginine (L-NMMA; 100 μM) or N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM), which are potent inhibitors of cNOS. Both compounds virtually abolished responses to histamine.

In conclusion, we have demonstrated that cGMP levels can be elevated by FNBE in cultured vascular cells via mechanisms independent of cNOS activation. More importantly the increase in cGMP may provide a possible explanation for the reduced ulcerogenic properties of FNBE observed in rats (Wallace *et al.*, 1994).

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72P DEFICIENT EXPRESSION, AXONAL TRANSPORT AND RESPONSE TO NERVE GROWTH FACTOR IN DIABETIC RATS: EFFECT OF ITS REPLACEMENT WITH RECOMBINANT PROTEIN

L.T. Diemel, P. Fernyhough, J. Hardy, W.J. Brewster, L. Mohiuddin & D.R. Tomlinson, Department of Pharmacology, Queen Mary and Westfield College, London, U.K.

Diabetic rats were treated with NGF (0.2, 0.5 and 1.0 mg/kg s.c. 3 times per week for the last 4 weeks of 8 weeks streptozotocin-diabetes). At the end of this time the sciatic nerve levels of NGF-like immunoreactivity (NGF-LI) were compared with those of CGRP-like immunoreactivity (CGRP-LI by ELISA and RIA respectively). Data (mean $\pm$ 1SD) are given in the table.

Group/Treatment (n)	NGF-LI (pg/cm nerve)	CGRP-LI (ng/cm nerve)
Controls (8)	$72\pm20^{b,e}$	3.1 ± 0.7^{b}
Diabetic untreated (10)	34 ± 12^{a}	$1.8\pm0.6^{a,c}$
Diabetic + insulin (8)	$62\pm18^{b,c}$	2.5 ± 0.3^{c}
Diabetic + NGF 0.2 mg/kg (7)	$107\pm51^{b,e}$	$4.2\pm1.7^{b,d}$
Diabetic + NGF 0.5 mg/kg (7)	$111\pm35^{b,d}$	$5.2\pm1.3^{b,d,f}$
Diabetic + NGF 1.0 mg/kg (8)	$198\pm106^{b,d,f}$	$5.2\pm1.3^{b,d,f}$
Levels of significance:-	a *vs.* b, c *vs.* d, e *vs.* f significant at $p<0.05$	

In a separate protocol retrograde axonal transport of NGF was examined using the two higher doses of NGF, given in a treatment schedule identical to that cited above. The left sciatic nerve was ligated (halothane recovery anaesthesia). Six hours later, vehicle-treated diabetic rats showed a significant reduction in the accumulation of NGF distal to the crush (45% of control, $p<0.05$). NGF levels in segments from the contralateral unligated nerve (47% of control, $p<0.05$) and dorsal root ganglia (43% of control, $p<0.01$) were significantly reduced compared to controls. Treatment with exogenous NGF reversed these deficits; levels of NGF in each nerve segment or pair of ganglia were raised above controls. NGF levels in foot skin, gastrocnemius and soleus muscle (target tissues for neurons in the sciatic nerve) were reduced in vehicle-treated diabetic rats compared to controls. Treatment with exogenous NGF reversed these deficits, but levels of NGF mRNA in soleus muscle and foot skin were also reduced and were not affected by exogenous NGF treatment, thereby demonstrating that increased levels of NGF in tissues of rats injected with the neurotrophin were derived by uptake and transport of the exogenous material. In another diabetic group, treatment with sustained release insulin implants (4IU/day) normalised all variables. Thus, experimental diabetes is associated with reduced synthesis of NGF in peripheral target tissues, manifest as a reduction in retrograde axonal transport of NGF from the periphery. Exogenous NGF is retrogradely transported to the dorsal root ganglia, where it can normalised deficient gene expression for CGRP.

73P EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISM ON SCIATIC NERVE FUNCTION IN EXPERIMENTAL DIABETES

E.J. Stevens & D.R. Tomlinson, William Harvey Research Institute, Department of Pharmacology, Queen Mary and Westfield College, London E1 4NS.

Experimental diabetes causes reductions in sciatic nerve motor conduction velocity (Tomlinson & Mayer, 1985) and laser Doppler flux (Stevens *et al.*, 1994). This study investigated the effects of endothelin (ET) receptor antagonism with the non-peptide, non-selective ($ET_A/_B$) compound bosentan (Clozel *et al.*, 1994) on these abnormalities. Rats with 5-6 weeks untreated streptozotocin-diabetes exhibited characteristic slowed nerve conduction velocity (mean±SD in m s^{-1}; 36.6±3.4) compared to age-matched control animals (42.7±2.4, $p<0.01$). Preventative treatment of diabetic rats with bosentan at 100mg^{-1} kg^{-1} day *po* attenuated this deficit (39.7±3.0). This treatment also significantly attenuated decreases in nerve laser Doppler flux (arbitrary units; 398±77 in controls and 197±64 in diabetics, $p<0.01$; 305±56 in bosentan-treated diabetics, $p<0.01$ versus controls and diabetics). Mean arterial pressures (MAP) were lower in diabetic rats (mmHg; 104±8) compared to control animals (119±15, $p<0.01$) but were not affected by bosentan treatment (100±11, $p<0.01$ *versus* controls). In control and untreated diabetic rats, 1nmol^{-1} kg *iv* ET-1 caused an initial hypotension (duration in s, 31±13 and 26±9, respectively; maximal change in MAP in mmHg, -27±13 and -25±7, respectively) followed by prolonged hypertension (maximal change in MAP, 52±18 and 31±5, respectively). Bosentan efficacy was demonstrated by inhibition of the hypotensive response to ET-1 in treated diabetic rats (5±2s; -4±2mmHg) although the hypertension was unaltered (32±9mmHg).

Acute *iv* infusion of 10mg^{-1} kg bosentan caused variable and transient rises in nerve laser Doppler flux in control (peak, 78±63) and untreated diabetic rats (93±77). Bosentan infusion blocked the hypotensive response to subsequent ET-1 infusion but only attenuated the later hypertension in control rats (change in MAP, 21±9 in controls, 29±10 in diabetics).

In summary, these data show that oral treatment of diabetic rats with an ET receptor antagonist can improve poor sciatic nerve perfusion and conduction. This suggests that the vasoconstrictor action of endogenous ET may contribute to peripheral nerve dysfunction in experimental diabetes.

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74P EFFECTS OF THE NON-PEPTIDE, NON-SELECTIVE ENDOTHELIN ANTAGONIST, BOSENTAN, ON REGIONAL HAEMODYNAMIC RESPONSES TO NG-MONOMETHYL-L-ARGININE (L-NMMA) IN CONSCIOUS RATS

S.M. Gardiner, P.A. Kemp, J.E. March & T. Bennett. Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham NG7 2UH.

In vitro data indicate that nitric oxide (NO) inhibits endothelin (ET) release (Boulanger & Lüscher, 1990), hence it is feasible that some of the regional haemodynamic effects of the NO synthase inhibitor, L-NMMA *in vivo* are due to ET release. We have assessed this possibility in conscious Long Evans rats (n = 8), chronically-instrumented with intravenous and intra-arterial catheters, and pulsed Doppler flow probes to monitor changes in renal, mesenteric and hindquarters flows. All surgery was carried out under sodium methohexitone anaesthesia (40-60 mg kg^{-1} i.p., supplemented as required). The protocol involved animals receiving a bolus dose of L-NMMA (30 mg kg^{-1} i.v.) on 4 consecutive days, but on the last day, the animals received bosentan (30 mg kg^{-1} i.v. Gardiner *et al.*, 1994) 10 min before L-NMMA. Results are summarized in Tables 1 and 2.

Table 1. Resting cardiovascular variables prior to administration of L-NMMA. Values are mean ± s.e. mean (n=8). R = renal, M = mesenteric, H = hindquarters vascular conductance (VC).

	Day 1	Day 2	Day 3	Day 4
HR (beats min^{-1})	334 ± 6	339 ± 9	339 ± 6	342 ± 6
BP (mmHg)	103 ± 2	105 ± 2	105 ± 1	111 ± 5
RVC (kHz mm Hg^{-1})10^3	61 ± 5	58 ± 6	61 ± 7	56 ± 5
MVC (kHz mm Hg^{-1})10^3	72 ± 9	71 ± 10	80 ± 10	81 ± 12
HVC (kHz mm Hg^{-1})10^3	42 ± 3	39 ± 2	37 ± 1	38 ± 3

Resting cardiovascular variables were not different on the 4 experimental days prior to administration of L-NMMA (Table 1). There was a progressive increase in the pressor and regional vasoconstrictor responses to L-NMMA on Days 1, 2 and 3 (Table 2). On Day 4, in the presence of bosentan, the renal and hindquarters vasoconstrictor responses to L-NMMA were less than on Days 2 and 3, but not different from those on Day 1, whereas the pressor and mesenteric vasoconstrictor responses were smaller than on any other day (Table 2).

Table 2. Cardiovascular responses to L-NMMA (30 mg kg^{-1}) in the absence (Days 1,2 and 3) and presence (Day 4) of bosentan (30 mg kg^{-1}). Values (mean ± s.e. mean; n = 8) are expressed as areas under or over curves (0-5 min). $^{a}P < 0.05$ vs Day 1; $^{b}P < 0.05$ vs Day 2; $^{c}P < 0.05$ vs Day 3 (Friedman's test).

	Day 1	Day 2	Day 3	Day 4
HR (beats)	-213 ± 32	-289 ± 30	-292 ± 48	-260 ± 26
BP (mmHg min)	144 ± 20	172 ± 12^{a}	190 ± 9^{ab}	127 ± 8^{abc}
RVC (% min)	-161 ± 17	-176 ± 11	-193 ± 6^{a}	-147 ± 5^{bc}
MVC (% min)	-233 ± 15	-249 ± 7	-250 ± 7^{a}	-216 ± 9^{abc}
HVC (% min)	-151 ± 12	-183 ± 12^{a}	-185 ± 14^{a}	-156 ± 16^{bc}

These results indicate that a component of the haemodynamic response to L-NMMA may be due to disinhibition of ET release.

Thanks to Dr M Clozel for the gift of bosentan.

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75P BQ123 INHIBITS [^{125}I]ET-1 AND [^{125}I]S6B BINDING WITH SUBNANOMOLAR AFFINITY BUT IS A MORE POTENT ANTAGONIST OF S6b VASOCONSTRICTION IN HUMAN ISOLATED BLOOD VESSELS

J.J. Maguire, R.E. Kuc, B.A. Rous, F. Fitzgerald* & A.P. Davenport. Clinical Pharmacology Unit, University of Cambridge, Box 110, Level 2, F&G Block, Addenbrooke's Hospital, Cambridge, CB2 2QQ, U.K. *Amersham International plc, Amersham, Bucks.

Endothelin (ET) antagonists appear to be more effective against *in vitro* responses to sarafotoxin 6b (S6b) than against ET-1 in human vascular preparations (Bax *et al.*, 1993; Bodelsson and Stjemquist, 1993). One explanation is that the sarafotoxins and the endothelins mediate their effects through a heterogeneous receptor population. We have shown that micromolar concentrations of the ET_A selective antagonist BQ123 (cyclo(D-Trp-D-Asp-Pro-Val-Leu)) will antagonise ET-1 vasoconstriction in human isolated blood vessels, however this compound competes for [^{125}I]-ET-1 binding in the same tissues with sub-nanomolar affinity. To investigate these discrepancies further we have determined the potency of BQ123 as an antagonist of S6b-mediated vasoconstriction in human saphenous vein (SV) and compared it to the K_D obtained for BQ123 in competition binding experiments against [^{125}I]-S6b binding in SV homogenates. Additional experiments were carried out to determine whether differences in the rates of dissociation can explain the different sensitivity of these agonists to BQ123 in the organ bath.

Saturation binding assays: SV membranes were incubated for 2 hours (23°C) with increasing concentrations (15pM-2nM) of [^{125}I]-S6b (Amersham International). Competition binding assays: SV membranes were incubated for 2 hours (23°C) with 0.1nM [^{125}I]-S6b and increasing concentrations (20pM-100μM) of BQ123.

Dissociation experiments: 10μm sections of human aorta were incubated for 2h (23°C) with 1nM of either [^{125}I]-ET-1 or [^{125}I]-S6b. After washing, sections were immersed in HEPES buffer for increasing time periods of up to 4 hours. In each experiment non-specific binding was determined by inclusion of 1μM of the unlabelled peptides.

Organ bath experiments: 2mm rings of endothelium-denuded SV were suspended in organ baths containing oxygenated Krebs soln. (37°C). Cumulative dose response curves were constructed to S6b (10^{-10}-10^{-6}M) in the absence and presence of BQ123 (10-300nM) added 30 min prior to S6b. Experiments were terminated by addition of 50mM KCl and S6b responses expressed as a percentage of this maximum. Values of pA_2 were derived from the Schild data.

From the saturation data [^{125}I]-S6b has a K_D value of 0.2±0.01nM, a B_{max} value of 57.2±1.03 fmol/mg protein with a Hill slope of 0.98± 0.01. In competition experiments BQ123 competed for the binding of [^{125}I]-S6b in a biphasic manner with calculated K_D values $ET_{A=}$ 0.52±0.02nM, $ET_{B=}$11.2±0.31μM. These are comparable to values obtained for BQ123 against [^{125}I]-ET-1. (All values are mean ± s.e.mean, n=3). From the kinetic experiments it was apparent that whilst 20% of specifically bound [^{125}I]-ET-1 and [^{125}I]-S6b had dissociated after 4 hours there was no significant difference between the two peptides.

BQ123 caused rightward shifts of the S6b dose response curve with a pA_2 value of 8.42 and regression slope of 0.92 (n=4). The calculated K_B value of 4.8nM was 100 times better than that previously obtained in saphenous vein for antagonism of ET-1 vasoconstriction by BQ123 (Maguire and Davenport, 1993).

These data indicate that BQ123 competes for both [^{125}I]-ET-1 and [^{125}I]-S6b binding with sub-nanomolar affinity whereas BQ123 is 100 times more potent an antagonist of S6b vasoconstriction than of ET-1 vasoconstriction. This discrepancy does not appear to be due to a slower dissociation rate for ET-1. Further investigation is required to determine why it is more dfficult to antagonise the functional effects of ET-1 in human isolated blood vessel preparations.

We are grateful to the Theatre staff at Papworth Hospital for permission to collect tissue samples.

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76P PHOSPHORAMIDON INHIBITS THE *IN VIVO* CONVERSION OF BIG ET-1 TO ET IN MAN

C. Plumpton, W.G. Haynes[1], D.J. Webb[1] & A.P Davenport, Clinical Pharmacology Unit, University of Cambridge, Box 110, Level 2, F & G Block, Addenbrooke's Hospital, Cambridge, CB2 2QQ and [1]Dept of Medicine, Western General Hospital, Edinburgh, EH4 2XU

Big endothelin 1 (big ET-1) is much less effective than ET-1 in constricting human isolated blood vessels (Howard *et al*, 1992). When infused into human forearm arteries, the peptide causes slow onset dose-dependent vasoconstriction (Haynes & Webb, 1994). It is hypothesised that this is caused by the synthesis of biologically active mature peptide and an inactive C-terminal fragment (CTF) following cleavage of big ET-1 by an endothelin converting enzyme (ECE). Our aim was to determine whether immunoreactive (IR) ET-1 and CTF could be detected following infusion of big ET-1.

With local ethical approval, big ET-1 (50pmol/min) was infused into the brachial artery of the non-dominant arm of six healthy male subjects. Blood samples were taken prior to infusion and at 60 min from both arms. This was repeated on a second study day but in the presence of phosphoramidon (30 nmol/min). Plasma samples were solid phase extracted using C18 minicolumns and evaporated to dryness. Plasma concentrations of ET, big ET-1 and CTF were determined by radioimmunoassays using antisera raised against the C-terminii of ET and big ET-1 (Plumpton *et al*, 1993). Results are mean of six individuals ± s.e.mean. Data were analysed by ANOVAR.

Peptide	Arm	Pre Infusion	60 min
IR Big ET-1 (pM)	Infused	Not detected	386 ± 106
	Non-infused	Not detected	8.67 ± 2.99
IR ET (pM)	Infused	2.2 ± 0.73	7.50 ± 1.77
	Non-infused	2.17 ± 0.06	3.00 ± 0.93
IR CTF (pM)	Infused	0.17 ± 0.17	37.2 ± 13.0
	Non-infused	Not detected	0.67 ± 0.49

Table 1: Big ET-1 infusion

Infusion of big ET-1 over 0 to 50 min caused a -33% change in average blood flow (-49 to -17; 95% confidence intervals, Haynes & Webb, 1994). IR big ET-1 was not detectable in the pre-infusion samples but increased in both arms after 60 min. IR ET was detected in pre-infusion samples, arising from endogenous ET. These levels were signicantly increased in the samples from the infused arm but not the non-infused arm. In concordance with this the CTF concentrations also increased in the infused arm (P< 0.05).

Peptide	Arm	Pre Infusion	60 min
IR Big ET-1 (pM)	Infused	Not detected	97.2 ± 32.1
	Non-infused	Not detected	54.2 ± 31.1
IR ET (pM)	Infused	2.33 ± 0.62	1.83 ± 0.48
	Non-infused	3.17 ± 1.08	2.67 ± 0.76
IR CTF (pM)	Infused	0.17 ± 0.17	10.83 ± 3.83
	Non-infused	0.17 ± 0.17	7.00 ± 4.53

Table 2: Big ET-1 and phosphoramidon infusion

In the presence of phosphoramidon, there was no significant increase in IR ET in the arm infused with big ET-1 after 60 min. This is in agreement with the abolition of vasoconstriction observed in the vessel.

In the human brachial artery big ET-1 induced vasoconstriction is associated with a significant increase in IR ET and CTF after 60 min. This rise is blocked by coinfusion of phosphoramidon suggesting that a phosphoramidon sensitive ECE is at least in part responsible for the vasoconstrictive properties of big ET-1.

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77P CHARACTERIZATION OF ENDOTHELIN RECEPTORS MEDIATING CONTRACTION AND RELAXATION IN THE ISOLATED FUNDUS OF THE RAT STOMACH

Bruno Battistini, Graham H Allcock, Timothy D. Warner and John R. Vane. *The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, EC1M 6BQ, U.K.*

In rat stomach strips (RSS), endothelins (ET-1, ET-3) and sarafotoxin (SX) 6c cause contractions (Warner et al., 1993) while ET-1, but not ET-3, causes relaxations (Olsen and Weis, 1992). Here, we have used ET-1, ET-3, SX6c and IRL 1620 (selective ET_B receptor agonists; Williams et al., 1991; Takai et al., 1992, respectively) in addition to various antagonists to characterize ET receptors on RSS that mediate contraction and relaxation. To study the activity of endothelin-converting enzyme (ECE) within this preparation, we also assessed the contractile activity of three precursors, big $ET\text{-}1_{1\text{-}38}$, big $ET\text{-}1_{1\text{-}39}$ and big $ET\text{-}3_{1\text{-}41}$amide.

Male Wistar rats (250-300 g) were killed with thiopentone sodium (Sagatal, 120 mg kg^{-1} i.p.). The stomach was rapidly excised, placed in warmed (37°C) and oxygenated (95% O_2 : 5% CO_2) Krebs-Henseleit buffer solution (pH 7.4) and trimmed free of connective tissue and fat. Strips were prepared from the fundal part of the stomach (Vane, 1957). The tissues were mounted in 10 ml organ baths under tensions of 1.5 g and connected to isometric transducers (Hugo Sachs K30, Germany) coupled to a chart recorder (Graphtec WR3310, Japan). After equilibration for 60 min (with washing every 15 min), the tissues were contracted three times with 80 mM KCl, with a 30 min washout period between each contraction. Cumulative concentrations (0.1-300 nM) of agonists were added to the tissues. Only one agonist with or without one antagonist was tested in each preparation. The responses were standardized as the percentage of the mean contraction evoked by 80 mM KCl.

ET-1, ET-3, or selective ET_B agonists SX6c and IRL 1620 (10^{-10} - 3 x 10^{-7} M) induced equipotently concentration-dependent contractions of the RSS. The contractions were not antagonised by the ET_A antagonist BQ-123 (10^{-5} M) but were inhibited by the ET_B antagonist BQ-788 (10^{-5} M) or the mixed antagonist PD 145065 (10^{-5} M). Application of ET-1, ET-3, SX6c or IRL 1620 (3 x 10^{-7} M) produced contractions that peaked within 1-3 min. Human big $ET\text{-}1_{1\text{-}38}$ (3 x 10^{-7} M) produced contractions that were of a similar size to those of ET-1 (3 x 10^{-7} M) but that took 8-10 times longer to develop. Big $ET\text{-}3_{1\text{-}41}$amide (3 x 10^{-7} M) induced a contraction that was 6% of that of ET-3 (3 x 10^{-7} M). In RSS precontracted with PGE_2 (3 x 10^{-8} M), ET-1 (3 x 10^{-9} M) produced a transient (<2 min) relaxation (approx. 40% of the induced tone). This relaxation was blocked by BQ-123 (10^{-5} M), suggesting that it was mediated by ET_A receptors. The relaxation was changed from transient to sustained in the presence of the ET_B antagonist BQ-788 (10^{-5} M).

Thus, isolated strips of the rat stomach contain a population of ET_B receptors that mediate contraction, ET_A receptors that mediate relaxation and an endothelin-converting enzyme (ECE) that is more active in converting big ET-1 into ET-1 than big ET-3 into ET-3.

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78P ENDOTHELIN-1 CONTRIBUTES TO THE *EX VIVO* INCREASE IN PERFUSION PRESSURE IN HEARTS TAKEN FROM IL-2-TREATED RATS

P. Klemm*, T. Hohlfeld#, R. Corder, T.D. Warner, C. Thiemermann and J. R. Vane. The William Harvey Research Institute, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ; *Department of Pharmacology, Cassella AG, Frankfurt/Main, Germany; #Department of Pharmacology, University of Düsseldorf, Germany.

Treatment with interleukin-2 (IL-2) or IL-2 plus lymphokine-activated killer (LAK) cells produces disease regression in patients with advanced metastatic melanoma or renal cell cancer (Rosenberg et al., 1994). Unfortunately, this is associated with a greatly elevated risk of myocardial infarction (MI) via an unknown mechanism. As IL-2 enhances the release of the potent vasoconstrictor endothelin-1 (ET-1) from intact blood vessels (Miyamori et al., 1991), we have used the ET_A receptor selective antagonist, FR 139317, to investigate whether ET-1 mediates some of the cardiac events following administration of IL-2 to the rat.

Male Wistar rats (230-260 g) were anaesthetized (sodium thiopentone, 120 mg kg^{-1}, i.p.) and the trachea cannulated to facilitate respiration, the right carotid artery to measure blood pressure and the right jugular vein for the administration of drugs. After a 30 min stabilization period, animals were given IL-2 ($2x10^6$ U, bolus, i.v.) or vehicle. 240 min later rats were killed by cutting the diaphragm and the hearts immediately removed for perfusion, by the Langendorff technique, with warmed (37°C) and gassed (95% O_2: 5%CO_2) Krebs' buffer (12 ml min^{-1}). The ET_A receptor-selective antagonist, FR 139317 (Sogabe et al., 1992) (0.3 mg kg^{-1} min^{-1}, i.v.), or vehicle, was infused constantly from 1 min before the injection of IL-2 and was present in the Krebs' buffer (FR 139317, 1 μM) throughout the subsequent *in vitro* experiments.

Hearts from control rats had an initial perfusion pressure of 47±2 mm Hg, which increased slightly during 120 min of perfusion (Table 1). However, hearts taken from IL-2 treated rats developed greatly elevated perfusion pressures (Table 1). This increase was significantly prevented by FR 139317.

Treatment	Coronary Perfusion Pressure at 0, 30, 60, 90 and 120 min (mm Hg)				
	0	30	60	90	120
Control	47 ± 2	52 ± 1	52 ± 1	56 ± 1	55 ± 2
IL-2 (240 min)	51± 1	125 ± 5*	131 ± 9*	128 ± 8*	135 ± 6*
IL-2 + FR 139317	55 ± 1	100 ± 3#	105 ± 6#	95 ± 3#	85 ± 4#

Table 1. Values are mean ± S.E.M (n=6). * $p<0.05$ vs. control, # $p<0.05$ vs. IL-2 by ANOVA and Bonferoni test, as appropriate.

Thus, IL-2 treatment *in vivo* causes a marked increase in coronary perfusion pressure *ex vivo* of which a large part is mediated by ET-1. Therefore, coronary vasoconstriction secondary to an increased production of ET-1 may contribute to MI seen in IL-2 treated patients.

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79P EFFECTS OF BQ-123 ON CONTRACTIONS INDUCED BY ENDOTHELIN-1 OR SARAFOTOXIN 6b IN THE HUMAN MIDDLE MENINGEAL ARTERY

W.B. Stam*[1], C.J.J. Avezaat[2] & P.R. Saxena[1], Departments of Pharmacology[1] and Neurosurgery[2], Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

Elevated plasma levels of endothelin-1 (ET-1) have been observed in patients during migraine attacks and this increase was greatest in the first phase of the attack (Gallai *et al.*,1994). Since the meningeal vasculature has been suggested as a likely source of the migraine headache, we investigated the effect of the ET_A receptor antagonist BQ-123 on contractile responses of human middle meningeal artery (HMA) induced by ET-1 or sarafotoxin 6b (Sf6b) (both 10^{-11}-3.10^{-7} M). HMA segments (2-3 mm) were suspended in an organ bath (15 ml) for isometric tension measurements. For each agonist concentration response curves (CRC) were constructed in parallel in the absence or presence of BQ-123 (10^{-8}, 10^{-7} and/or 10^{-6} M); n=4-5. Both ET-1 and Sf6b contracted HMA in a concentration dependent manner (Fig. 1). The maximal response and the potency of ET-1 and Sf6b, respectively, were: E_{max}, 154.2±27.5 vs 78.8±10.2 % of maximal response to KCl (40 mM); pEC_{50}: 9.02±0.14 vs 8.14±0.22. In the presence of BQ-123 (10^{-8} and 10^{-7} M), there was a non-significant shift in the CRC to ET-1. However, in the lower concentration range (10^{-11}- 3.10^{-9} M), BQ-123 (10^{-7} M) caused a significant rightward shift; the dose ratio, based on this part of the curve, was 2.92±0.5. It therefore seems that contractions to low concentrations of ET-1 are apparently mediated by an ET_A receptor, while at higher concentrations a receptor insensitive to BQ-123 is more involved. The CRC to Sf6b was also shifted to the right by BQ-123 (10^{-7} and 10^{-6} M). When the CRC to Sf6b in the presence of BQ-123 (10^{-7} M) was extrapolated to the maximum of its control curve, a significant rightward shift was observed; the dose ratio being 27.8±9.5. Since BQ-123 (10^{-7} M) caused a more pronounced shift in the CRC to Sf6b than to ET-1 (lower concentration range), it appears that Sf6b is either selective for BQ-123-sensitive receptor or it may act on a receptor different from those activated ET-1.

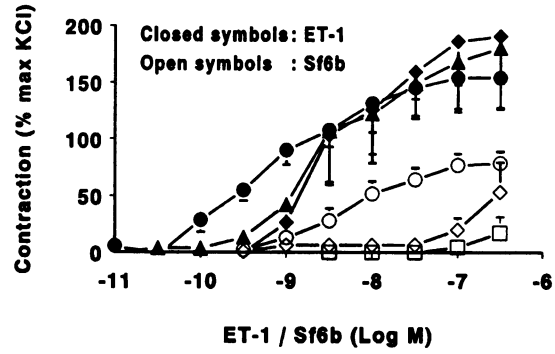


Fig. 1: Contractions of HMA by ET-1 and Sf6b in absence (circles) or presence of BQ 123; 10^{-8} M (triangle), 10^{-7} M (diamonds), and 10^{-6} M (squares).

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80P ANTAGONISM OF ENDOTHELIN A-RECEPTORS, BUT NOT ENDOTHELIN B-RECEPTORS, ATTENUATES THE REGIONAL AND SYSTEMIC HAEMODYNAMIC CHANGES TO ENDOTHELIN-1 IN THE ANAESTHETISED RAT

Graham H. Allcock, Timothy D. Warner and John R. Vane, *The William Harvey Research Institute, St. Bartholomews Hospital Medical College, Charterhouse Square, London, EC1M 6BQ.U.K.*

Endothelin-1 (ET-1) produces vasoconstriction, via activation of ETA and ETB receptors on vascular smooth muscle, and vasodilatation, via ETB receptors on endothelial cells (Warner et al., 1994). Here we have used the ETA receptor selective antagonist, BQ-123, the ETB receptor selective antagonist, BQ-788 and the ETA/ETB receptor non-selective antagonist, PD 145065 (Warner et al., 1994; Ishikawa et al., 1994) to study the roles of these receptors in mediating haemodynamic changes induced by an infusion of ET-1.

Male Wistar rats (330-430 g) were anaesthetised (thiopentone sodium, 120 mg/kg i.p.) and a cannula introduced into the left ventricle, via the carotid artery, for the administration of ^{57}Co labelled microspheres. Arteries were also cannulated to take a reference blood sample (for the calculation of total peripheral resistance, TPR and cardiac output, CO; McDevitt and Nies, 1976) and for the measurement of mean arterial blood pressure (MAP), and veins for the administration of drugs. After a 30 min equilibration period the rats received hexamethonium (10mg/kg, i.v., over 5 min) and 20 min later an infusion of ET-1 (10pmol/kg/min) together with an antagonist (10nmol/kg/min) or its vehicle was begun. Control rats received only hexamethonium. The microspheres were injected 70 min after the onset of the ET-1 infusion.

BQ-123 attenuated the effects of ET-1 on CO, TPR, and on renal and skin vascular resistances. Conversely, BQ-788 tended to enhance the effects of ET-1, and PD 145065 was without effect (see Table 1). Thus, BQ-123 , a selective ETA receptor antagonist, inhibits the pressor and vascular constrictor effects of ET-1 more actively than PD 145065. As BQ-788 potentiates, for instance, the renal constrictor effects of ET-1, and tends to increase the effects of ET-1 on TPR this suggests that despite their ability to mediate vasoconstrictions the predominant role of ETB receptors in the rat circulation is to limit the pressor effects of ET-1.

*Table 1. Cardiovascular parameters at the time of microsphere injection (n=4-5). † P<0.05 compared to control values, * P<0.05 compared to ET-1 determined by an unpaired t test or Mann Witney U test, as appropriate.*

	Control	ET-1	ET-1 + BQ-123	ET-1 + BQ-788	ET-1 + PD 145065
increase in MAP (mmHg)	0.7±7.2	57.3±6.6†	30.5±2.5*†	66.3±12.4†	61.4±6.3†
CO (ml.min^{-1})	102.6±11.3	57.7±9.5†	88.1±11.3*	35.9±5.3†	64.3±9.6†
TPR(mmHg.ml^{-1}.min.100g body weight)	3.24±0.6	10±0.8†	5.21±0.76*	14.84±2.08†	8.68±1.46†
Organ vascular resistances					
Heart (mmHg.min.ml^{-1})	22.5±4.5	35.4±4.8	45.9±12.7	46±0.7	43.1±15.3
Brain (mmHg.min.ml^{-1})	60.7±11.3	66.6±13.4	76.9±17.9	77.7±13.3	81.9±15.5
Right kidney (mmHg.min.ml^{-1})	12.9±0.6	121.2±29.2†	30.1±3.6*†	214±14.5*†	82.5±19.1†
small intestine (mmHg.min.ml^{-1})	5.5±0.7	23.1±3.2†	19.9±2.6†	31.8±2†	18.8±4.1†
skin (mmHg.min.ml^{-1})	13.2±2.9	46±13.3†	13.1±1.1*	81.9±12†	28.9±4.6†

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81P INDUCIBLE PROSTANOID SYNTHESIS MAY ACCOUNT FOR THE HYPERACTIVITY OF ENDOTHELIUM-DEPENDENT CONTRACTING FACTORS IN SHR AORTA

A. Zerrouk, M. Auguet, P.-E. Chabrier and P. Braquet, Institut Henri Beaufour Research Labs., ZA de Courtaboeuf - 1, ave des Tropiques 91952 LES ULIS CEDEX - France

Endothelium derived relaxing (nitric oxide) and contracting (prostaglandins) factors play a role in the local regulation of the vascular tone of the Wistar kyoto (WKY) and the spontaneously hypertensive rats (SHR). Like nitric oxide synthases, cyclooxygenases which metabolizes arachidonic acid to the prostaglandin endoperoxides PGG_2 and PGH_2 exist in a constitutive (COX-1) and an inducible (COX-2) isoforms. Thus, the aim of this study was to investigate whether endothelium derived prostanoids in SHR aorta are due to the constitutive or the inducible form of the enzyme.

Thoracic aortae endothelium-intact or denuded rings, 3-4 mm long, from male SHR and WKY rats (14 weeks old) were suspended (2g tension) in organ baths containing Krebs-Henseleit solution warmed at 37°C and gassed with 95%/5% O_2/CO_2 for the recording of isometric tension. After equilibration (60 min) in the bath, preparations were subjected to phenylephrine (10^{-6} M) and carbachol (10^{-5} M) in order to verify the integrity of endothelium (Time 0 incubation onset). Then, Angiotensin II (AII) was used as contractile agonist exquisitely sensitive to the action of the factors released by endothelium (Zerrouk et al., 1994).

After 30 min incubation, cumulative addition of AII (10^{-10}-10^{-6} M, n=6) caused concentration-dependent contraction. The tension Emax ratio SHR/WKY was 0.27 in rings with endothelium. After 180 min incubation, the contractile response to AII was enhanced only in SHR intact rings (tension Emax ratio = 5.8). No enhanced responses to AII were observed in SHR denuded rings and in WKY rings (intact or denuded). The time dependent hyperresponsiveness to AII is also observed after addition of a single dose of AII (10^{-7} M), only in SHR intact rings (+215 ± 38 %, n=6). This phenomenon could not be attributed to a reduced release of nitric oxide as previously shown (Zerrouk et al., 1994). Contractions to AII in SHR intact rings are largely reduced by agents acting on the cyclo-oxygenase pathway : aspirin ($5\ 10^{-4}$ M; -78 ± 5 %, n=8) and a TP receptor antagonist - SQ29,548 - (10^{-6} M; -95 ± 2 %, n=5). After 180 minutes incubation time, SHR aorta does not express newly functional TP receptors that mediate hypersensitivity to AII, because in 30 or 180 min incubated rings from SHR, the contractile sensitivity to the thromboxane mimetic U46,619 was similar. The hyperactivity to AII in SHR requires de novo protein synthesis since cycloheximide ($7.1\ 10^{-4}$ M; -28.5 ± 12 %, n=12) and moreover actinomycin D (10^{-5} M, -52.5 ± 9.6 %, n=8) reduced the constrictor response to the peptide after prolonged incubation. A role for a time dependent increased expression of COX-2 was assesed by immersion of SHR intact rings with NS-398 (10^{-6} M), an inhibitor of COX-2. The developed tension to AII in incubated rings (30 min : 0.38 ± 0.06 g; 180 min = 0.86 ± 0.06 g, n=6) was inhibited by NS-398 (30 min = 0.18 ± 0.02; 180 min = 0.375 ± 0.06 g, n=6). It should be noted that after 30 min incubation, contractions to AII (10^{-7} M) in SHR (0.88 ± 0.15 g) and WKY (0.76 ± 0.05 g) intact rings were similarly reduced by aspirin ($5\ 10^{-4}$ M, SHR = -69 ± 9.4 %, n=6 ; WKY = -60 ± 9.3 %, n=6).

The results of the present study indicate that after short incubation time of intact rings, contractile reactivity to AII was similarly modulated by cyclooxygenase in both strains. However, strain differences between SHR and WKY were evidenced after prolonged incubation time, and resulted in SHR rings to an exaggerated AII contractile response due to overproduction of constrictor prostanoids by a most probably inducible endothelial cyclooxygenase.

Zerrouk, A., Auguet M., Chabrier P.E. and Braquet P. (1994) *Arch. Mal. Coeur*, **87**, 1001-1004.

82P PROSTANOID ANTAGONIST AH 6809 ANTAGONISES PGE_2 INHIBITION OF HUMAN NEUTROPHIL ACTIVATION

S.W.Li, K.McKechnie, R.J.Griffiths[1], N.K.Boughton-Smith, A.Blackham & P.Leff, Department of Pharmacology, Fisons plc, Pharmaceutical Division, Research and Development Laboratories, Bakewell Road, Loughborough, Leicestershire, LE11 0RH.

The potency order of prostaglandin E (EP) receptor agonists and activity of AH13205 (EP_2 selective agonist) on neutrophil superoxide production and cAMP levels, suggest the EP receptors on human neutrophils are of the EP_2 subtype (Wheeldon & Vardey, 1993 and Li *et al.*, 1993). There are, however, no selective antagonists for the EP_2 receptor. AH 6809 has been described as an antagonist for EP_1 and DP receptors (pA_2 values of 6.6-7.0, Coleman *et al.*, 1987, and 5.3, Keery & Lumley, 1988, respectively) and it has been stated that it is a weak or inactive antagonist ($pA_2 < 5.0$) at EP_2 receptors in the cat trachea and chick ileum (Coleman *et al.*, 1985). In the present study we have evaluated AH 6809 as an antagonist of PGE_2 in human neutrophils and compared it to other EP_1 (SC 19220, pA_2=5.4-5.7, Coleman *et al.*, 1987) and DP (BW A868C, pA_2=9.26, Giles *et al.*, 1993) receptor antagonists.

Human neutrophils were isolated from venous blood by centrifugation (450-470 g) with Polymorphprep (Nycomed) and pre-incubated with antagonists at room temperature (60 min) in phosphate buffered saline containing 0.9mM $CaCl_2$, 0.5mM $MgCl_2$ and 11mM glucose. Cytochalasin B (5µg ml^{-1}) and the agonists or vehicle were added and incubation continued for a further 5 min (37°C). Superoxide production (5 min, 37°C) was stimulated by the addition of FMLP (10^{-7} M) and measured as superoxide dismutase (90 U ml^{-1}) inhibitable reduction of ferri-cytochrome C (0.08mM). PGE_2 inhibition of FMLP stimulated superoxide production (pEC_{50}=6.9 ± 0.1, mean ± s.e.mean, n=12) was antagonised by AH 6809 (pA_2=7.04±0.06, slope=1.06±0.07, n=5 experiments) but not by SC 19220 (10^{-4} M). BW A868C (10^{-7} M) also failed to antagonise PGE_2, a concentration which caused a 2.38±0.09 log_{10} unit rightward-shift (mean ± s.e.mean, n=5) of the PGD_2 concentration-effect curve. Furthermore, the shift of the PGE_2 curve by AH 6809 (10^{-5} M) was unaffected by the presence of BW A868C (10^{-7} M, n=3). AH 6809 (10^{-5} M) did not antagonise inhibition of superoxide production mediated by the adenosine receptor agonist NECA or the adenylate cyclase activator forskolin (both n=3).

The results demonstrate a selective effect of AH 6809 on PGE_2-induced suppression of human neutrophil activation, an effect that cannot be attributed to an action on DP receptors. The EP receptor on human neutrophils, like those mediating relaxation of the cat trachea and chick ileum, is classified as EP_2 on the basis of agonist potency order. However its sensitivity to AH 6809 appears to distinguish it from those receptors. While it is tempting to suggest that these data provide evidence for a receptor subtype, the lack of detailed information in the literature on the action of AH 6809 in EP_2 receptor containing tissues means that experimental differences cannot be excluded.

AH 6809, SC 19220 and BW A868C were gifts from Glaxo, Searle and Wellcome respectively.

[1]Current address: Department of Immunology, Pfizer Central Research, Groton, CT 06340, USA.

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83P IONTOPHEORESIS OF ACETYLCHOLINE CAUSES PROSTANOID-MEDIATED VASODILATATION OF HUMAN SKIN MICROVESSELS

J. P. Noon, M. F. Hand, P. D. Jordan, J. Simpson, B. R. Walker & D. J. Webb. Department of Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK.

Dilatation of forearm resistance vessels during brachial artery infusion of acetylcholine is impaired in patients with hypertension (Panza 1990) and hypercholesterolaemia (Chowienczyk 1992). This has been attributed to impaired endothelial nitric oxide generation, but it is unclear whether or not this is a manifestation of global endothelial dysfunction. We have previously reported, in the skin microcirculation, that nitric oxide is more important in regulation of thermoregulatory blood flow than of nutritive flow (Noon 1994). In the present study, we assessed endothelial function in the skin microcirculation by measuring forearm dermal vasodilatation using laser Doppler flowmetry following transdermal iontophoresis of acetylcholine (ACh) or the endothelium-independent vasodilator, sodium nitroprusside (SNP). To establish the pharmacological mechanisms of cholinergic vasodilatation in these microvessels, we studied 6 healthy males, on separate occasions, before and after brachial artery infusion of noradrenaline (at doses ranging from 60 - 480 nmol/min), and L-N^G-monomethyl-arginine (L-NMMA; 4 μmol/min for 20 min), an inhibitor of nitric oxide synthesis, and before and after systemic administration of aspirin (600 mg bolus iv), an inhibitor of cyclo-oxygenase. Results are presented as mean ± s.e.mean.

L-NMMA and noradrenaline reduced forearm blood flow by 43±2% and by 44±2% respectively, but neither had any influence on the iontophoretic response to ACh or SNP. In contrast, aspirin had no effect on the SNP response, but inhibited the ACh response: basal flux was 32±2 AU without and 35±10 AU with aspirin; and maximal stimulated flux was 473±80 AU without and 222±43 with aspirin [P<0.01] (Fig. 1).

We conclude that the predominant mediator of cholinergic vasodilatation in the dermal microvessels of healthy humans is prostacyclin rather than nitric oxide. From our data, other, as yet undetermined mediators, may also be involved, such as the putative endothelium-derived hyperpolarising factor. This non-invasive technique can now be applied in larger numbers of hypertensive and hypercholesterolaemic patients to establish whether their defect in endothelium-dependent vasodilatation is specific to resistance vessels and/or to the L-arginine:nitric oxide pathway.

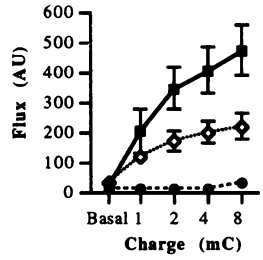


Figure 1. Responses in skin flux to iontophoresis of acetylcholine before (filled squares) and after (open squares) aspirin 600 mg iv bolus. Response to vehicle also shown (filled circles).

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84P THE EFFECT OF NON-PROSTANOID PROSTACYCLIN MIMETICS ON RAT NEUTROPHIL AGGREGATION AND CYCLIC AMP PRODUCTION

H. Wise & B.S. Chow, Department of Pharmacology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

Aggregation of rat peritoneal neutrophils stimulated by FMLP is inhibited by IP-agonists such as cicaprost and iloprost (Wise & Jones, 1994). The non-prostanoid PGI_2 mimetics octimibate, BMY-42393 and BMY-45778 behave as partial IP-receptor agonists yet still produce maximal inhibition of platelet aggregation (Meanwell *et al.*, 1994). In contrast octimibate behaved as an antagonist of both iloprost and PGE_2 in the human monocyte cell line Mono Mac 6 (Wise *et al.*, 1991). Therefore the aim of this study was to test these compounds on rat neutrophils.

Rat peritoneal neutrophils were harvested 19h following injection of thioglycollate (i.p.) and purified over a Ficoll-paque density gradient. Neutrophils were tested at 37°C in HEPES-buffered saline plus $CaCl_2$ (1mM), glucose (10mM) and indomethacin (3μM). Neutrophil aggregation (5 x 10^6 cells/ml) was measured with a Chrono-log platelet aggregometer. Test compounds were added 2 min before an EC_{50} concentration of FMLP (16nM). Adenylate cyclase activity was assayed by the conversion of [^{3}H]-ATP to [^{3}H]-cylic AMP. Neutrophils (1 x 10^7 cells/ml) were incubated for 10 min in buffer further supplemented with rolipram (0.1mM) followed by test compound for 10 min.

Table 1. Effects of IP-agonists on rat neutrophils

Drug (1μM)	FMLP-Aggregation (% Control)	[^{3}H]-cAMP (% Control)
Cicaprost	49 ± 5 ***	362 ± 28 ***
Iloprost	38 ± 3 ***	370 ± 25 ***
BMY-45778	54 ± 4 ***	255 ± 32 **
BMY-42393	52 ± 5 ***	178 ± 5 ***
Octimibate	57 ± 5 ***	126 ± 13

** $P < 0.01$, *** $P < 0.001$ compared (Student's unpaired t test) with controls. Results are mean ± s.e.m., n = 3-5.

The results of this study indicate that although the non-prostanoid PGI_2 mimetics, particularly octimibate, are relatively weak activators of adenylate cyclase they all function as full agonists with regard to inhibition of FMLP-stimulated neutrophil aggregation (Table 1).

We are grateful to Dr.N.A. Meanwell for the gifts of octimibate, BMY-42393 and BMY-45778.

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85P THE EFFECTS OF PHENYTOIN ON FREQUENCY-DEPENDENT CHANGES IN AMPA, NMDA, $GABA_A$ AND $GABA_B$-RECEPTOR-MEDIATED SYNAPTIC POTENTIALS IN THE RAT ENTORHINAL CORTEX *IN VITRO*

A. Dhillon* and R.S.G. Jones
Department of Pharmacology, University of Oxford, Oxford.

Excitatory synaptic responses of neurones in layer V of the rat entorhinal cortex (EC) consist of a fast component mediated by glutamate receptors of the AMPA/kainate type and a slower component mediated by NMDA receptors (Jones and Heinemann, 1988). Both components show a facilitation if activated at frequencies of 1-3 Hz and we have recently shown that certain anticonvulsant drugs can reduce this frequency-dependent facilitation of glutamate transmission Jones and Dhillon, 1993). In the present study we looked at the effects of the anticonvulsant drug, phenytoin on frequency-dependent changes in pharmacologically isolated components of the synaptic responses. Intracellular recordings were made from neurones in layer V of the EC in a slice preparation from the rat brain, maintained *in vitro* (Jones and Heinemann, 1988). Synaptic responses were evoked by stimulating intracortical pathways and components of the responses mediated by AMPA, NMDA, $GABA_A$ and $GABA_B$-receptors were isolated using combinations of appropriate receptor antagonists (CNQX, 2-AP5, bicuculline and CGP55845, respectively)

The amplitude of the fast AMPA-receptor mediated EPSP increased by 59 ± 24 % (mean ± SEM) when the frequency of stimulation was increased from 0.2 to 2 Hz (n=4). In the presence of phenytoin (100 μM) the increase was 43 ± 15%. This decrease in the degree of facilitation was not significant. The isolated NMDA-receptor mediated EPSP showed a facilitation of 37 ± 8% in the control situation whereas in the presence of phenytoin the increase was only 7 ± 10% (n=6). The difference between drug and control situations was significant ($p<0.01$, paired t-test).

In contrast to the frequency dependent facilitation of glutamate transmission, synaptic inhibition mediated by GABA displays a frequency dependent decrement over a similar stimulation range. GABA-dependent IPSPs were isolated by blocking glutamate receptors with CNQX and 2-AP5 and stimulating close to the recording site. The fast $GABA_A$ IPSP decreased in amplitude by 46 ± 6% when the frequency of stimulation was increased from 0.2 to 2 Hz. In the presence of phenytoin the frequency decrement in the IPSP was almost identical (-45 ± 8%, n=4). The slow $GABA_B$-receptor mediated IPSP also faded at the higher stimulation rate (-73 ± 4 %). The degree of frequency dependent fading was less when phenytoin was added (-54 ± 18 %, n=4) but the difference was not significant.

Thus, the ability of phenytoin to reduce frequency dependent facilitation of glutamate transmission in the EC was largely due to an effect on the slow NMDA component. In addition, there was a tendency for the drug to reduce the frequency-dependent decrement in the slow $GABA_B$-inhibition. Since these components normally show a considerable temporal overlap, the overall effect of the drug should be to decrease slow excitatory effects within the synaptic network. Such an effect could contribute to the anticonvulsant actions of phenytoin.

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AD is an MRC Scholar

86P THE INVOLVEMENT OF CALCIUM CHANNEL SUBTYPES IN GLUTAMATE RELEASE FROM CULTURED RAT CEREBELLAR GRANULE NEURONES AND ITS MODULATION BY $GABA_B$ RECEPTOR ACTIVATION

E. Huston & A.C. Dolphin, Department of Pharmacology, Royal Free Hospital Medical School, Rowland Hill Street, London NW3 2PF.

Cerebellar granule neurones, when grown in primary cell culture, can be stimulated, by potassium (K^+) depolarisation, to release [^{3}H]-glutamate, newly synthesised from [^{3}H]-glutamine. We have previously shown this release to be dependent on the entry of calcium (Ca^{2+}) through a number of subtypes of high voltage activated Ca^{2+} channels (Huston and Dolphin, 1994). Inhibiton of glutamate release in these neurons, by the $GABA_B$ receptor agonist, (-)-baclofen, has been described previously (Huston et al, 1990). It has been postulated that this modulation may result, at least in part, from inhibition of calcium influx through voltage-activated calcium channels. We now investigate which Ca^{2+} channel sub-types may be involved in the modulation of K^+-stimulated [^{3}H]-glutamate release by (-)-baclofen, from cerebellar granule neurones.

The methods described in Huston et al (1990, 1993) were used to study the release of [^{3}H]-glutamate. When cells were stimulated by 4μM ionomycin the ensuing glutamate release was much reduced as compared to K^+-stimulated release and was not modulated by (-)-baclofen (100μM). We have examined the ability of 100μM (-)-baclofen to modulate 40mM K^+-stimulated glutamate release following selective blockade of Ca^{2+} channel sub-types by the dihydropyridine antagonist, nicardipine (1μM), the N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (ωCgTx GVIA, 1μM) and the P-type Ca^{2+} channel blocker, ω-agatoxin IVA (ωAga IVA, 200nM). In the presence of either nicardipine or ωAga IVA, glutamate release was inhibited by (-)-Baclofen to a similar extent as in control cells. In contrast, inhibition of glutamate release by (-)-baclofen was reduced from 30±3% in control cells to 21±3% in cells pre-incubated with ωCgTx GVIA (mean±SEM, $p<0.05$, n=16).

We have previously shown that a large component of K^+-stimulated glutamate release still remains after block of the N-, P-, and L-type Ca^{2+} channels (Huston and Dolphin, 1994). It has been suggested that the novel peptide toxin ω-conotoxin MVIIC (ωCTx MVIIC) inhibits Q, as well as N- and P-type Ca^{2+} channels in cerebellar granule neurones (Zhang et al, 1993). We have previously shown that at a maximal concentration of 5μM, ωCTx MVIIC inhibits release of glutamate by 50-60% (Huston and Dolphin, 1994). Although this release can be blocked further by the addition of the L-type Ca^{2+} channel blocker, nicardipine (1μM), inhibition is not complete and a significant proportion of Cd^{2+} sensitive glutamate release still remains. In the presence of ωCTx MVIIC, 50mM K^+-stimulated [^{3}H]-glutamate release from cerebellar granule neurones can no longer be modulated by activation of the $GABA_B$ receptors by (-)-baclofen. This result agrees with the finding that, whereas, in the presence of ωCgTx GVIA inhibition of K^+-stimulated glutamate release by (-)-baclofen is reduced, in the presence of both ωCgTx GVIA and nicardipine, inhibition of release by (-)-baclofen is increased to 37±3%.

These results suggest the presence of multiple Ca^{2+}-channel subtypes at the presynaptic terminal in cerebellar granule neurones, which are able support the release of newly synthesised [^{3}H]-glutamate. They also suggest that modulation of this glutamate release by $GABA_B$ receptor activation involves interaction with ωCTx MVIIC-sensitive Ca^{2+}-channel subtypes.

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87P CAPSAICIN-INDUCED MECHANICAL HYPERALGESIA IN THE RAT: INVOLVEMENT OF NK_1, BRADYKININ B_1 AND B_2 AND INTERLEUKIN-1 RECEPTORS

A. J. Davis & M.N. Perkins. Sandoz Institute for Medical Research, Gower Place, London WC1E 6BN

Previously we have shown that Freund's complete adjuvant and a variety of cytokines can induce a bradykinin B1-receptor mediated hyperalgesia in the rat (Davis and Perkins, 1994a,b). We now report on the involvement of bradykinin B1 and B2 and interleukin-1 receptors in the induction and maintenance of mechanical hyperalgesia induced by the neurotoxin capsaicin.

Capsaicin was injected (100µl) into a single knee joint of female Sprague-Dawley rats (80-100g) under brief enflurane anaesthesia and the level of hyperalgesia assessed 1, 4, 6 and 24h later by measuring the load tolerated by the treated limb as described previously (Davis & Perkins 1994). Capsaicin (10nmol) produced a reduction in load tolerated which fell from control levels of 95±3g to 69±4g at 1h, 67±3g at 4h and 73±3g at 6h post administration (mean± s.e.mean, $P<0.05$, ANOVA followed by Tukey' test).

Co-administration of the capsaicin receptor antagonist capsazepine (0.5-5nmol; Bevan *et al.*, 1992) or the NK1 receptor antagonists CP 99994 (100nmol) or RP 67580 (1nmol) with capsaicin (10nmol) blocked the subsequent development of hyperalgesia. At 4h post administration of 10nmol capsaicin, animals tolerated 62±1g compared to 91±2g after co-administration with capsazepine or 87±2g after CP 99994 and 94±3g after RP 67580 ($P<0.01$, Tukey's test).

Co-administration of capsaicin (10nmol) with the B2 receptor antagonist icatibant (5pmol) or the B1 receptor antagonist desArg9Leu8BK (0.5nmol) prevented the development of the hyperalgesia over the entire timecourse. After capsaicin alone animals tolerated 67±4g, 64±1g and 68±3g at 1, 4 and 6h compared to 88±3g, 86±2g and 81±3g after icatibant or 92±4, 90±2 and 88±5 after desArg9Leu8BK. Administration of icatibant (100pmol/kg i.v.) or desArg9Leu8BK (10nmol/kg iv) 3.5h after capsaicin (10nmol) also caused a significant reversal of hyperalgesia when assessed 30min later.

Recombinant human interleukin-1 receptor antagonist 0.1 µg (IL-1ra), when co-administered with capsaicin, significantly reduced the development of hyperalgesia over the entire time course, with animals tolerating 66±3g. 58±5 and 63±2g 1,4 and 6h after capsaicin alone compared to 83±2g, 92±3 and 74±3g after capsaicin and IL-1ra ($P<0.01$, Tukey's test).

Indomethacin (1mg/kg sc) pretreatment had no effect on the level of capsacin-induced hyperalgesia. However administration of desArg9Leu8BK (10nmol/kg iv) to animals 4h after pretreatment with capsaicin and indomethacin had no effect on the level of hyperalgesia.

These data suggest that after intra-articular administration of capsaicin NK1, B1, B2 and IL-1 receptors are involved in the hyperalgesia, however pretreatment with indomethacin removes the B1 component of capsaicin-induced hyperalgesia.

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88P ELECTROPHYSIOLOGICAL CHARACTERISATION OF TACHYKININ RECEPTORS IN THE RAT NUCLEUS TRACTUS SOLITARIUS *IN VITRO*: NEUROKININ A MAY ACT AT AN NK_1 RECEPTOR SUBTYPE

K. A. Maubach, *R. M. Hagan and R. S. G. Jones
Department of Pharmacology, University of Oxford, Oxford and *Glaxo Research and Development, Ware, Hertfordshire.

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are endogenous tachykinins and are the prototypic ligands for NK1, NK2 and NK3 receptors. They may play a role as neurotransmitters in the nucleus tractus solitarius (NTS) in the brainstem. There is a high density of binding sites for all three endogenous agonists in the NTS (Mantyh *et al*, 1989). *In vitro* electrophysiological studies have shown that NTS neurones are powerfully excited by both SP and NKA (Maubach *et al*, 1994). We have determined the receptors responsible for these effects using specific agonists and antagonists. Slices of rat brainstem were maintained at the interface between artificial CSF containing a cocktail of peptidase inhibitors (1.2 ml/min at 34°C) and moistened carbogen gas (95% O_2, 5% CO_2). Intracellular voltage recordings were made from neurones in the NTS and drugs applied by bath perfusion.

The mean (± SEM) membrane potential and input resistance of a sample (n=41) of neurones were 63.9 ± 1.4 mV and 120.81 ± 5.1 MΩ. SP depolarised 75% of neurones (by 4.0 ± 0.7 mV at 250 nM, n=5), NKA was more effective, depolarising 92% of neurones (by 7.6 ± 1.0 mV at 250 nM, n=9) whereas NKB had no effect (up to 3 µM). These responses were concentration-dependent (10 nM-1 µM) and were often accompanied by an increase in input resistance. Other agonists also depolarised NTS neurones and EC_{50} values for the NK1 receptor agonists SP, SPOMe, septide and GR73632, were 773 ± 146 nM (n=3), 310 ± 190 nM (n=2), 20 ± 2 nM (n=2) and 40 ± 13 nM (n=4), respectively. The EC_{50} for NKA was 167 ± 44 nM (n=6). In contrast, the NK2 agonist, GR64349 and NK3 receptor agonists (NKB and senktide) had no effect (up to 3 µM). GR82334 (5 µM), an NK1 receptor antagonist greatly reduced responses to 50 nM septide (100%, n=2) and GR73632 (85 ± 5%, n=6). Another NK1 antagonist, CP99994 (5 µM) had similar effects (69 ± 7 % , n=2 and 82 ± 10% , n=3). Surprisingly, neither antagonist (up to 10 µM) had any effect on responses to SP or SPOMe. Responses to NK1 agonists were unaffected by the NK2 receptor antagonist, GR159897 (up to 10 µM). Responses to NKA (100 nM) were also unaffected by the NK2 antagonist but reduced by the NK1 antagonists, GR82334 (91 ± 10%, n=3) and CP99994 (72 ± 2%, n=3), both at 5 µM

Thus, the excitation of NTS neurones by septide and GR73632 may be mediated by NK1 receptors but the lesser potency of SP and SPOMe could indicate the existence of subtypes of NK1 receptor. The blockade of responses to septide and GR73632 but not SP and SPOMe by NK1 antagonists would support this view. The greater potency of NKA compared to SP may suggest that NKA plays a more predominant role as the endogenous mediator in the NTS. The effects of NKA may not be mediated via NK2 receptors, however, since another NK2 receptor agonist, GR64349 had little effect and responses to NKA were not blocked by an NK2 antagonist. The blockade of responses to NKA by NK1 receptor antagonists suggests that NKA may act at the novel NK1-like receptor in the NTS.

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89P RADIOLIGAND BINDING STUDIES WITH THE SELECTIVE SOMATOSTATIN SSTR2 RECEPTOR LIGAND [I^{125}]-BIM23027 IN RAT STRIATUM AND HIPPOCAMPUS

S. Holloway, W. Feniuk, A.D. Michel and P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ.

BIM23027 is a highly selective ligand for the recombinant somatostatin SSTR2 receptor (Raynor *et al.*, 1993, Castro *et al.*, 1994). Preliminary competition studies using Tyr^{11}-somatostatin $(SRIF)_{14}$ in rat striatal and hippocampal membranes showed that BIM23027 (10nM) maximally inhibited only 70 ± 9% and 35 ± 3% of total SRIF binding, respectively. We have examined these binding sites in more detail using the novel radioligand [I^{125}]-BIM23027 (specific activity of 2000Ci/mmol; Amersham) for the first time.

Rat striatal and hippocampal membranes were prepared in 50mM TRIS HCl buffer (pH 7.4 at 21°C) containing 5mM $MgCl_2$, soyabean trypsin inhibitor 1μg/ml, leupeptin 10μg/ml and bacitracin 0.2mg/ml. Association experiments were performed incubating 0.1nM [I^{125}]-BIM23027 for increasing periods (0-240min). In dissociation experiments, membranes were incubated with radioligand for 1h before dissociation was initiated with 1μM SRIF in a volume of 0.5ml for 0-240min. Saturation studies were carried out by incubating membranes with increasing concentrations of radioligand and competition studies by varying the concentration of competing drug. Binding parameters were determined using iterative curve fitting (SigmaPlot V40, Jandel). Non specific binding (25-45%) was determined as that remaining bound to the filters in the presence of 1μM SRIF. All values quoted are the geometric mean (95% confidence limits) or arithmetic mean ± s.e. mean from at least 3 determinations, measured in triplicate.

Binding of [I^{125}]-BIM23027 (0.1nM) to hippocampal and striatal membranes was time-dependent, reaching steady state within 60min at 21°C with observed association rate constants (K_{obs}) of 0.023±0.005 and 0.052±0.006min^{-1}, respectively. The dissociation rate constant (K_{-1}) was determined as 0.010±0.001 and 0.012±0.001 min^{-1}, yielding a calculated association rate constant of 0.162±0.06 and 0.622±0.09 $min^{-1}nM^{-1}$. These parameters gave kinetic K_d values of 0.06 and 0.019nM for the hippocampus and striatum, respectively. Saturation binding analysis yielded K_d values of 0.171±0.044nM (B_{max} 241±32 fmol/mg protein) for hippocampal membranes and 0.047±0.002nM (B_{max} 78±8 fmol/mg protein) for striatal membranes. Competition data using some SRIF receptor selective ligands (Raynor *et al.*, 1993) are summarised in the table below.

	Striatum		Hippocampus	
	IC_{50} (nM)	n_H	IC_{50} (nM)	n_H
SRIF	0.17 [0.08-0.33]	1.02±0.12	0.17 [0.13-0.23]	0.89±0.05
$SRIF_{28}$	0.31 [0.10-0.92]	0.92±0.10	0.30 [0.07-1.38]	0.92±0.13
BIM23027	0.11 [0.07-0.18]	0.87±0.06	0.20 [0.06-0.70]	0.74±0.11
iodoBIM23027	0.19 [0.07-0.55]	0.90±0.10	0.22 [0.10-0.48]	0.87±0.03
seglitide	0.09 [0.07-0.12]	0.90±0.06	0.13 [0.07-0.21]	0.90±0.05
L-362,855	1.07 [0.28-4.11]	0.73±0.18	0.99 [0.12-8.54]	0.74±0.09
BIM23056	>300		>300	

The results from these studies suggest that the SRIF receptors labelled by [I^{125}]-BIM23027 in the striatum and hippocampus are similar and iodination of BIM23207 does not alter its affinity for this receptor. The order of binding affinities suggests that the major site labelled by BIM23027 in the hippocampus and striatum most closely resembles the recombinant SSTR2 receptor. [I^{125}]-BIM23027 appears to be a useful new radioligand for studying the distribution of SRIF receptors. Autoradiographic studies in whole brain are also in progress.

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90P HUMAN BRAIN SOMATOSTATIN SS-1/SS-2 SITES EXPRESS OPERATIONAL CHARACTERISTICS OF RECOMBINANT HUMAN sst2/sst1 RECEPTORS, RESPECTIVELY

D. Hoyer, C. Bruns, J. Schloos & A. Probst*. Preclinical Research, SANDOZ Pharma Ltd, 360/604, CH 4002 Basel, and * Institute of Pathology, CH 4056 Basel, Switzerland.

Two subtypes of somatostatin (SRIF) receptors (SS-1 and SS-2) have been described on the basis of radioligand binding studies (Reubi, 1985; Reubi & Maurer, 1986) although five SRIF receptor cDNAs (SSTR1 - SSTR5, now called sst1 - sst5) have recently been cloned (Hoyer *et al*, 1994). In the present study, the pharmacology of human cerebral cortex SS-1 and SS-2 binding sites has been compared with that of human recombinant sst1 and sst2 receptors.

Human cortical SS-1 and SS-2 sites were labelled with [^{125}I]204-090 (Tyr^3-octreotide) and [^{125}I]Tyr^{11}-$SRIF_{14}$ (in the presence of 120 mM NaCl) respectively, as described by Reubi, 1985 and Reubi & Maurer, 1986. Human recombinant sst1 and sst2 receptors expressed stably in HEK 293 or transiently in CHO cells were labelled with [^{125}I]Tyr^{11}-$SRIF_{14}$ and [^{125}I]204-090, respectively (Hoyer *et al*, 1994). Non specific binding was determined by addition of 1 μM SRF-14. Competition studies were carried out and pK_d's (-log M, ± s.e.mean, n≥3) determined for a range of SRIF receptor selective ligands (Table 1):

The operational profiles of SS-1 and sst2 sites, and those of SS-2 and sst1 sites, correlated significantly ($r = 0.980$ and 0.962, $p < 0.001$, $n = 11$, respectively). SS-1 or SS-2 binding correlated less or not significantly with sst3, sst4 or sst5 receptor binding. The present data demonstrate that SS-1 and SS-2 sites identified in human cerebral cortex, correspond respectively to sst2 and sst1 receptors.

Table I

Drug	SS-1	sst2	SS-2	sst1
SRIF-14	10.1±0.3	10.5±0.2	8.3±0.2	8.7±0.1
SRIF-28	9.7±0.2	10.3±0.2	7.7±0.0	8.2±0.1
octreotide	8.6±0.2	9.9±0.2	6.8±0.1	6.5±0.1
somatuline	8.9±0.2	9.7±0.2	6.3±0.1	6.1±0.0
seglitide	10.1±0.3	10.6±0.2	4.9±0.1	4.6±0.0
octastatin	8.9±0.1	10.2±0.3	5.9±0.1	6.3±0.1
CGP 23996	8.8±0.2	9.6±0.2	7.5±0.1	8.0±0.1
SA	5.4±0.1	5.9±0.2	5.5±0.3	5.9±0.1
L363,301	8.3±0.1	9.3±0.2	5.1±0.1	5.3±0.1
BIM 23052	7.8±0.1	8.7±0.1	6.4±0.1	7.2±0.1
BIM 23056	6.1±0.1	6.7±0.1	6.0±0.1	6.4±0.1

SA = c[Aha-Phe-D-Trp-Lys-Thr(Bzl)]; BIM 23052 = D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2; BIM 23056 = D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2; CGP 23996 = c[Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser]; L-363,301 = c[pro-Phe-D-Trp-Lys-Thr-Phe].

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M. Corsi, G. Dal Forno, F. Th. M. van Amsterdam, A. Feriani, A. Ursini, E. Ratti, G. Gaviraghi and D. G. Trist, Glaxo Research Laboratories, 37135 Verona, Italy.

A novel CCK-B receptor antagonist, GV150013 ((R)-+-N-[(Adamantane-1-methyl)-2,4-dioxo-5-phenyl-2,3,4,5-tetrahydro-1H-1,5 benzodiazepin-3-yl]-N'phenylurea, fig. 1) has been characterized in peripheral tissues known to contain CCK-A and CCK-B receptor subtypes.

Guinea pig ileum longitudinal myenteric plexus (GPILMP), has been prepared as described by Dal Forno et al. (1992). Contractions were induced by the selective CCK-A and CCK-B agonists N-acetyl derivative of A71623 (NAA) and CCK-4, respectively. With the latter agonist, the Krebs solution contained the selective CCK-A receptor antagonist L-364,718 at 10nM. Guinea pig gall bladder (GPGB) was set up as reported by Corsi et al. (1992) and contractions were obtained by CCK-8S. The isolated rat gastric mucosa (RGM) was prepared as indicated by Reeves and Stables (1985) and acid secretion as generated by pentagastrin in the presence of 2.7 μM indomethacin. The equilibration time for GV150013 used was one hour and responses were expressed as percentage of the contraction obtained by 30μM DMPP (GPILMP), by 100mM KCl (GPGB), or as percentage of secretion obtained by 30 μM histamine (RGM). In GPILMP, NAA (0.001-10μM) and CCK-4 (0.01-300μM) induced concentration-dependent contractions with a potency (EC_{50}) of 10±0.2nM and 158± 23 nM respectively. GV150013 at 3μM antagonised NAA-induced contractions with a pK_B of 5.9 ±0.2 (s.e.; n=3). CCK-4-induced contractions were concentration dependently antagonized by GV150013 (0.01-0.1μM). However, the antagonism showed a concentration-related depression of the maximal response. A combined dose ratio-analysis using L-365,260 as a standard CCK-B receptor antagonist revealed that both compounds acted on the same receptor site. Experiments carried out with 0.01μM of GV150013 indicated that the antagonism was reversible after the tissues were washed-out for 120min . A pK_B value of 8.9±0.3 (s.e., n=4-6) was obtained for GV150013 using a non-competitive analysis. In GPGB, CCK-8S (0.0003-1μM) induced contractions with a potency (EC_{50}) and a maximal response of 1.7±0.1 nM and 150±2 %, respectively. GV150013 at 3μM antagonized CCK-8S-induced contractions with a pK_B of 5.8±0.1 (s.e., n=3). These data agree with radioligand binding data obtained in Guinea Pig cortex membranes for CCK-B (pKi=9.15 ±0.04) and in rat pancreas for CCK-A (pKi=5.83±0.05). In RGM, pentagastrin (0.001-10μM) induced an acid gastric secretion with a potency (EC_{50}) of 0.02±0.002μM and a maximal response of 66.4± 8.2%. GV150013 (0.1 and 0.3 μM) antagonized pentagastrin-induced acid secretion with an apparent pK_B of 7.40 ±0.2 (s.e., n=3-10). These results indicate that GV150013 has a nanomolar potency for the CCK-B receptors present in GPILMP, while its selectivity versus the CCK-A receptors present in both GPILMP and GPGB reaches a thousand fold. Furthermore, GV150013 seems to discriminate between the CCK-B receptor present in the ileum and the gastrin/CCK-B receptors of the rat gastric mucosa.

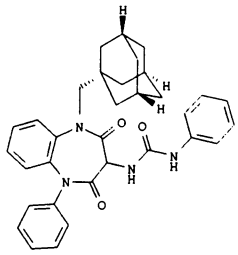


Fig. 1 GV150013

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92P ANXIOLYTIC ACTIVITY OF GV 150013, A NEW POTENT AND SELECTIVE CHOLECYSTOKININ-B (CCK-B) ANTAGONIST

A.Reggiani, P.A.Gerrard, G.Maraia, S.Melotto, E.Ratti, G.Gaviraghi & D.G. Trist, Glaxo Research Laboratories, via Fleming 4, Verona, Italy

Large evidence indicate that central CCK-B receptors are involved in anxiety and CCK-B antagonists could become a novel class of anxiolytic agent (Harro et al, 1993). GV 150013 (GV) has been recently identified as a potent and selective CCK-B antagonist (Corsi et al, this meeting). Therefore, the present study was undertaken to investigate the anxiolytic potential of the compound.

GV was tested in the following animal models: mouse light/dark box, rat social interaction and marmoset "human threat" test respectively. The experiments were performed as described by Costall et al, 1991. Briefly, in the light/dark box the time spent in the white section of the box was measured over a 300 sec period, in the rat social interaction the time spent interacting was measured over a 600 sec period and in the " human threat" the number of postures was measured over a 120 sec period of time. All data were analyzed by ANOVA followed by Dunnett. Experiments were performed blind.

In the mouse, a dose dependent anxiolytic effect was found in the range of doses between 0.01-3 μg/kg po (n=15 each dose). The first significantly effective dose was 0.3 μg/kg po (128.2 ± 6.9 sec vs 170.5 ± 9.7; $P< 0.01$) and an ED_{50}= 0.05 μg/kg po (0.001-0.205, C.L. 95%) was estimated. Comparative studies with reference compounds such as diazepam and PD 134308 (PD), another CCK-B antagonist, indicate that efficacy (i.e. maximal effect) of GV was similar to these compounds, whereas the rank order of potency was GV = PD >> diazepam (≈ 3000 times less). Moreover, the duration of action of GV was longer compared to the other compounds tested at equieffective doses. In fact, at 0.3 μg/kg po a significant effect was seen up to 6 hrs, whereas for PD it was less than 2 hrs (at 0.3 μg/kg po) and for diazepam (at 1 mg/kg po) it was less than 4 hrs. In the rat social interaction test, GV was tested after the experimental induction of anxiogenesis with FG 7142 (FG, 10 mg/kg ip). In this model, the initial baseline of interaction of 130.3 ± 10.3 sec, was reduced to 83.2 ± 6.6 sec by FG administration ($P < 0.01$). GV was able to reverse this anxiogenesis in the range of doses between 0.3-30 μg/kg po (n=15 each dose). The first significantly effective dose was 3 μg/kg po (115.7 ± 8.3, $P < 0.01$ vs FG). The efficacy of the compound was similar to chlordiazepoxide (CDP) and slightly higher than that of PD. Finally, GV was found effective in the "human threat" test in marmoset. The doses tested were in the range of 0.1 and 1 μg/kg sc (n=4 each dose), and the first significantly effective dose was 0.3 μg/kg sc (from 12.3 ± 0.5 to 6.8 ± 0.8, $P < 0.01$). The efficacy of GV was similar to PD and only slightly lower than CDP, probably because the latter compound has a sedative effect not shared by the other anxiolytic.

In conclusion, the present studies show that the selective CCK-B antagonist GV 150013 has a potent and long lasting anxiolytic activity in different animal models of anxiety.

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Sandy Hogg, Emma Fluck & Sandra E. File, Psychopharmacology Research Unit, UMDS, Guy's Hospital, London SE1 9RT

In chicks the duration of tonic immobility (TI) is significantly lower in animals which have been regularly and gently handled than in unhandled birds (Jones & Waddington, 1992). Since, in the rat, handling-induced behavioural differences are accompanied by a significant increase in benzodiazepine (BDZ) receptor number (Andrews et al. 1992) we compared BDZ binding in forebrain of handled (H) and unhandled (U) chicks.

Day old male ISA brown chicks were divided into H (handled twice daily for 10 days) and U groups (n=8/group). TI was assessed by placing them on their backs and manually restraining them for 5s. The number of inductions (N) required to produce TI > 5s and the duration (D) of the first TI which lasted longer than 5s were recorded; max N=5, D=300s. Saturation binding of [^{3}H]-flunitrazepam (FLU, 0.05-12nM), with diazepam (3μM) to define non-specific binding, was performed in a well washed membrane preparation from forebrain (5 mg/ml) according to the method of Hogg et al. (1994). K_D (nM) andB_{max} (fmol/mg protein) were calculated. GABA (10^{-7}-10^{-4}M) significantly enhanced FLU binding (concentration effect $p<0.05$, analysis of variance).

It would appear that, in contrast to the rat, BDZ binding changes do not underlie the handling-induced behavioural differences in the chick. This may represent an important species difference. However, quail selectively bred for long and short TI responses do exhibit differences in BDZ binding (Hogg et al., 1994), suggesting a different neurochemical basis for genetically determined and experimentally induced TI differences in poultry.

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Table 1. TI median ± semi-interquartile range and [^{3}H]-flunitrazepam binding (mean ± sem) in handled and unhandled chicks. H vs U, *P<0.001 Mann Whitney U test.

	TI		[^{3}H]-flunitrazepam		GABA enhancement (% of control)			
	N	D	K_D	B_{max}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
H	4.0±0.2	3.5±1.4	1.7±0.1	2183±126	102.6±2.7	111.4±3.7	116.1±4.7	116.4±5.6
U	1.0±0.1*	48.5±9.0*	1.7±0.1	2044±193	98.5±4.2	109.0±5.3	115.2±4.6	112.8±7.6

94P STIMULATION OF CENTRAL ADENOSINE A_1 RECEPTORS CAN INDUCE BODY JERKS IN MICE

R. Anderson, S. Close, M.J. Sheehan and P. Strong. Dept. of Cellular Sciences, Glaxo Research and Development Ltd., Ware, Herts, SG12 ODP.

In mice, central administration of adenosine A_1 receptor-selective agonists produces a range of physiological effects including reduced locomotor activity (Barraco *et al* 1983), sedation and hypothermia. During the course of an investigation of the hypothermic effects of adenosine agonists (Anderson *et al*, 1994) we noted a previously unreported body jerking response. We have now studied the characteristics of this novel response using a range of adenosine receptor-selective ligands following intracerebro-ventricular (i.c.v.) and intraperitoneal (i.p.) injection.

Adenosine agonists were administered i.c.v. in conscious, male, weanling (8-12g) CRH mice. Antagonists were administered i.p. 10 min prior to agonist. Body jerks were counted for 30 min following agonist administration. The non-selective adenosine receptor agonist 5'-N-ethyl-carboxamidoadenosine (NECA), the adenosine A_1 receptor-selective agonists, cyclopentyladenosine (CPA), N^6-(9R-phenylisopropyl)-adenosine (R-PIA), N-(1S, trans)-[2-hydroxycyclopentyl] adenosine (GR79236; all at 0.01-1 µg) and the A_3 receptor agonist N^6-(4-aminophenylethyl) adenosine (APNEA; 0.1-3µg) produced body jerks. This dose-dependent response consisted of discrete twitches of the body and limbs and proved variable between animals (e.g. for NECA at 0.3µg, jerks for individual mice were 87, 4, 6, 59, 48, 84). The maximum number of jerks produced by each agonist was also variable but all the compounds displayed a similar threshold of activity (0.01-0.3µg). Potency values (ED_{100}, defined as dose (µg) causing a total of 100 jerks/6 mice/30min) were as follows: NECA 0.05, GR79236 0.1, CPA 0.23, R-PIA >1, APNEA 0.34. However, the adenosine A_{2a} and A_{2b}-selective agonists 2-[p-(2-carboxyethyl)phenethylamino]-5'-N-ethyl-carboxamido-adenosine (CGS-21680, 0.1-10µg i.c.v.) and N-[(2-methylphenyl) methyl]adenosine (metrifudil, 0.01-1µg i.c.v.) produced only very few or no body jerks respectively at the highest doses tested (e.g. CGS-21680 (10µg) caused a total of 4 jerks in 6 mice). The body jerking response to the adenosine A_1 receptor-selective agonist GR79236 was dose-dependently antagonised by either the non-selective adenosine receptor antagonist 8-phenyltheophylline (1-10 mg/kg ip; 42 fold rightward shift at 10 mg/kg, measured at half-maximal response) or the adenosine A_1 receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 6 fold shift at 0.1 mg/kg). In addition, the body jerking response to APNEA was similarly blocked by DPCPX (5 fold shift at 0.1 mg/kg), suggesting that A_3 receptors were not involved.

Thus, these data describe a novel excitatory behavioural response mediated by activation of adenosine A_1 receptors.

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95P DIFFERENTIAL EXPRESSION OF ADENOSINE RECEPTORS COUPLED TO cAMP ACCUMULATION IN TYPE-1 AND TYPE-2 ASTROCYTES

M-C. Peakman & S. J. Hill, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

In previous work we have demonstrated the presence of adenosine A_1 and A_{2b} receptor subtypes coupled to adenylyl cyclase in primary cultures of rat astrocytes containing mixed type-1 and type-2 populations (Peakman and Hill, 1994). The aim of this study was to establish whether type-1 and type-2 astrocytes differ in their expression of adenosine receptor subtypes coupled to cAMP accumulation.

Primary cultures of rat astrocytes (Ruck *et al.*, 1991), maintained for 10-15 days *in vitro*, were manipulated to prepare secondary cultures enriched for type-1 or type-2 astrocytes using a modification of the method described by Levison and McCarthy (1991). Briefly, O-2A progenitor cells were dislodged by shaking the cultures at 350 rpm for 18 hours at 37°C on an orbital shaker and were seeded into 24 well cluster dishes. The remaining type-1 astrocyte monolayers were trypsinised and seeded into 24 well cluster dishes. Cells were prelabelled with 8-[^{3}H]adenine and [^{3}H]cAMP generated was isolated by sequential dowex alumina chromatography and quantified by scintillation counting (Peakman and Hill, 1994)

The adenosine receptor agonist, 5'-N-ethylcarboxamido-adenosine (NECA) caused a concentration-dependent increase in the accumulation of [^{3}H]cAMP in type-1 astrocyte-enriched cultures. The mean EC_{50} ± standard error calculated for this effect was 1.2 ± 0.4 μM (n = 3) and a maximal response of 13.5 ± 2.8 fold (n = 3) over a mean basal level of 4,135 ± 305 dpm (n = 3) was achieved with 0.1mM NECA. The cyclic AMP response to 10 μM NECA was antagonised in a concentration-dependent manner by xanthine amine congener (XAC) and PD115,199 (Peakman and Hill, 1994) with apparent K_d values of 8.8 ± 3.5nM (n = 3) and 121.8 ± 43.6nM (n = 4) respectively. These values are in close agreement with those obtained for the A_{2b} receptor in mixed astrocyte cultures (Peakman and Hill, 1994). The A_1 receptor-selective agonist, N^6-cyclopentyladenosine (CPA), however, at concentrations up to 1 μM, caused no significant inhibition of forskolin (1 μM, n = 5)- or of isoprenaline (0.1 μM, n = 3)-mediated cAMP accumulation in type-1 astrocytes.

NECA (10 μM) caused a significant accumulation (5.8 ±2.1 fold over basal, n = 6, $p<0.01$ 2way ANOVAR) of [^{3}H]cAMP in type-2 astrocyte-enriched cultures. A mean basal level of 646 ± 147 dpm was elevated to 6,020 ± 2,460 dpm by 10 min stimulation with NECA (n = 6). This response was significantly antagonised by XAC (1μM, 79.2 ± 0.9% inhibition, n = 3, $p<0.01$, 2 way ANOVAR) and PD115,199 (10 μM, 55.5 ± 12.8% inhibition, n = 3, $p<0.01$, 2 way ANOVAR). Forskolin (1 μM) elevated cyclic AMP accumulation in type-2 astrocytes (5.7 ± 1.1 fold over basal, n = 6, $p<0.01$, 2 way ANOVAR) and this response was significantly inhibited by CPA (0.1μM, 62.7 ± 12.2% inhibition, n = 6, $p<0.01$, 2 way ANOVAR). The inhibitory response to 0.1 μM CPA was antagonised (0.1 μM CPA, 9.3 ± 15.6% inhibition of the response to 1 μM forskolin, n = 6, $p>0.05$, 2 way ANOVAR) in the presence of the A_1 receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (0.1 μM).

These data imply that type-1 (and probably type-2) astrocytes express adenosine receptors of the A_{2b} subtype, whereas A_1 receptors, negatively coupled to cAMP accumulation are only expressed by type-2 astrocytes.

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96P DHP-SENSITIVE CALCIUM CHANNEL BLOCKADE PREVENTS THE SUPPRESSION OF LIPOGENESIS IN ETHANOL WITHDRAWAL

Z. Hughes, M. Al Qatari and P.V. Taberner, Department of Pharmacology, University of Bristol, School of Medical Sciences, University Walk, Bristol BS81TD, U.K.

Dihydropyridine-sensitive (DHP) binding sites are known to increase in the CNS during chronic ethanol drinking (Littleton *et al.*, 1990). Furthermore, the concurrent administration of DHP antagonists prevents the development of ethanol tolerance and also ameliorates the withdrawal syndrome (Whittington *et al.*, 1991). We have already shown that chronic ethanol drinking produces adaptive increases in adipocyte fatty acid synthesis and lipolysis which are reversed in withdrawal (Al-Qatari *et al.*, 1991). Since calcium is an important regulator of several key steps in intracellular lipid metabolism we have determined the effects of the DHP Ca^{++} channel antagonist PN 200-110 and the agonist BAY K 8644 on adipose tissue fatty acid synthesis in ethanol withdrawal.

Groups of 6-8 male TO mice were given 20% ethanol solution as drinking fluid for 4 - 6 weeks (equivalent to 10-15 g ethanol kg^{-1} day^{-1}); controls received water. *In vivo* lipogenesis rates were estimated by measuring the incorporation of ^3H into adipose tissue fatty acids over 1 hr following IP injection of ^3H_2O (Mercer & Trayhurn, 1983). Assays were performed at the same time (between 09.30 and 11.00 h) each day. PN 200-110 (10 mg kg^{-1}) was given IP at the time of withdrawal (t = 0) or at 8 or 16 h subsequently. Lipogenesis, expressed as μg atoms H incorporated h^{-1} g fat-free tissue $weight^{-1}$, was measured over the time periods t = 0 - 1, 8 - 9 and 24 - 25 h from withdrawal. BAY K 8644 (2mg kg^{-1}), where appropriate, was given concurrently with the PN 200-110. Controls received injections of vehicle at the equivalent time points. Differences between groups (mean ± s.e.mean) were analysed by Students t-test.

Chronic ethanol treatment slightly increased the brown adipose tissue lipogenesis rate compared to controls but. at 9 h withdrawal, lipogenesis was significantly decreased to 37.0 ± 5.4 from 132 ± 7.8 at t = 0 ($P < 0.001$), returning to near the control (non-drinking) value (117 ± 8.2) by 24 h (106 ± 9.8). PN 200-110 given at t = 0 prevented the decrease observed at 9 h withdrawal. Administration of PN 200-110 at 8 h, immediately before the lipogenesis assay, did not alter the lipogenic rate compared to vehicle-treated animals. BAY K 8644, given together with PN 200-110 at t = 0, blocked the effect of PN 200-110 so that lipogenesis subsequently remained suppressed at 9 h (34.0 ± 8.8). When administered at the same doses to control (non-drinking) mice, BAY K 8644 slightly increased lipogenesis, but PN 200-110 was without effect.

We conclude that the metabolic adaptation responsible for the suppression of lipogenesis in ethanol withdrawal occurs early in the withdrawal phase and may be dependent upon calcium entry into adipocytes.

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97P CALCIUM STORE DEPLETION BY CYCLOPIAZONIC ACID ACTIVATES A NON-SELECTIVE CATION CURRENT IN SINGLE SMOOTH MUSCLE CELLS FROM THE MOUSE ANOCOCCYGEUS

C.P. Wayman, A. Gibson, I. McFadzean & J.F. Tucker, Pharmacology Group, Division of Biomedical Sciences, King's College London, Manresa Road, London SW3 6LX.

Cyclopiazonic acid (CPA), an agent which inhibits Ca^{2+}-ATPase activity in sarcoplasmic reticulum and thus depletes intracellular Ca^{2+} stores in a receptor-independent manner, produces contractions of the mouse anococcygeus muscle (Gibson *et al.*, 1994). These contractions persist in the presence of nifedipine (1μM) but are abolished on the removal of extracellular Ca^{2+}, suggesting that they are mediated by Ca^{2+} influx *via* a non-voltage-dependent pathway. In the present study, we have obtained electrophysiological evidence for such a process.

Single smooth muscle cells were prepared by an enzymatic dissociation method based on that described for rat anococcygeus cells (McFadzean & England, 1992). Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique. The extracellular solution bathing the cells contained *inter alia* tetraethylammonium chloride (TEA; 30mM), $CaCl_2$ (10mM) and nifedipine (1μM). The intracellular solution contained (in mM) CsCl 130, TEA 20, $MgCl_2$ 1.0, HEPES 10, EGTA 5, $CaCl_2$ 1.17 (free intracellular Ca^{2+} 100nM) and was supplemented with ATP 0.5 and GTP 0.5 (pH 7.2).

CPA (5μM) induced an inward current (amplitude 2.4±0.3pA at a holding potential of -40mV) in 52 out of 69 cells to which it was applied. The response was concentration-dependent (1-20μM CPA) and was reversed by nickel (100μM; n=7), cadmium (100μM; n=6) and the imidazole derivative SKF96365 (10μM; n=8; Merritt *et al.*, 1990). It was not however reversed by either lanthanum (400μM; n=6) or gadolinium (50μM; n=5). Current-voltage (I-V) relationships for the cells, before and after CPA, were constructed by ramping the membrane potential from -100mV to +60mV over 1 second. The I-V relationship for the CPA-induced current (obtained by subtracting the control I-V relationship from that obtained in the presence of CPA) was linear over the potential range tested, and reversed at 1.7±1.6mV (n=22). The reversal potential was not changed (0.8±1.1mV; n=8) when the extracellular chloride concentration was lowered from 148mM to 63mM, indicating that the CPA-induced current is not a Ca^{2+}-dependent chloride current but is most likely due to activation of a non-selective cation conductance.

In conclusion, we have described a non-selective cation current which is activated by intracellular Ca^{2+} store depletion, the pharmacology of which corresponds well with that of the CPA-induced contraction in this tissue, and, by extension, to store regulated Ca^{2+} influx.

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98P THE ACTION OF HYDROCHLOROTHIAZIDE ON FORCE AND INTRACELLULAR Ca^{2+} IN GUINEA-PIG ISOLATED MESENTERIC SMALL ARTERIES

P. Pickkers & A.D.Hughes, Department of Clinical Pharmacology, St. Mary's Hospital Medical School, ICSTM, London W2 1NY, UK.

Thiazide diuretics are widely used in the treatment of essential hypertension. Although hydrochlorothiazide is a diuretic, long term administration of thiazides reduces peripheral vascular resistance *in vivo* (Van Brummelen et al., 1980). Recently hydrochlorothiazide has been shown to relax vascular smooth muscle *in vitro* (Calder et al., 1992). We have therefore examined the effect of hydrochlorothiazide on tone and intracellular Ca^{2+} ($[Ca^{2+}]_i$) in isolated vascular smooth muscle.

Small arteries (~300μm internal diameter) were isolated from guinea-pig mesentery and mounted in a myograph to allow simultaneous measurements of isometric force and $[Ca^{2+}]_i$ (Jensen et al., 1992). Vessels were loaded with the Ca^{2+} indicator, fura-2, by incubating them in modified Krebs' physiological salt solution (PSS) containing 5μM fura-2/AM for 2h at 37°C. $[Ca^{2+}]_i$ was measured as the ratio of fluorescence at 510nm evoked by excitation at 340 and 380nm. Increases in force and $[Ca^{2+}]_i$ were normalized by expressing them as % increase in response to a depolarizing potassium solution (KPSS). Relaxation or reduction of $[Ca^{2+}]_i$ was measured as % reduction of agonist-induced force or $[Ca^{2+}]_i$. Data are means±s.e.mean of n observations.

In PSS hydrochlorothiazide (30μM) relaxed 10μM noradrenaline-contracted arteries by 85±16% (n=9); this relaxation was associated with a fall in $[Ca^{2+}]_i$ of 62±11%. (n=9). The effect of hydrochlorothiazide (30μM) was much reduced in vessels contracted with 10μM noradrenaline in the presence of KPSS (relaxation = -1±9%; n=5, reduction in $[Ca^{2+}]_i$ = 19±9%; n=5). The effect of hydrochlorothiazide on noradrenaline-induced force was inhibited in PSS by 20 min preincubation with charybdotoxin (100nM) (relaxation = 9±9%, reduction in $[Ca^{2+}]_i$ = 22±17; n=4). Preincubation of vessels with hydrochlorothiazide (30μM) for 20 min reduced noradrenaline-induced increases in force and $[Ca^{2+}]_i$ from 69±11% to 29±16% (force) and 50±4% to 35 ± 17% ($[Ca^{2+}]_i$). In contrast preincubation with hydrochlorothiazide (30μM) had little effect on the peak force or rise in $[Ca^{2+}]_i$ induced by caffeine (10mM) (caffeine alone = 38 ± 13% (force), 20 ± 6% ($[Ca^{2+}]_i$); n=5, caffeine + hydrochlorothiazide = 38 ± 22% (force), 18 ± 6% ($[Ca^{2+}]_i$; n=5).

Hydrochlorothiazide relaxes noradrenaline-induced force and reduces $[Ca^{2+}]_i$ in physiological saline, but fails to affect force or $[Ca^{2+}]_i$ induced by noradrenaline under depolarizing conditions. The effect of hydrochlorothiazide is inhibited by charybdotoxin, a blocker of Ca^{2+}-activated potassium channels (K_{Ca}) channels. These findings suggest that hydrochlorothiazide may relax vascular smooth muscle by an action involving K_{Ca} channels.

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99P COUPLING BETWEEN STIMULATION OF ATP RECEPTORS, INCREASE IN INTRACELLULAR Ca^{2+} AND CONTRACTION IN SMALL MESENTERIC RESISTANCE ARTERY OF THE RAT

R. Andriantsitohaina, G.J.L. Lagaud & J.C. Stoclet. Laboratoire de Pharmacologie et Physiopathologie Cellulaires, Université Louis Pasteur de Strasbourg, URA CNRS 600 Faculté de Pharmacie, BP 24, 67401 Illkirch-Cedex, FRANCE

Adenosine triphosphate (ATP) is a major mediator involved in sympathetic neurogenic contraction in small mesenteric arteries of the rat in which it produces its effect via the activation of P_{2x} purinoceptors (Juul et al. 1993). It has been reported that the contraction induced by ATP is associated with depolarization and a rise in intracellular Ca^{2+} ions ($[Ca^{2+}]_i$) in these resistance arteries (Juul et al., 1992). However, the mechanisms by which ATP produces this increase of $[Ca^{2+}]_i$ is not fully understood. These mechanisms were investigated in branch II or III of superior mesenteric arteries dissected from 12-14 week old male Wistar rats. Arterial segments (1.5-2 mm) were removed and mounted on a myograph filled with physiological salt solution (PSS) (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 0.4, $NaHCO_3$ 14.9, $MgSO_4$ 1.17, $CaCl_2$ 2.5, glucose 5.5) continuously kept at 37°C and gassed with a mixture of 95% O_2, 5% CO_2 (pH 7.4). Changes in contraction and $[Ca^{2+}]_i$ were determined simultaneously in vessels loaded with the calcium-indicator fura-2. [^{3}H]-D-*myo*-inositol 1,4,5 trisphosphate (IP_3) was measured in the presence of lithium (10 mM) in vessels exposed to ATP (15 s), after pre-incubation with [^{3}H]-D-*myo*-inositol for 18 h. Pertussis toxin (PTX) (600ng/ml) was used to investigate the possible involvement of G protein in the mechanism of action of ATP. All the experiments were carried out on vessels with intact endothelium. ATP (1 to 3000 µM) produced concentration-dependent increases in both $[Ca^{2+}]_i$ and contraction. Maximal responses were reached at 3 mM. In normal PSS, the increase in $[Ca^{2+}]_i$ and contraction produced by 1 mM ATP were inhibited by 70% in the presence of the voltage-dependent calcium channel blocker, nitrendipine (1 µM) and were abolished in the presence of the receptor-mediated calcium entry blocker, SK&F 96365 (30 µM). After depletion of the intracellular calcium stores (by repeated exposure of the vessels with noradrenaline (10 µM) in Ca^{2+}-free medium containing 2 mM EGTA), contractile responses elicited by exogenous calcium (3 mM) in the presence of 3 mM ATP were abolished by both nitrendipine (1 µM) and SK&F 96365 (30 µM). In Ca^{2+}-free medium (containing 0.5 mM EGTA), ATP was able to elicit a rise in both $[Ca^{2+}]_i$ and contraction only at a concentration of 3 mM. At this concentration, ATP also stimulated the accumulation of IP_3 from 16.1 ± 3.2 to 60.5 ± 5.5 dpm/µg DNA (n = 3; P<0.01). In Ca^{2+}-free medium, caffeine (10 mM) induced a fast transient increase in $[Ca^{2+}]_i$ and contraction. Under the same experimental condition, the rise in $[Ca^{2+}]_i$ and contraction produced by ATP (3 mM) were both completely abolished after stimulation of the vessels with 10 mM caffeine. By contrast, the responses to caffeine were not affected by prior stimulation with 3 mM ATP. Also in Ca^{2+}-free medium, thapsigargin (1 µM), an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, had no effect on either $[Ca^{2+}]_i$ or contraction when added to unstimulated vessels, but it decreased by 65 % the responses elicited by ATP (3 mM). Treatment of the vessels for 12 h with PTX (600 ng/ml) inhibited the contractile responses to ATP at 1 and 3 mM by 30% and 20% respectively. The PTX treatment abolished the contractile response to the exogenous Ca^{2+} (3 mM) in vessels exposed to ATP (3 mM), after depletion of the intracellular Ca^{2+} stores. By contrast, it did not affect the contractions produced either by 3 mM ATP or 10 mM caffeine, in Ca^{2+}-free medium.

These results indicate that, in rat small mesenteric resistance arteries, the increase in $[Ca^{2+}]_i$ and contraction elicited by ATP are due 1- to an influx of Ca^{2+} through both voltage-dependent calcium channels sensitive to nitrendipine and receptor-operated calcium channels sensitive to SK&F 96365, and 2- to the release of Ca^{2+} from an internal source via the production of IP_3. In addition, the results suggest the existence of two intracellular Ca^{2+} pools releasable by ATP in these resistance arteries. These two intracellular pools were both caffeine-sensitive, but only one of them was sensitive to thapsigargin. Furthermore, caffeine released another independent intracellular Ca^{2+} pool which is insensitive to ATP. Finally, the results suggest that a PTX-sensitive G protein mediates the effect of ATP on external calcium-dependent contractions in the small mesenteric resistance arteries of the rat.

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100P THE *IN VITRO* PHARMACOLOGY OF ZM241385, A NOVEL, NON-XANTHINE, A_{2A} SELECTIVE ADENOSINE ANTAGONIST

S.M. Poucher, M.G.Collis, J.R.Keddie, S.M. Stoggall, P. Singh, P.W.R.Caulkett & G.Jones.
Cardiovascular and Metabolism Dept., ZENECA Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG.

Adenosine receptors have been classified into at least four different subtypes, A_1, A_{2a}, A_{2b} and A_3 (Collis & Hourani, 1993). A more complete understanding of the role of the various adenosine receptor subtypes in specific physiological responses has been limited by the lack of selective and specific receptor antagonists. Currently the most A_{2a}/A_1 selective antagonist is 8-(3-chlorostyryl)caffeine which has 520-fold and 22-fold A_{2a}/A_1 selectivity in binding and functional tests respectively (Jacobson *et al*, 1993). In this communication we report studies of the antagonist properties of a novel compound, ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol), at A_{2a} adenosine receptor subtypes.

Affinity of ZM241385 at A_{2a} and A_1 binding sites was assessed in a rat phaeochromocytoma PC12 cell line (Williams *et al* 1986) and in rat cortex (Bruns *et al*, 1980) respectively. Evaluation of antagonism of adenosine receptor mediated functional responses was further investigated using the guinea pig atria (A_1 receptor, Collis, 1983),the guinea pig Langendorff heart (A_{2a} receptor, Ueeda et al, 1991) and guinea-pig aortae (A_{2b} receptor, Hargreaves *et al*, 1991). ZM 241385 has a low potency both against displacing [^{3}H] R- PIA(R-phenylisopropyladenosine) from guinea pig cerebral cortex membranes (pIC_{50} = 5.69, 95% confidence limit, cl, 5.57-5.81) and blocking the bradycardic action of 2-CADO (2-chloroadenosine, pA_2=5.95,cl 5.72-6.18, Schild slope = 1.041) in the guinea pig atria. ZM 241385 has high binding affinity (pIC_{50} = 9.52, cl, 9.02-10.02) in displacing tritiated NECA (5'-N-ethylcarboxamidoadenosine) from PC12 cell membranes and displayed high potency (pA_2 = 9.02, cl 9.20-8.80, Schild slope 0.786) in attenuating coronary vasodilatation of the isolated guinea-pig Langendorff heart produced by CGS 21680 2-[p-(2-carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidoadenosine). ZM 241385 has a relatively low potency (pA_2=7.06,cl 6.92-7.19, Schild slope = 1.071) in blocking the vasorelaxant effects of adenosine in the guinea-pig aorta compared to its antagonism of vasodilator responses in the guinea pig Langendorff heart.

Thus in ligand binding ZM241385 demonstrated 6760-fold A_{2a}/A_1 selectivity and the functional A_{2a}/A_1 selectivity was 1170-fold. Although ZM241385 demonstrated a somewhat less A_{2a}/A_{2b} selectivity this was still around 90-fold. In conclusion, ZM241385 is a novel, non-xanthine A_{2a} selective adenosine receptor antagonist in both rat phaeochromocytoma PC12 cell membranes, rat cerebral cortex membranes and guinea-pig isolated tissue preparations.

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101P BINDING SITES FOR α,β-METHYLENE ATP ARE PRESENT IN THE VERTEBRATE RETINA

M.J. Neal,* J.R. Cunningham & S.J. Paterson, Department of Pharmacology, UMDS, St Thomas' Hospital, London, SE1 7EH

Adenosine is co-localised with acetylcholine in displaced amacrine cells in the rabbit retina (Blazynski, 1989) and since adenosine is rapidly phosphorylated to ATP in the rabbit retina (Perez *et al.*, 1986), it is possible that ATP and acetylcholine are co-localised in these central neurones. Using a rabbit eye-cup preparation we have shown recently that co-released ATP modulates the light-evoked release of ACh from cholinergic neurones (Neal & Cunningham, 1994). In the present study we show that the retina possesses binding sites for α,β-methylene ATP (α,β-meATP), a P_{2X}-purinoceptor agonist.

Washed membranes were prepared from the brain and retina of male Wistar rats, New Zealand white rabbits and Dunkin-Hartley guinea-pigs in 50mM Tris.HCl (pH 7.4) (Kosterlitz *et al.*, 1981). Homogenates were incubated with [^{3}H]α,β-meATP (1nM) for 30 min at 4°C or 25°C in a final volume of 250μl. Samples were filtered over 20mM tetrasodium pyrophosphate treated filters and washed with Tris.HCl (pH7.4) at 4°C. In preliminary experiments, the binding of [^{3}H]α,β-meATP in rabbit retina was found to be greater at 25° C (422±68 fmol mg^{-1} protein, n=5) than 4°C (194±27 fmol mg^{-1} protein, n=4). The inclusion of 3mM $CaCl_2$ (Michel & Humphrey, 1994) in the incubation medium had no effect on binding at 25°C but increased the binding at 4°C to 255±27 fmol mg^{-1} protein (n=4). Similar results were obtained in rat brain. Since the highest levels of binding were found at 25°C in the absence of added ions, these conditions were used for all subsequent experiments.

When the binding of [^{3}H]α,β-meATP was compared in the retina and brain of several species, the binding in the retina was 2.5 to 5.4 times higher than that in the brain (Table 1).

Table 1 Binding of [^{3}H]α,β-meATP (1nM) in brain and retina at 25°C.

	binding (fmol mg^{-1} protein)	
Species	Brain	Retina
rat	94±9 (23)	238±45 (4)
rabbit	57±14 (4)	308±44 (22)
guinea-pig	63±14 (5)	235±41 (9)

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102P THE EFFECT OF FPL 67085 ON ADP-INDUCED PLATELET AGGREGATION *EX VIVO* IN THE URETHANE-ANAESTHETISED RAT: A COMPARISON WITH ORAL ASPIRIN AND TICLOPIDINE

J.A. Clegg, L. Fraser-Rae, R.G. Humphries & M.J. Robertson, Department of Pharmacology, Fisons plc, Pharmaceutical Division, Research & Development Laboratories, Bakewell Road, Loughborough, LE11 0RH.

The P_{2T}-purinoceptor antagonist, FPL 67085, is a potent, specific and selective inhibitor of adenosine diphosphate (ADP)-induced platelet aggregation *in vitro* and *ex vivo* (Humphries *et al.*, 1994). In the present study, in anaesthetized rats, the effect of intravenous (i.v.) infusion of FPL 67085 on ADP-induced platelet aggregation *ex vivo* was compared with that of oral (p.o.) treatment with ticlopidine, an inhibitor of ADP-induced platelet aggregation with an ill-defined mechanism (Defryn *et al.*, 1991), or aspirin. Male rats (Sprague-Dawley, 450-600 g) were anaesthetized with urethane (40% w/v, 3 ml kg^{-1}). ADP (0.03-1000 μM)-induced platelet aggregation was measured by an impedance method in heparinized (10 U ml^{-1}) carotid arterial blood samples (1 ml). These were obtained at time-matched intervals during infusion of vehicle (saline, 0.03 ml min^{-1} i.v., n=5) or, in separate groups of animals, 1 of 3 doses of FPL 67085 (1, 10, 100 μg kg^{-1} min^{-1} i.v., n=5-6). Further groups of rats received either aspirin (100 mg kg^{-1} day^{-1} p.o, 3 days, n=6), a maximally tolerated dose of ticlopidine (200 mg kg^{-1} day^{-1} p.o., 5 days, n=6) or vehicle (5% arabic gum p.o., n=5) prior to anaesthesia. Effectiveness of aspirin pre-treatment was assessed by abolition of arachidonic acid (1 mM)-induced platelet aggregation *ex vivo*. ADP produced concentration-dependent platelet aggregation with a geometric mean ED_{50} of 0.28 μM (0.18 - 0.38) in control animals (Figure 1). The ADP concentration-response curve was displaced to the right in a dose-dependent manner by FPL 67085 (dose ratios of 5 (4-11), 65 (38-154) and 488 (393-607) at 1, 10 and 100 μg kg^{-1} min^{-1} i.v., respectively), with a trend to depression of the maximum response. Treatment of rats with either aspirin or ticlopidine produced approximately 2-fold rightward displacements (dose ratios 1.4 (0.8-2.4) and 1.6 (0.8-2.7), respectively) of the ADP concentration-response curve.

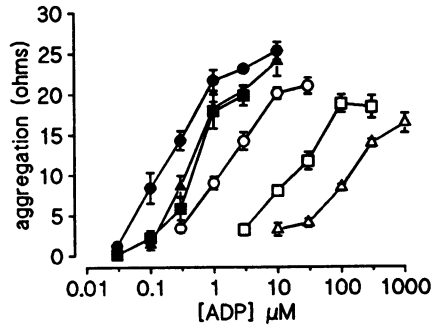


Figure 1: The effect of FPL 67085 (○ 1, □ 10, △ 100 μg kg^{-1} min^{-1} i.v.), vehicle (●), ticlopidine (▲) or aspirin (■) on ADP-induced platelet aggregation ex vivo. Values are means ± s.e. (n = 5-6)

In summary, FPL 67085 is a more effective inhibitor of ADP-induced platelet aggregation *ex vivo* than aspirin or ticlopidine in the anaesthetized rat. We have previously shown that FPL 67085 is a more efficacious anti-thrombotic agent than p.o. aspirin in the anaesthetized dog (Robertson *et al.*, 1994). However, it remains to be determined whether these differences will translate into a superior anti-thrombotic profile for FPL 67085 in man.

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103P DOES PROTEIN PHOSPHATASE 1 REGULATE NEUROTRANSMITTER RELEASE IN POSTGANGLIONIC SYMPATHETIC NERVE TERMINALS IN THE GUINEA-PIG VAS DEFERENS?

D.B. Elrick and T. C. Cunnane, University Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT.

Phosphorylation of specific nerve terminal proteins is well established as an important mechanism in modulating neurotransmitter release (Walaas and Greengard, 1991). The release of ATP from postganglionic sympathetic neurones innervating the guinea-pig vas deferens is powerfully enhanced by agents which activate protein kinase C (Wardell and Cunnane, 1994). The effects of these phosphorylation changes are relatively short-lived, suggesting that dephosphorylation reactions must return the substrates to a resting dephosphorylated state when stimulation ceases. In the present study, the effects of the protein phosphatase inhibitors calyculin A and okadaic acid on neurotransmitter release mechanisms in sympathetic nerve terminals have been investigated.

Conventional intracellular and focal extracellular recordings were made in guinea-pig isolated vas deferens as previously described (Brock and Cunnane, 1988). Excitatory junction potentials (EJPs) evoked by trains of 10 stimuli at 1 Hz were markedly reduced in amplitude in the presence of calyculin A (50 - 300 nM). The mean amplitude of the 10th EJP in a train was reduced from 12.6 ± 1.6 mV in controls to 5.3 ± 1.0 mV (n = 5) by calyculin A (100 nM). The pattern of facilitation of EJP amplitudes during trains was unaffected by calyculin A (100 nM). The amplitude distribution of extracellularly recorded spontaneous excitatory junction currents (SEJCs) was unaffected by calyculin A (300 nM) but their frequency of occurrence was reduced by 41.5 % (n = 3). Averaged EJC amplitudes evoked by trains of 30 stimuli at 1 Hz were reduced from 19.8 ± 3.4 μV in controls to 9.0 ± 1.7 μV (n = 3) in calyculin A (300 nM). This decrease in mean amplitude was due to an increase in the numbers of failures. The nerve terminal impulse was unaffected by calyculin A (300 nM). Okadaic acid (1 μM) produced a modest yet significant ($P < 0.05$) inhibition of EJP amplitudes, the 10th EJP in a train being reduced from 20.6 ± 1.2 mV in control to 16.1 ± 0.6 mV (n = 3).

In conclusion, calyculin A reduces neurotransmitter release from postganglionic sympathetic neurones in the guinea-pig vas deferens, without affecting the pattern of facilitation. The inhibitory effect of calyculin A has a prejunctional origin and lies at the level of depolarisation-secretion coupling. Calyculin A has been shown to be 30 - 250 times more potent than okadaic acid as an inhibitor of protein phosphatase 1, whilst these agents are equipotent as inhibitors of protein phosphatase 2A (Ishihara *et al.*, 1989). Comparison of the effects of these agents in this preparation suggests that inhibition of protein phosphatase 1 mediates the reduction in neurotransmitter release produced by calyculin A.

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104P THE EFFECT OF ECTO-ATPASE INHIBITION ON AGONIST POTENCY ORDERS AT P_{2Y} AND P_{2X} PURINOCEPTORS

McKechnie K.C.W., Crack B E., Dainty I A., and Leff P., Department of Pharmacology, Fisons Plc, Research & Development Laboratories, Bakewell Road, Loughborough, Leicestershire LE11 ORH.

FPL 67156 (6-N,N-diethyl-D-β,γ-dibromo-methyleneATP) is a recently discovered selective inhibitor of ecto-ATPase (Beukers *et al.*, 1994). Previous studies have shown that in functional tissues, FPL 67156 causes concentration effect (E/[A]) curves to the hydrolysable agonists ATP and UTP to be displaced to the left (Crack *et al.* 1994). The purpose of the present study was to determine the effect of FPL 67156 on agonist potency orders at P_{2X} - and P_{2Y} - purinoceptors, respectively.

P_{2X}-mediated contractions were studied in the rabbit ear artery. Isolated ring preparations from male New Zealand White rabbits were prepared for isometric force recordings and maintained in Krebs buffer containing indomethacin (2.8μM) at 37°C and gassed with 95%O_2/5%CO_2. Cumulative E/[A] curves were constructed to the standard agonist α,β-methylene-ATP (α,β-meATP). Preparations were then washed and 70 minutes later a second E/[A] curve to one of the test agonists was constructed in the presence or absence of FPL 67156 (300μM). Data were fitted to a logistic function for potency information.The relative potencies (R.P.) were calculated with respect to α,β-meATP ($p[A_{50}]$= 6.59±0.05). P_{2Y}-mediated endothelium dependent relaxations of pre-constricted guinea-pig aortic rings were obtained as described elsewhere (Dainty *et al.*, 1991). The first E/[A] curve to the test agonist was constructed and the tissues then washed. 60 minutes later a second E/[A] curve was constructed in the presence or absence of FPL 67156 (300μM). Relative potencies were calculated with respect to 2meSATP ($p[A_{50}]$=7.75± 0.09).

At P_{2X} -purinoceptors in the rabbit ear artery the potency order obtained was:

α,β–meATP>>ATPγS>2meSATP>ATP>UTP

In the presence of FPL 67156 the potency order was changed to:

α,β–meATP>2meSATP=ATP>ATPγS>UTP

At P_{2Y} purinoceptors the potency order was:

2meSATP>>ATPγS>ATP>>αβmeATP,UTP

In the presence of FPL 67156 the potency order was not changed although the absolute potencies of ATPγS and ATP, but not 2meSATP did increase. These data suggest that in both the rabbit ear artery and the guinea-pig aorta the potencies of hydrolysable agonists at P_2-receptors are affected by ecto-ATPase activity. That the potency order at P_{2Y} receptors in the guinea pig aorta was not altered in the presence of FPL 67156 may reflect different degrees of ecto-ATPase activity in the two tissues.

The change in potency orders observed in the rabbit ear artery, and the leftwards displacements in the agonist curves found in both tissues in the presence of ecto-ATPase inhibition, demonstrates that agonist potency orders currently held to designate P_2- purinoceptors needs to be reassessed.

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Table 1.

Agonist	P_{2X} R.P. [95% C.L.] Control	+FPL67156	Agonist	P_{2Y} R.P. [95% C.L.] Control	+FPL 67156
α,β–meATP	1.0	1.0	2meSATP	1.0	1.0
ATPGS	0.0079 [0.0047-0.0132]	0.0123 [0.006 - 0.0256]	ATPGS	0.0068 [0.0054 - 0.0086]	0.0759 [0.0578 - 0.0916]
2meSATP	0.0052 [0.0027-0.01]	0.0468 [0.0218 - 0.1]	ATP	0.0039 [0.0013 - 0.0071]	0.0298 [0.0071 - 0.1244]
ATP	0.0005 [0.0002-0.001]	0.0282 [0.0059 - 0.1348]	αβ–meATP	inactive	inactive
UTP	<0.0001	0.0005 [0.0003 - 0.0010]	UTP	inactive	inactive

105P AUGMENTED SENSORY-MOTOR VASODILATATION OF RAT MESENTERIC ARTERIES AFTER CHRONIC ANTAGONISM OF P_1-PURINOCEPTORS WITH 1,3-DIPROPYL-8-SULFO-PHENYLXANTHINE (DPSPX)

V. Ralevic, A. Rubino & G. Burnstock, Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower St., London WC1E 6BT

Continous perfusion of the non-selective adenosine P_1-purinoceptor antagonist 1,3-dipropyl-8-sulfophenylxanthine (DPSPX) *in vivo* causes hypertension and structural changes in the cardiovascular system of rats (Azevedo & Osswald, 1992). In the rat mesenteric artery this is expressed as hypertrophy and hyperplasia of vascular smooth muscle cells. The functional consequences of these vascular changes have not been examined. Hence, the function of sensory-motor nerves, sympathetic nerves, endothelium and smooth muscle after DPSPX-treatment was examined in the isolated perfused mesenteric arterial bed of the rat.

Mesenteric arterial beds were isolated from rats which had received constant infusion for 7 days of either DPSPX (30 μg/kg/h) or saline using Alzet osmotic minipumps. Preparations were perfused at 5 ml/min with warmed (37°C), gassed (95% O_2/5% CO_2) Krebs' solution (Ralevic *et al.*, 1994). Responses were measured as changes in perfusion pressure (mmHg). Sensory-motor vasodilatation to electrical field stimulation (EFS; 1-12 Hz, 60V, 0.1ms, 30s; in the presence of guanethidine, 3μM) and endothelium-dependent and -independent vasodilatation was examined during methoxamine (3-30 μM)-raised tone. Vasoconstrictor responses to EFS of sympathetic nerves (4-32 Hz, 90V, 1ms, 30s) and to sympathetic agonists were examined at basal tone.

Frequency-dependent sensory-motor vasodilatation was markedly augmented in DPSPX-treated rats. At 8Hz relaxation was 38.34 ± 4.76% (n = 5) in controls and 65.92 ± 3.68% (n = 5) in DPSPX-treated preparations ($P < 0.01$; Student's *t* test). Adenosine (3 μM) attenuated sensory-motor vasodilatation to a similar degree in DPSPX- and saline-treated preparations at all frequencies; at 8Hz responses were inhibited by 59.89 ± 1.5% and 45.46 ± 8.59% respectively. Dose-dependent vasodilatation to the sensory transmitter calcitonin gene-related peptide (CGRP; 5-50 pmol) and to the sensory neurotoxin capsaicin (50-500 pmol) were similar between the two groups. Dose-dependent endothelially-mediated vasodilatation to acetylcholine and endothelium-independent vasodilatation to sodium nitroprusside were also unchanged. Vasoconstriction to EFS of sympathetic nerves was similar between the two groups. Adenosine (10 and 30 μM) inhibited sympathetic constrictor repsonses equally in DPSPX and control preparations. Constrictions to exogenous noradrenaline and adenosine 5'-triphosphate were similar between the groups.

In conclusion, prolonged antagonism of P_1-purinoceptors with DPSPX *in vivo* causes a selective increase in mesenteric sensory-motor vasodilatation that is not due to a defect in prejunctional inhibition via P_1-purinoceptors, or to a change in postjunctional receptors for CGRP, or to functional changes in the vascular smooth muscle. Possible mechanisms will be discussed.

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106P CLASSIFICATION OF P_2-PURINOCEPTORS ON A HUMAN PROSTATE CANCER CELL LINE (PC3 CELLS)

I.A. Dainty, M. Franklin & K.C.W. McKechnie, (introduced by P. Leff) Department of Pharmacology, Fisons Research and Development Laboratories, Bakewell Road, Loughborough, Leicestershire, LE11 0RH.

ATP and other nucleotides have been shown to increase $[Ca^{2+}]_i$ in human prostate cancer (PC3) cells (Fang *et al.*, 1992). Fang's study used a limited number of nucleotides at single concentrations. In the present study full concentration-effect (E/[A]) curves of a range of nucleotides and nucleosides were constructed to obtain an agonist potency order. The influence of ecto-ATPases can change agonist potency orders (McKechnie *et al.*, 1994). The effect of an ecto-ATPase inhibitor on agonist potency order in these cells has been tested. Suramin, which may identify sub-types of P_{2U}-purinoceptors (Dainty *et al.*, 1994), was also used.

Changes in $[Ca^{2+}]_i$ were measured using standard fluorescent techniques (Fura-2 or, in suramin studies Fluo-3). Fluorescence was measured in cuvettes containing a 2ml suspension of cells. Each cuvette was used for a single determination. Fluorescence was measured for 5s and then for at least 30s after addition of a compound. E/[A] curves (1000-0.1μM) were constructed in 1 $\log_{10}$ unit increments. Suramin and compounds with no agonist activity were tested as antagonists: E/[A] curves were constructed in the presence of the putative antagonist added 10s before the agonist. Responses were expressed as a % of that to 10μM UTP.

Compounds showing agonism (Table 1) gave a potency order of: UTP≥ATP>ATPγS>ITP>UDP≥ADP>TTP>XTP>GTP>CTP
Nucleotides and nucleosides showing no agonism at 1mM did not antagonise UTP. The response to 100μM of αβmeATP and 2meSATP were < 15% of UTP and did not affect subsequent UTP responses. The P_{2T}-purinoceptor antagonist FPL 66096 (Humphries *et al.*, 1994) had no effect on the ADP response. 300μM FPL 67156 (McKechnie *et al.*, 1994) caused no leftward shifts of E/[A] curves to ATP, UTP and ATPγS. Suramin caused over three fold rightward shifts of the UTP curve. Under the conditions used the curves in the presence of suramin were incomplete at 100μM so exact quantification is not possible.

Agonist	α	$p[A_{50}]$	Agonist	α	$p[A_{50}]$
UTP	98.5 (4.6)	6.2 (0.1)	ITP	108.8 (11.3)	5.2 (0.2)
ATP	100.3 (6.1)	5.9 (0.1)	ADP	100.2 (0.6)	4.5 (0.0)
ATPγS	100.6 (12.3)	5.4 (0.2)	UDP	108.2 (28.4)	4.4 (0.2)

Table 1 Values shown are mean (s.e. mean) n= 4-8. Curves to TTP, XTP, GTP and CTP were incomplete at 1mM

This study demonstrates the presence of a purinoceptor mediating increases in $[Ca^{2+}]_i$ in PC3 cells. The potency of ATP and the lack of effect of adenosine suggest that this is a P_2-purinoceptor. The poor activity of αβmeATP and 2meSATP indicates an absence or low reserve of P_{2X}- and P_{2Y}-purinoceptors. P_{2T}-purinoceptor activity is precluded since FPL 66096 did not affect ADP. Thus, the general potency order suggests the presence of a P_{2U}-purinoceptor on these cells. As is the case in the rat aorta, the P_{2U}-purinoceptor is a suramin sensitive subtype (Dainty et al., 1994). The lack of effect of FPL 67156 indicates that this is the true potency order for the human P_{2U}-purinoceptor present in PC3 cells.

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107P DIADENOSINE POLYPHOSPHATES DIFFERENTIALLY ACTIVATE P_{2X}- AND P_{2Y}-PURINOCEPTORS IN THE GUINEA-PIG UROGENITAL AND GASTROINTESTINAL TRACTS

C.H.V. Hoyle, A.U. Ziganshin[1] & G. Burnstock, Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT and [1]Kazan Medical Institute, Butlerov St., Kazan.

The pharmacological activity of a series of P^1,P^n-bis(5'-adenosine)oligophosphates, where n ranges from 2 to 6 (Ap_2A to Ap_6A), was examined in isolated preparations of the guinea-pig urinary bladder and vas deferens, which both possess P_{2X}-purinocpetors, and the taenia coli, which possesses P_{2Y}-purinoceptors. Tissues were maintained in a standard Krebs' solution at 37 ± 0.5 °C, gassed with 95% O_2 / 5% CO_2 in 10 ml organ baths. Concentration response relationships were determined for all the compounds. In the bladder and vas deferens the curves did not plateau, so relative potencies were determined from the negative log concentration that produced an increase in tension of 40 $mg.mg^{-1}$ tissue (pC_{40}, Table 1). In the taenia coli all the compounds could produce a complete loss of tone induced by carbachol (50 nM), thus relative potencies were compared using pD_2 (-log EC_{50}) values (Table 1). The rank orders of potency of the dinucleotides at the vas deferens, urinary bladder and taenia coli were: $Ap_5A > Ap_6A > Ap_4A > Ap_3A$, $Ap_5A > Ap_6A = Ap_4A > Ap_3A$, and $Ap_3A > Ap_4A > Ap_5A > Ap_6A > Ap_2A$, respectively. At the P_{2X}-purinoceptors Ap_2A was inactive up to 1 mM. pA_2 values were estimated from pK_B values. In the vas deferens and urinary bladder the P_{2X}-purinoceptor antagonist, pyridoxal phosphate-6-azophenyl disulphonic acid (PPADS), 10 μM, produced such a severe antagonism that pK_B values could not be determined. For PPADS at a concentration of 3 μM the estimated pA_2 values against Ap_5A and ATP in the vas deferens were 6.3 ± 0.16 (4) and 6.4 ± 0.33 (4), respectively. In the taenia coli the non-selective P_2-purinoceptor antagonist, suramin (100 μM), antagonised ATP and all the dinucleotides, except Ap_2A, with a similar estimated pA_2 (Table 1). These results indicate that the P_{2X}-purinoceptors prefer long-chain to short-chain diadenosine oligophosphates, and that the P_{2Y} receptor is less discriminative, although it is selectively activated by Ap_3A rather than Ap_5A.

Table 1. Data given as mean ± s.e. mean (n)

	ATP	Ap_2A	Ap_3A	Ap_4A	Ap_5A	Ap_6A
pC_{40} vas	3.9 ± 0.3 (4)	- (4)	3.4 ± 0.2 (4)	5.2 ± 0.2 (10)	6.9 ± 0.3 (10)	5.5 ± 0.2 (8)
pC_{40} bladder	4.5 ± 0.2 (4)	- (4)	3.7 ± 0.2 (4)	5.2 ± 0.2 (4)	6.9 ± 0.2 (6)	5.8 ± 0.2 (4)
pD_2 taenia	6.2 ± 0.2 (7)	4.9 ± 0.1 (6)	6.6 ± 0.1 (5)	6.0 ± 0.1 (5)	5.5 ± 0.1 (6)	5.5 ± 0.1 (7)
pA_2 suramin	5.3 ± 0.1 (2)	4.3 ± 0.3 (2)	5.2 ± 0.2 (2)	5.0 ± 0.1 (2)	4.9 ± 0.1 (2)	5.0 ± 0.1 (2)

108P UNIBLUE A (UBA) BUT NOT COOMASSIE BRILLIANT BLUE G (BB) ANTAGONISES α,β-METHYLENE ATP- BUT NOT UTP-EVOKED DEPOLARISATIONS OF RAT SUPERIOR CERVICAL GANGLIA

G.P. Connolly & P.J. Harrison. Department of Physiology, University College London, Gower Street, London. WC1E. 6BT.

We have investigated the effects of BB (a putative antagonist of P_2-purinoceptors; Soltoff *et al.*, 1989) and UBA (an analogue of reactive blue 2) on the depolarisation of rat SCG evoked by alpha-beta-methylene-adenosine 5'-triphosphate (α,β-MeATP) and uridine 5'-triphosphate (UTP) using methods described before (Connolly *et al.*, 1993). Ganglia were placed in a chamber, perfused with medium and the d.c. potential recorded across a greased barrier. Adenosine, α,β-MeATP or UTP were added to the perfusate for 2 min and muscarine or K^+ for 1 min with ≥ 20 min between applications. Responses of 4 ganglia in the absence (control) and presence of a dye (≥ 30 min incubation) were compared for statistical significance (* = $P<0.05$ and ** = $P<0.01$; paired t-test). Responses are expresed as a % of the control response and summarised in Table 1. BB and UBA did not alter depolarisations evoked by muscarine or K^+, and only UBA reduced hyperpolarisations evoked by adenosine. Depolarisations mediated by α,β-MeATP (a specific P_{2X}-purinoceptor agonist) were reduced in the presence of UBA but not by BB. Depolarisations evoked by UTP were significantly enhanced by UBA and unaltered by BB (Table 1).

Our results are consistent with the proposal that UBA and reactive blue 2 (Connolly & Harrison, 1994) but not BB are antagonists of P_{2X}-purinoceptors and that UBA distinguishes between receptors for UTP and α,β-MeATP on the rat SCG. The depression by UBA of hyperpolarisations evoked by AD suggests that as well as being an antagonist at P_2-purinoceptors, UBA is also an antagonist at P_1-purinoceptors. UBA shares with RB2 (Connolly & Harrison, 1994) and suramin an ability to enhance UTP-evoked depolarisations. The ability of UBA, BB, suramin and reactive blue 2 to inhibit α,β-MeATP-evoked depolarisations but potentiate UTP-evoked depolarisations may indicate that these two phenomena are coupled via some common mechanism.

In conclusion these results further justify our proposal that on rat SCG, UTP and ATP activate seperate receptors, i.e. a pyrimidinoceptor and an P_2-purinoceptor respectively.

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Table 1. Effect of UBA and BB on the response of the rat SCG evoked by depolarising agents and adenosine (% control).

Treatment\ Agent	αβMeATP (100μM)	UTP (100μM)	AD (100μM)	Muscarine (100nM)	K^+(5mM)
UBA(300μM)	-56 ± 7*	78 ± 27*	-78 ± 5*	-41 ± 15	53 ± 19
BB (10uM)	-19 ± 12	18 ± 6	5 ± 25	-50 ± 35	-15 ± 12

109P ENHANCEMENT OF SYMPATHETIC, PURINERGIC NEUROTRANSMISSION BY A NOVEL ECTO-ATPase INHIBITOR IN GUINEA-PIG ISOLATED VAS DEFERENS

P. Sneddon, H. Walker, P. Leff[1] & C. Kennedy, Department of Physiology & Pharmacology, University of Strathclyde, Glasgow G1 1XW and [1]Department of Pharmacology, Fisons PLC, Loughborough, LE11 ORH

In smooth muscle, ecto-ATPase enzymes rapidly inactivate exogenous ATP, but their effect on endogenous, neuronally-released ATP is not known. We have studied the role of ecto-ATPases in modulating purinergic responses in the guinea-pig vas deferens using a novel and selective ecto-ATPase inhibitor, FPL 67156 (6-N,N,-diethyl-D-β,γ-dibromomethylene ATP, Leff *et al.*, 1994). In this tissue excitatory junction potentials (e.j.ps) and the initial, phasic component of the contraction in response to sympathetic nerve stimulation is mediated by ATP, acting via P_{2X}-purinoceptors and the secondary, tonic component is mediated by noradrenaline (NA), acting via α_1-adrenoceptors.

Guinea-pig isolated vas deferentia were bathed in Krebs' solution at 35°C, bubbled with 95% O_2 5% CO_2. Contractions were evoked by field stimulation (supramaximal voltage, 0.5 ms) at 1-8 Hz for 20 s or by adding ATP, NA or α,β-methyleneATP (α,β-meATP) to the bathing solution. Standard intracellular recording techniques were used to record e.j.p.s elicited by field stimulation (submaximal voltage, 0.3 ms) at 1 Hz. Data were analysed using Student's paired t-test and * indicates $P < 0.05$.

FPL 67156 (20 μM) significantly increased the magnitude of the purinergic component of neurogenic contractions at 1 and 2 Hz, but had less effect at 4 Hz and no effect at 8 Hz (Table 1). Contractions to ATP (100 μM) were more than doubled in size in the presence of FPL 67156, but those to NA (10 μM) and α,β-meATP (10 μM) were unchanged. The effects of FPL 67156 were rapid in onset and rapidly reversed on washout.

Table 1. Effect of FPL 67156 on contractions (g) of guinea-pig isolated vas deferens.

	Control	+ FPL 67156 (20 μM)	
1 Hz	0.79 ± 0.44	1.21 ± 0.64*	(n = 8)
2 Hz	0.89 ± 0.18	1.44 ± 0.28*	(n = 13)
4 Hz	0.83 ± 0.21	1.04 ± 0.30	(n = 6)
8 Hz	1.11 ± 0.43	1.07 ± 0.60	(n = 4)
ATP (100 μM)	0.68 ± 0.18	1.37 ± 0.34*	(n = 9)
NA (10 μM)	1.60 ± 0.24	1.60 ± 0.23	(n = 4)
α,β-meATP (10 μM)	1.45 ± 0.55	1.46 ± 0.61	(n = 4)

FPL 67156 (50 μM) produced a small, but significant enhancement of the fully facilitated e.j.p. magnitude (from 13.0 ± 1.9 to 14.6 ± 2.1 mV), e.j.p. rise time (from 24.1 ± 1.9 to 29.3 ± 2.7 ms) and the time for the e.j.p. to decay to 50% of its peak value (from 125.1 ± 8.4 to 142.3 ± 10.2 ms), (n = 6 for each).

These results indicate that the ecto-ATPase inhibitor FPL 67156 enhances purinergic responses in guinea-pig isolated vas deferens, suggesting that ecto-ATPases normally modulate the actions of neuronally-released ATP in this muscle.

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110P SYSTEMIC MODULATION OF INTERFERON-γ AND TUMOUR NECROSIS FACTOR IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

A. Hutchings, V. Gibson, N. Lad, D. Bradshaw & R.F.G. Booth. AI Biology, Roche Research Centre, Welwyn Garden City, AL7 3AY

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterised by focal areas of demyelination leading to neurological deficit. Pathology has revealed the presence of interferon-gamma (IFNγ) and tumour necrosis factor (TNF) producing cells in MS lesions (Hofman *et al.*, 1989; Traugott & Lebon, 1988). We have investigated the effect of modulating systemic IFNγ and TNF levels using two cytokine receptor-IgG fusion proteins in EAE, an animal model for MS. Ro 45-2155 is a chimaeric construct comprising the murine IFNγ receptor (Kurschner *et al.*, 1992) and part of the heavy chain of murine IgG (IFNγR-IgG) whilst Ro 45-2081 is a similar construct comprising the soluble part of the human p55 TNF receptor (Lesslauer *et al.*, 1991; Loetscher *et al.*, 1991) and the heavy chain of human IgG (TNFp55-IgG). EAE was induced in AHH/R rats and Biozzi AB/H mice by inoculation of a spinal cord homogenate in Freund's adjuvant. The animals were weighed and evaluated daily for neurological signs (expressed as a clinical score). All control groups were dosed with phosphate buffered saline.

In the rat model treatment with 2 mg/kg *i.p.* IFNγR-IgG (on the day of induction and every 5 days thereafter to day 20) had no significant effect on the maximum mean clinical score (1.8 ± 0.2 *vs.* control 2.0 ± 0.3, ns) and disease incidence (10/10 *vs.* control 10/10), but increased the maximum mean body weight loss to 24.9 ± 2.5 g (control 18.5 ± 1.4 g, $p<0.05$). However treatment with 10 mg/kg *i.p.* TNFp55-IgG daily from day 8 to day 18 post-induction significantly reduced the clinical score to 1.3 ± 0.3 (control 2.7 ± 0.2, $p<0.05$) but had little effect on disease incidence (7/10 *vs.* control 10/10) or maximum mean body weight loss (14.7 ± 3.6 g *vs.* control 24.0 ± 4.3 g, ns). A similar profile was observed in the mouse model where treatment of 3 mg/kg *i.v.* IFNγR-IgG (on the day of induction and every 5 days thereafter) had little effect on clinical score (3.3 ± 0.6 *vs.* control 3.8 ± 0.1, ns), body weight loss (9.0 ± 3.7 g *vs.* control 6.8 ± 1.6 g, ns) or disease incidence (10/10 *vs.* control 10/10). Treatment with 7 mg/kg *i.p.* TNFp55-IgG daily from day 10 to day 30 post-induction reduced both the mean clinical score (0.9 ± 0.5 *vs.* control 2.2 ± 0.4, $p<0.05$) and body weight loss (1.2 ± 0.9 g *vs.* control 3.7 ± 1.5 g, $p<0.02$) but had little effect on disease incidence (6/10 *vs.* control 10/10). Although IFNγR-IgG had no significant effects in these models, it has been demonstrated to inhibit clinical scores in adoptive transfer models of EAE (Wekerle, personal communication). These results suggest that systemic administration of cytokine inhibitors can modify inflammation within the CNS.

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111P EVIDENCE THAT HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 PROTEIN gp120 REDUCES THE EXPRESSION OF NEURONAL NITRIC OXIDE SYNTHASE AND INCREASES NGF IN THE HIPPOCAMPUS OF RAT

G. Bagetta[1], M.T. Corasaniti[1], A.M. Paoletti[1], A. Finazzi-Agrò[2], F. Angelucci[3], L. Aloe[3] & G. Nisticò[1], [1]"Mondino-Tor Vergata" Neurobiol. Center, Dept. of Biology, and of [2]Exp. Med. and Biochem. Sci., University of Rome "Tor Vergata", 00173 Rome; [3]CNR Institute of Neurobiol., 00137 Rome

It has been suggested that the human immunodeficiency virus type 1 (HIV-1) coat glycoprotein gp120 may be the aetiologic agent of the reported human brain neuronal loss because it causes death of several types of neurones maintained in culture (see Lipton et al., 1992). Neuronal cell death caused by gp120 involves excessive Ca^{2+} entry into neurones via N-methyl-D-aspartate (NMDA) receptor associated cation channel and through voltage operated Ca^{2+} channels since NMDA antagonists and Ca^{2+} channel blockers prevent neuronal cell death (see Lipton, 1992). Recently, it has been shown that exposure of cortical neurones in culture to gp120 increases nitric oxide (NO), a highly reactive radical species, which seems to participate in the mechanisms of gp120-induced death because this was abolished by inhibition of NO synthesis (Dawson et al., 1993). Here we report evidence demonstrating that, intracerebroventricular (i.c.v.) microinfusion of gp120 for 14 days reduces the expression of the neuronal type of NO synthase (nNOS) and this is paralleled by significant increase of nerve growth factor (NGF) in the hippocampus of rat.

Recombinant gp120 (100 ng day^{-1}) or bovine serum albumin (BSA; 300 ng day^{-1}) were microinfused i.c.v. in rats for up to 14 days via mini-osmotic pumps (Alza mod. 2002; 0.5 ul h^{-1} rate) or by single, daily iniection of freshly dissolved HIV-1 coat protein as previously reported (see Bagetta et al., 1994). The expression of nNOS mRNA, the nNOS activity (i.e. conversion of [^{3}H]arginine into [^{3}H]citrulline) and the level of NGF were measured in the rat hippocampus as detailed elsewhere (Bagetta et al., 1993; Bracci-Laudiero et al., 1992).

Northern blot analysis of total RNA, extracted from the hippocampus of rats treated for 14 days with gp120, revealed a 2-fold reduction of the 10.5 kb nNOS mRNA signal as compared to the signal observed in control, BSA-treated animals (n=8). This effect was paralleled by a decrease of nNOS activity which was (gp120; 72±7 pmol of [3H]citrulline mg^{-1} protein min^{-1}) significant ($P<0.01$ vs control) in comparison to the control (BSA-treated; 118±5 pmol of [3H]citrulline mg^{-1} protein min^{-1}) enzyme activity (n=12). The reduction of nNOS expression induced by gp120 was accompanied by an approximately 30% increase in hippocampal NGF (3.5±0.4 ng g^{-1}tissue; n=5) over control (2.8±0.2 ng g^{-1}tissue; n=11). No consistent changes in nNOS mRNA expression (n=8 rats) and enzyme activity (n=12), as well as in NGF levels (n=10) were seen after 1 day and 7 days of treatment with gp120.

Financial support from the VII AIDS Project (Italian Ministry of Health, Rome) is gratefully acknowledged.

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112P INVESTIGATIONS INTO [^{3}H]L-ARGININE UPTAKE AND NITRIC OXIDE SYNTHASE ACTIVITY IN PRIMARY CULTURES OF HYPOTHALAMIC NEURONES

J. Wayte, J.C. Buckingham & A.M. Cowell. Dept. of Pharmacology, Charing Cross & Westminster Medical School, London W6.

Immunohistochemical studies, using antisera to nitric oxide synthase (NOS) and NADPH diaphorase staining as a neuronal marker for NOS, have shown NOS to be present in magnocellular and to a lesser extent parvicellular neurones of the supraoptic and paraventricular nuclei of the hypothalamus (Bredt *et al.* 1990, Arevalo *et al.* 1992). The precise role of NOS in the hypothalamus has not yet been determined but it has been suggested that NO may be involved in the modulation of magnocellular hormone release.

To examine further the role of NOS within this tissue, we have used the method of Kiedrowski *et al.* (1992) to investigate the uptake of [^{3}H]L-arginine and subsequent production of [^{3}H]L-citrulline in primary cultures of hypothalamic neurones. Cultures were initiated from rat tissue obtained at embryonic day 18 and maintained in a defined medium in 15mm^2 plates as described previously (Murray & Gillies 1993). Cells for study were used between days 3 and 12 *in vitro*.

Briefly cells were incubated with 200µl of [^{3}H]L-arginine (10µCi/ml) in incubation medium at 37^{0}C for 10 - 60 min. Uptake was terminated by rapid removal of the substrate and rinsing with cold incubation medium devoid of glucose and calcium. Cells were solubilised with 0.3M perchloric acid and the amounts of [^{3}H]L-arginine and [^{3}H]L-citrulline in the neutralised extracts were determined in parallel. [^{3}H]L-citrulline was measured as described by Salter *et al.* (1991) and is expressed as the% of incorporated radioactivity which was converted. For each treatment group 6-8 wells were used and results are expressed as mean ± s.e.m.

[^{3}H]L-arginine uptake was observed in cells from 3 days *in vitro* and this effect appeared to be time dependent. The % uptake of substrate and % converted to [^{3}H]L-citrulline respectively after 10, 20 and 30 min were 13.28±0.15 and 2.08±0.27%; 19.15±0.28% and 2.53±0.06%; 22.87±0.44% and 3.24±0.10%. Experiments performed on cells from day 5 and day 10 *in vitro* showed a similar pattern of uptake and [^{3}H]citrulline formation. For cells from day 5 *in vitro* at 10, 12.5, 15, 30 and 60 min of incubation the % uptake of [^{3}H]L-arginine was 14.24±0.19%, 16.79±0.21%, 18.15±0.20%, 26.72±0.30% and 34.77±0.33% respectively, with corresponding values for conversion to [^{3}H]L-citrulline of 1.53±0.06%, 1.803±0.04%, 1.86±0.06%, 2.519±0.08% and 2.77±0.04%.

L-arginine displays affinity for the basic amino acid transporter and in this preparation [^{3}H]L-arginine uptake was inhibited by the basic amino acids L-lysine and L-ornithine. Using a 12.5 min incubation time, uptake was 16.9±0.5% for control; 14.2±0.5% ($p<0.01$ cf control, Student's t-test), 10.1±0.4%($p<0.001$) and 4.6±0.23%($p<0.001$) for 10µM, 100µM and 1mM L-lysine and 14.5±0.83% (NS), 10.3±0.24% ($p<0.001$) and 4.3±0.06% ($p<0.001$) for 10µM, 100µM and 1mM L-ornithine. [^{3}H]L-arginine uptake was calcium dependent since omission of calcium from the incubation medium resulted in a 51.2% decrease in [^{3}H]L-arginine uptake ($p<0.001$). The NOS inhibitor Nw-nitro-L-arginine benzyl ester (L-NABE; 1mM) produced a 35% decrease ($p<0.01$) in [^{3}H]L-citrulline formation. These results show that NOS activity is detectable in primary hypothalamic neurone cultures and suggest that this preparation provides a suitable model for examining further the role of NO in hypothalamic function.

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113P NITRIC OXIDE, RELEASED FROM ROUSSIN'S BLACK SALT, STIMULATES TRITIUM RELEASE FROM [^{3}H]DOPAMINE-LOADED STRIATAL SLICES

T.L. Stewart, M.D. Black, A.D. Michel, & P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ

From studies using hydroxylamine it has been claimed that nitric oxide (NO) stimulates [^{3}H] dopamine release from the striatum *in vitro* in an extracellular-calcium-dependent manner (Lonart *et al*, 1992). However, using fast cyclic voltammetry we have shown that illumination of the photosensitive NO donor Roussins Black Salt (RBS) increases endogenous dopamine *in vitro* in an extracellular-calcium-independent manner (Black *et al*, 1994). In view of these contradictory observations we have studied the effects of RBS on tritium release from tritiated dopamine loaded striatal slices.

Cross chopped striatal slices (350 µM x 350 µM) were prepared from male AHA Sprague-Dawley rats (200-300 g). Slices were loaded with [^{3}H] dopamine (7 nM) and then placed in a superfusion chamber maintained at 37°C superfused (0.5 ml min^{-1}) with modified Krebs containing 10 µM EDTA and the amine uptake inhibitor, nomifensine (10 µM). The experiment was performed under darkened conditions. RBS (30 µM) was added to the superfusion medium for 30 min and washed off for 30 min prior to the start of collection of superfusate. The brain slices were illuminated using a tungsten-halogen light source for a 4 min period. Release of tritium in response to illumination was measured in 3 x 4 min fractions over a 12 min period from the start of illumination. Data is expressed as fractional release i.e. % of total tritium remaining in the tissue. Results were analysed by an un-paired Student's t-test.

Basal tritium efflux in a 12 min period was 8.31 ± 1.23% (n=4) and was not affected by illumination (8.25 ± 1.14%; n=4). In tissues incubated with RBS the basal efflux was 11.24 ± 0.97% (n=18) which was not significantly different from control. However, when slices pre-incubated with RBS were illuminated, tritium efflux significantly increased ($P<0.05$) with measurable release between 0-4 min (6.10 ± 0.53%), peaking at 4-8 min (9.43 ± 0.9%) and subsiding to basal levels within 12-16 min (4.55 ± 0.51%). The total tritium released during the 12 min period from the start of illumination was 21.26 ± 1.87%. Oxyhaemoglobin (10 µM), included in the superfusion medium for 20 min before and during illumination did not affect the basal tritium efflux but significantly decreased the stimulated release by between 60-80 % (13.46 ± 1.25%; n=7). In contrast, removal of extracellular calcium did not significantly modify the light-induced tritium efflux (24.26 ± 1.89; n=6).

The present study demonstrates that photoactivation of RBS, increases tritium release from the rat striatum *in vitro*. Inhibition of this release by oxyhaemoglobin suggests that the release is NO-mediated. Interestingly, removal of extracellular calcium did not decrease this presumed NO-stimulated dopamine efflux, which is entirely in keeping with previous observations (Black *et al.*, 1994; Black & Humphrey, 1994). These data are consistent with the recent report that NO can cause a calcium-independent release of glutamate from hippocampal synaptosomes (Meffert *et al*, 1994).

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114P 7-NITRO INDAZOLE DOES NOT PROTECT AGAINST QUINOLINIC ACID-INDUCED STRIATAL TOXICITY IN THE RAT

G.M. MacKenzie, P. Jenner & C.D. Marsden, Neurodegenerative Diseases Research Centre, Pharmacology Group, Biomedical Sciences Division, King's College, Manresa Road, London, U.K.

Nitric oxide (NO) may be involved in excitatory amino acid-induced toxicity. However, N^G-nitro-L-arginine methyl ester (L-NAME) does not protect against striatal toxicity following intrastriatal injection of the NMDA receptor agonist, quinolinic acid (QA) (MacKenzie *et. al.*, 1994). A protective effect may be masked by the accompanying cardiovascular effects of L-NAME. In contrast, 7-nitro indazole (7-NI) inhibits NOS without increasing blood pressure when administered *in vivo* (Moore *et. al.*, 1993) and so may be more appropriate for assessing the role of NO in QA-induced toxicity. We have now investigated the effect of 7-NI on striatal neuronal loss following intrastriatal injection of QA.

Male Wistar rats (180-220g) received 7-NI (30mg/kg, i.p.) or 6ml/kg arachis oil 0.5h prior to stereotaxic application of QA (30nmoles, coordinates: A +8.4mm, L +2.6mm relative to lambda, V -4.5mm relative to the dura) and then every 4h for 20h. Neuronal loss in striatal slices (30µm) of rats killed 14 days after QA administration was determined by histochemical staining for NADPH diaphorase (Thomas and Pearse, 1961) and acetylcholinesterase (AChE) (Karnovsky and Roots, 1964). Neurones were counted in the non-lesioned and lesioned sides of striatal sections taken from the level of injection site and 4 levels posterior to the injection site and the total number of neurones in the 5 levels calculated. NOS activity was determined in striatal homogenates prepared from the non-lesioned and lesioned sides of rats by conversion of [^{3}H]-L-arginine to [^{3}H]-citrulline and expressed as pmol citrulline/mg protein/15min (Moore *et. al.*, 1993).

Intrastriatal injection of QA resulted in a marked loss of NADPH diaphorase-positive neurones and striatal NOS activity and a mild loss of AChE-positive neurones. 7-NI administration did not significantly influence the neurotoxic effects of QA (Table 1).

This data does not support a role for NO in QA-induced neurodegeneration in the striatum.

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Table 1: Effect of 7-NI on QA-induced striatal toxicity.

	NADPH Diaphorase		AChE		NOS activity	
	N/L	L	N/L	L	N/L	L
QA/arachis oil	785 ± 38	405 ± 63*	665 ± 23	516 ± 21*	4.19 ± 0.10	0.91 ± 0.11*
QA/7-NI	872 ± 51	416 ± 33*	703 ± 23	588 ± 20*	4.26 ± 0.14	1.44 ± 0.17*

Data are means ± sem, n=6-7. *$p<0.05$ compared to N/L side, 2 way ANOVA, Dunn's test). N/L, non-lesioned side; L, lesioned side.

115P Nω-NITRO-L-ARGININE METHYL ESTER, A NITRIC OXIDE SYNTHESIS INHIBITOR, CAN MODULATE ACUTE AND CHRONIC MORPHINE-INDUCED PROLACTIN SECRETION

A. Matton, S. Engelborghs, F. Bollengier, E. Finné, L. Vanhaelst. (A.G. Dupont)
Dept of Pharmacology, Vrije Universiteit Brussel (V.U.B.), Laarbeeklaan 103, B-1090 Brussels, Belgium.

Repeated administration of morphine induces tolerance to most of its effects, including the prolactin (PRL) inducing capacity. Several studies have demonstrated that N-methyl-D-aspartate (NMDA) antagonists can attenuate the development of morphine tolerance. Since NMDA receptor stimulation can result in the synthesis of nitric oxide (NO), inhibitors of this NO synthesis might be able to modulate in turn the development of morphine tolerance.
The aim of this study was to examine whether an inhibitor of NO synthesis, in this case Nω-nitro-L-arginine (L-NAME), could modulate the PRL enhancing effect of morphine in acute and tolerance conditions.
In vivo experiments were performed using male Wistar rats outfitted with a permanent intravenous catheter. This rat model allowed intravenous morphine administration as well as blood sampling for PRL determination without causing stress. One hour before a first blood sampling four groups of rats received an i.p. administration of either saline or L-NAME 1, 10 or 30 mg/kg as a pretreatment. Each of the pretreated groups was subdivided into four groups of twelve animals. After the first blood sampling each group received intravenously either saline or various concentrations of morphine (3, 6 or 12 mg/kg). For the tolerance condition, rats received 3 or 6 mg/kg morphine 90 minutes after a first administration of 3 or 6 mg/kg respectively. Rats that received 12 mg/kg morphine at first, got a second dose of 6 mg/kg.
L-NAME had no effect on basal PRL secretion. All L-NAME pretreated rats showed a significantly higher PRL peak after administration of 6 mg/kg of morphine than rats that received no L-NAME pretreatment. When rats received 3 mg/kg morphine, the PRL peak was only significantly potentiated in rats pretreated with 10 and 30 mg/kg L-NAME. The potentiating effect of L-NAME seemed dose-dependent. In the case of an administration of 12 mg/kg morphine, PRL peaks were dramatically increased in rats pretreated with the highest dose of L-NAME, namely 30 mg/kg. Moreover within 90 min after this single morphine administration all twelve animals of this group died. Hence, in this group, tolerance condition could not be evaluated anymore.
When rats were pretreated with the lowest dose of L-NAME (1 mg/kg) the tolerance induced by the highest morphine administration (12 mg/kg) was not significantly affected. In all other L-NAME pretreated rats tolerance for a second administration of morphine was significantly attenuated in a dose-dependent way.
From these results it can be concluded that L-NAME can modulate acute morphine-induced PRL secretion as well as the tolerance to the PRL-inducing effect of morphine. Since L-NAME had no effect on basal PRL secretion, it probably interferes with opiate regulated mechanisms.

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116P SIMILARITIES BETWEEN THE EFFECT OF REPEATED ECS AND ACUTE MK-801 ON DOPAMINE-MEDIATED BEHAVIOUR IN THE RAT

S. Smith & T. Sharp, University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE.

Electroconvulsive therapy over several weeks is an effective treatment of severe depression but its mechanism of action remains unclear. In rodents, repeated electroconvulsive shock (ECS) enhances the behavioural activation induced by systemic administration of dopamine agonists such as apomorphine, an effect mediated at least in part via the nucleus accumbens (Green & Nutt, 1987). Interestingly, acute administration of antagonists of the NMDA glutamate receptor such as MK-801 also enhances the behavioural response to dopamine agonists (Carlsson & Carlsson, 1989). Here we compare the effects of repeated ECS and acute injection of MK-801 on dopamine-mediated behaviour in the rat. The dopamine receptor agonists SKF 38393 (D1) and quinpirole (D2/3) were injected either systemically or locally into the nucleus accumbens.

Groups of male Sprague-Dawley rats (250-300 g) were administered one of the following; repeated ECS (5 ECS under halothane in 10 days, 150 V, 1 s), repeated sham-ECS (halothane only), acute MK-801 (0.05 mg/kg s.c.) or saline. Twenty four hours after the last ECS/sham, or 30 min after MK-801/saline, animals received a dopamine agonist challenge and behavioural responses were monitored by direct observation and/or automated ambulation meters. Observed behaviours (sniffing, locomotion, rearing, grooming and intense grooming) were scored as absent or present during a 20 s window every min for 5 min. This was repeated every 10 min for 60 min and an accumulated score was obtained (max. score, 30). Further groups of rats with guide cannulae implanted into the nucleus accumbens received bilateral injections of dopamine agonists both before and after treatment with repeated ECS, sham-ECS or acute MK-801. Ambulation was then monitored automatically for 60 min. Behavioural scores and ambulation counts were analysed statistically using the Mann-Whitney U-test or Student's t-test (* $P \leq 0.05$), respectively. Mean ± s.e.mean (n) values are given.

Repeated ECS (compared to sham) had no statistically significant effect on any measure of behavioural activation induced by SKF 38393 (7.5 mg/kg s.c.) or quinpirole (0.25 mg/kg s.c.) alone, though there was a trend for increased sniffing and grooming after SKF 38393. However, repeated ECS markedly increased both the locomotor score [sham: 1.4±0.5 (6), ECS: 7.9±1.2* (6)] and ambulation counts [sham: 299±55 (6), ECS: 1089±269* (6)] induced by concomitant administration of SKF 38393 (7.5 mg/kg s.c.) and quinpirole (0.25 mg/kg s.c.). Subsequent experiments were carried out with the dopamine agonists administered in combination. Intra-accumbens injection of SKF 38393 (1 µg/side) with quinpirole (0.4 µg/side) induced marked locomotor activation which was enhanced by repeated ECS [ambulation counts pre-ECS: 8124±2502 (7), post-ECS: 19024±4374* (7)] but not repeated sham-ECS [pre-sham: 6682±690 (6), post-sham: 7436±1475 (6)]. The behavioural response to systemically administered SKF 38393 (7.5 mg/kg s.c.) with quinpirole (0.25 mg/kg s.c.) was also markedly enhanced by acute pretreatment with MK-801; locomotor score [saline: 5.2±1.2 (6), MK-801: 20.2±1.9* (6)], ambulation counts [saline: 617±71 (6), MK-801: 2064±179* (6)]. Similarly, pretreatment with MK-801 caused a clear-cut increase in the locomotor response to intra-accumbens injection of SKF 38393 (1 µg/side) with quinpirole (0.4 µg/side) [ambulation counts pre-MK801: 7944±1556 (5), post-MK-801: 14386±3061* (5)].

Our results indicate that both repeated ECS and acute administration of MK-801 enhance the behavioural response to concomitant injection of D1 and D2/3 receptor agonists, either systemically or directly into the nucleus accumbens. These results further highlight the similarities between the neuropharmacological effects of repeated administration of ECS and acute administration of an NMDA receptor antagonist.

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117P SECRETONEURIN-INDUCED RELEASE OF DOPAMINE AND 5-HT *IN VIVO*: A MICRODIALYSIS STUDY

E. Agneter, H.H. Sitte, R. Fischer-Colbrie[1], H. Winkler[1], E.A. Singer. Institute of Pharmacology Vienna, Währingerstr. 13a, A-1090 and [1]Innsbruck, Peter-Mayr Str. 1a, A-6020.

Secretoneurin (SN, 33-amino acids) is a novel peptide derived by proteolysis of secretogranin II (chromogranin C). High concentrations of SN, comparable to those of other neuropeptides, were found in different brain regions in rat (Marksteiner et al. 1993a) and human (Marksteiner et al. 1993b) brain. In superfused rat brain slices SN can be released from large dense core vesicels where it is co-stored with classical neurotransmitters, with high potassium or electrical stimulation (Troger et al. 1994). SN itself releases dopamine from superfused rat striatal slices, (Saria et al. 1993). Thus the biochemical and morphological findings suggest a role for SN as a neuropeptide.

In order to ascertain such a role we investigated the neurotransmiter liberating effects of SN in vivo. Dopamine (DA) and serotonin (5-HT) release were monitored by means of intrastriatal (DA) and intrahippocampal (5-HT and DA) microdialysis in the awake, freely moving rat. In addition the main metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA) were measured. To control for the specifity of the SN effect we also tested other peptides derived from rat secretogranin II (LF-19, EL-17; see Kirchmaier et al.1993), as well as human SN.

In the striatum SN caused a concentration-dependent increase in the extracellular concentrations of DA (10 µmol/l SN resulted in an increase of DA of about 5 times the baseline values; baseline amounted to 45 ± 6 fmol/20 min). An EC_{50} of 6 µmol/l for SN was estimated. In contrast to the short-lasting effect of SN on transmitter release in vitro the releasing effect in vivo was sustained as long as the peptide was present in the dialysis medium (2 or 4 hours). However the DA metabolites DOPAC and HVA did not show any significant change. Human SN also increased DA release but to a lesser degree.The time course of its action, however was the same as with rat SN. The secretogranin II derived peptides LF-19 and EL-17 (up to 30 µmol/l) did not show any effect.

In the hippocampus baseline values of DA and 5-HT amounted to 6 ± 3 fmol/20 min and 34 ± 6 fmol/20 min, respectively. SN also increased the extracellular concentrations of DA and 5-HT (10 µmol/l SN by a fcactor of 3 and 2, respectively). The present results show for the first time the transmitter releasing effects of SN in vivo adding weight to the characterisation of SN as new neuropeptide.

This study was supported by the Medizinisch-wissenschaftlichen Fonds des Bürgermeisters der Bundeshauptstadt Wien.

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118P FETAL MESENCEPHALIC GRAFTS REVERSE INCREASED EXTRACELLULAR MET-ENKEPHALIN AND NEUROTENSIN RELEASE FOLLOWING A NIGROSTRIATAL LESION IN THE RAT

M.L.de Ceballos[1],B.Brera[2] & J.J. López Lozano[2]. [1]Dept.Neuropathol., Cajal Institute,CSIC,Doctor Arce,37,28023 Madrid;[2]Dept.Neurol.,Clinica Puerta de Hierro, Madrid, Spain.

There are increased striatal met-enkephalin (met-enk) and neurotensin (NT) levels following a nigrostriatal lesion with 6-hydroxydopamine (6-OHDA) in the rat (Thal et al., 1983;Taylor et al., 1992). It is unknown whether increased peptide levels are translated into increased release or the effects of a fetal mesencephalic graft. We have studied basal and K^+-evoked striatal release of met-enk and NT "in vivo" in unilaterally 6-OHDA lesioned rats and in lesioned rats grafted with fetal mesencephalic cells.

Male Wistar rats (250-280 g) were lesioned with 6-OHDA injected into the substantia nigra. Six months postlesion rats received a fetal graft (E14). Success of the lesion and of the fetal grafts was assessed by amphetamine-induced rotations (5 mg/kg, ip, 30 min). Two months postgraft microdialysis probes were implanted in the striatum of anaesthesized rats and perfused with artificial cerebrospinal fluid (2µl/min) and 15 min fractions were collected after 1 h stabilisation. After collecting 4 fractions the perfusion medium was switched to one containing 100 mM K^+ for 10 min. Peptides were measured by selective radioimmunoassays.

In partially lesioned rats basal NT and K^+ evoked met-enk and NT release was increased vs. sham lesioned/sham grafted rats (Table 1). In lesioned rats both basal and K^+ evoked met-enk and NT levels in microdialysates were increased. In succesfully grafted rats the increase in peptide release returned to control levels (Table 1).

In conclusion there is increased met-enk and NT release as measured by microdialysis following a nigrostriatal lesion. A fetal mesencephalic graft is able to reverse decreased extracellular dopamine metabolites and increased neuropeptide release.

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Table 1. Striatal concentration (fmol/30 µl)

		Met-enk		NT	
	rpm	Basal	K^+-evoked	Basal	K^+-evoked
Control		24.5±2.5	36.9± 3.9	40.5±6.7	67.8± 8.7
6-OHDA(partial)	0	33.4±4.6	98.5±22.8*	75.9±9.9*	103.7±10.8*
6-OHDA	13	36.4±4.2*	57.7± 7.9*	60.4±6.3*	96.3± 9.9*
6-OHDA+graft	0	29.2±3.6	46.6± 7.1	56.2±9.4	56.2±10.8

$p<0.05$ Student's t test vs. controls:sham lesion/sham graft;n=6-8

119P INHIBITION OF α-KETOGLUTARATE DEHYDROGENASE BY ISOQUINOLINE DERIVATIVES

K.St.P. McNaught, P. Jenner, B. Testa, A. Carotti & C.D. Marsden, Neurodegenerative Diseases Research Centre, Pharmacology Group, Biomedical Sciences Division, King's College, Manresa Road, London SW3 6LX, UK.

Defects in complex I and α-ketoglutarate dehydrogenase (α-KGDH) occur in the substantia nigra in Parkinson's disease (PD) (Schapira *et al.*, 1990; Mizuno *et al.*, 1994). Isoquinoline derivatives implicated in the cause of PD as endogenous neurotoxins are potent inhibitors of complex I (McNaught *et al.*, 1994), but their effects on α-KGDH and other mitochondrial non-respiratory chain enzymes are unknown. We now examine the effects of some isoquinoline derivatives on citrate synthase (CS), glutamate dehydrogenase (GDH) and α-KGDH.

Mitochondria were isolated from the forebrains of adult male Wistar rats (200-250 g) as previously described (Lai and Clark, 1979) and subjected to 3 cycles of freeze/thawing immediately before assay to produce mitochondrial particles. All enzymes were assayed at 37 °C (except for GDH which was assayed at 26 °C) in a final reaction volume of 1 ml by standard spectrophotometric techniques. The compounds examined were 1-methyl-4-phenylpyridinium (MPP^+), 1,2,3,4-tetrahydroisoquinoline (THIQ), isoquinoline (IQ), N-methyl-isoquinolinium ($N\text{-}MeIQ^+$), N-methyltetrahydroisoquinoline (N-MeTHIQ) and salsolinol. Each compound (0.1-20 mM) was added to the reaction mixtures and allowed to equilibrate for 1 min before the reaction was initiated by the addition of mitochondrial particles. Reaction rates were measured for 10 min and enzymatic activities determined from the initial linear part of the activity profile and expressed as nmol/min/mg. Assays were performed in triplicate and data presented as mean ± SEM.

None of the compounds inhibited CS or GDH activity. However, α-KGDH was concentration-dependently inhibited by all compounds with varying potencies. At 10 mM, MPP^+, THIQ, IQ, $N\text{-}MeIQ^+$, N-MeTHIQ and salsolinol inhibited α-KGDH activity by 37.6, 39.8, 75.5, 76.2, 77.8 and 26.2%, respectively (Table).

Table. Effects of isoquinoline derivative and MPP^+ (10mM) on mitochondrial non-respiratory chain enzymes.

	Mitochondrial Enzymatic Activity (nmol/min/mg)		
Compound	CS	GDH	α-KGDH
Control	2729 ± 30	436 ± 2	116 ± 1
MPP^+	2720 ± 17	407 ± 3	72 ± 7*
THIQ	2659 ± 66	475 ± 4	70 ± 2*
IQ	2699 ± 57	439 ± 5	28 ± 7*
$N\text{-}MeIQ^+$	2833 ± 41	421 ± 6	28 ± 1*
N-MeTHIQ	2791 ± 107	433 ± 4	26 ± 1*
Salsolinol	2854 ± 17	431 ± 10	86 ± 2*

* $p < 0.05$; Mann-Whitney *U*-test

MPP^+ was previously shown to inhibit α-KGDH (Mizuno *et al.*, 1987), but this is the first report of inhibition of α-KGDH by isoquinoline derivatives. These findings may represent additional neurotoxic mechanisms underlying mitochondrial dysfunction.

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120P EFFECTS OF OLANZAPINE AND ICI 204,636 ON BEHAVIOURAL RESPONSES TO THE 'D1-LIKE' DOPAMINE RECEPTOR AGONIST A 68930: COMPARISONS WITH CLOZAPINE

Aaron M. Deveney* & John L. Waddington, Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, St. Stephen's Green, Dublin 2, Ireland.

There are at least three current hypotheses for the superior clinical efficacy and low extrapyramidal side effect liability of the atypical antipsychotic clozapine relative to typical D_2 antagonist neuroleptics: combination of 5-HT_2 with modest D_2 antagonism, preferential D_4 antagonism, and preferential attenuation of D_1 function. We have previously described clozapine to influence behavioural responses to D_2 agonism via D_1:D_2 interactions in a manner similar to that of the selective D_1 antagonist SCH 23390, rather than of the selective D_2 antagonist YM 09151-2, and have reported (Deveney & Waddington, 1994) a generally similar profile for olanzapine but not ICI 204,636, two new putative atypical antipsychotics (Moore *et al*, 1992; Migler *et al*, 1993). The effects of these agents on behavioural responses to the new isochroman selective D_1-like agonist A 68930 (Daly & Waddington, 1993) have now been studied.

As described previously (Deveney & Waddington, 1994), rats [n=8 per group] were challenged s.c. with vehicle or A 68930, or with A 68930 following pretreatment with SCH 23390, YM 09151-2, clozapine, olanzapine or ICI 204,636 and resultant behavioural responses assessed. A 68930, 0.0625-4.0 mg/kg, induced grooming and vacuous chewing at lower doses which declined at higher doses; there was some induction of chewing and, particularly at higher doses, of sniffing. Grooming to 0.5 mg/kg A 68930 was readily blocked by 0.01-1.0 mg/kg SCH 23390 [-100%, $P<0.001$] and attenuated by 0.005-0.5 mg/kg YM 09151-2 [-81%, $P<0.001$]; vacuous chewing was not influenced by SCH 23390, but was decreased by 0.005 mg/kg YM 09151-2 [-59%, $P<0.05$] while higher doses tended to release this response. Grooming to A 68930 was readily blocked by 4.0-36.0 mg/kg clozapine [-97%, $P< 0.001$], 0.4-10.0 mg/kg olanzapine [-97%, $P< 0.001$] and 4.0-36.0 mg/kg ICI 204,636 [-81%, $P<0.001$]; vacuous chewing was not influenced by clozapine, while olanzapine released this response at 0.4 mg/kg [+300%, $P<0.01$] and ICI 204,636 exerted a general release of vacuous chewing [+245%, $P<0.01$].

In contrast to their more modest action to influence responsivity to selective D_2 agonism (Deveney & Waddington, 1994), olanzapine and ICI 204,636, like clozapine, each readily blocked typical D_1 agonist-induced grooming without reducing vacuous chewing; this would indicate further some preferential attenuation of D_1-mediated function by these putative atypical antipsychotics. However, the action of olanzapine and particularly of ICI 204,636 to release additional episodes of vacuous chewing to A 68930 indicates some divergence from a wholly clozapine-like profile, the basis of which remains to be clarified.

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121P CATALEPSY, BRAIN DOPAMINE METABOLISM AND THE INTERACTION BETWEEN HALOPERIDOL AND 8-OH-DPAT

Heidi L. Andersen and Ian C. Kilpatrick, Dept. of Pharmacology, Medical School, University Walk, Bristol, BS8 1TD.

The mechanism by which the 5-HT_{1A} agonist, 8-OH-DPAT, prevents haloperidol-induced catalepsy is unknown (Invernizzi *et al.*, 1988; Hinds & Kilpatrick, 1994). Here, we examined whether this action could be reflected by an influence on dopamine metabolism in the caudate-putamen, nucleus accumbens and/or medial prefrontal cortex.

Adult male Porton rats (250-300g) were injected with 8-OH-DPAT (76 or 760 nmol kg^{-1} s.c.) or its vehicle, 30 min after a prior injection of haloperidol (2.66 μmol kg^{-1} i.p.) or its vehicle. Catalepsy was assessed by a vertical grid test 45 min after the second injection and the three dopamine-rich brain areas were then removed (48 min after the second injection) and analysed for dopamine and its main metabolites, DOPAC and HVA as described in detail by Kilpatrick *et al.* (1986).

Haloperidol alone caused a robust cataleptic response (mean 111 s). 8-OH-DPAT alone (760 but not 76 nmol kg^{-1}) induced a characteristic syndrome that included head-weaving and a flattened body posture. After combined haloperidol and 8-OH-DPAT (76 or 760 nmol kg^{-1}), catalepsy was prevented in 30% and 100% of cases, respectively.

Haloperidol significantly increased the DOPAC and HVA contents of all three tissues (Table 1). Given alone, only the lower dose of 8-OH-DPAT caused a significant biochemical change - a doubling of cortical DOPAC. Regional dopamine concentrations were unaltered by any treatment. In the cases where catalepsy was prevented by either dose of 8-OH-DPAT, the increases in DOPAC and HVA were consistently reduced in the caudate-putamen. This pattern was true for the rise in cortical HVA but only in response to the lower dose of 8-OH-DPAT. In contrast, neither dose of 8-OH-DPAT affected haloperidol-induced rises in any accumbens dopamine metabolite or in cortical DOPAC. Importantly, when catalepsy was not prevented, 8-OH-DPAT did not influence the neurochemical responses to haloperidol in any region.

These results suggest that 8-OH-DPAT prevents haloperidol-induced catalepsy by a mechanism that reverses the typical, compensatory increases in meso-striatal and/or meso-cortical dopamine metabolism.

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Table 1. Regional DOPAC and HVA concentrations (*pmol mg^{-1}* wet tissue; mean ± s.e. mean) in control animals and the influence of various combinations of vehicle, haloperidol (2.66 μmol kg^{-1}) and 8-OH-DPAT (760 nmol kg^{-1}).

	Caudate-putamen		*Nucleus accumbens*		*Medial prefrontal cortex*	
	DOPAC	HVA	DOPAC	HVA	DOPAC	HVA
Vehicle + Vehicle	10.98 ± 0.63	5.69 ± 0.38	7.17 ± 0.45	2.38 ± 0.27	0.17 ± 0.03	0.25 ± 0.06
Haloperidol + Vehicle	+217 ***	+319 ***	+197 ***	+451 ***	+453 **	+712 **
Vehicle + 8-OH-DPAT	-2	-4	+25	+38	+70	-4
Haloperidol + 8-OH-DPAT	+59 ***	+104 ***	+262 ***	+532 ***	+412 **	+556 **

Values of drug combinations represent the % change with respect to the Vehicle + Vehicle treatment. All groups contain six determinations. Asterisks ** and *** indicate the probability level from Student's *t*-test of P<0.01 and P<0.001, respectively.

122P PLATELET ACTIVATION BY CLOT-ASSOCIATED THROMBIN: INHIBITION BY ARGATROBAN, A SPECIFIC THROMBIN INHIBITOR

C.N. Berry, C. Lunven, C. Lecoffre, D. O'Brien, C. Gauffeny[1] & N.O. Roome[1]. Dept. of Preclinical Research, Synthélabo Recherche, 31 Avenue P.V. Couturier, 92225 Bagneux, France. [1]Dept. of Drug Safety. Synthélabo Recherche, 2-8 route de Rouen, 78440 Gargenville, France.

During clot formation, thrombin is incorporated into the clot and retains its activity; it is resistant to inhibition by heparin:antithrombin III, but not to low molecular weight thrombin inhibitors such as PPACK (Weitz et al, 1990) and Argatroban (Berry et al, 1994). We show here that clot-associated thrombin induces platelet aggregation, and the effects of Argatroban, a specific, active-site thrombin inhibitor compared with heparin (in the presence of antithrombin III) on this aggregation.

Fibrin clots were prepared by incubating human fibrinogen (4.8 $mg.ml^{-1}$) with varying concentrations of human thrombin (3 - 12 NIH $units.ml^{-1}$) in 10 mM Hepes-buffer, pH 7.4 containing 16 mM $CaCl_2$. The clots were washed 5 times with 2 ml 0.9% NaCl. Their ability to aggregate rabbit platelets (washed in the presence of prostacyclin and resuspended in Tyrode's solution) was compared to that of thrombin in solution (0.1 - 0.4 NIH $Units.ml^{-1}$), by counting the number of single (non-aggregated) platelets in 50μl aliquots taken at different times after the addition of the clot to 0.55 ml platelet suspension using a cell counter. At the end of each incubation, the reaction medium was immunoassayed for thromboxane B_2 (TXB_2) after subtracting the levels found in 50μl time zero aliquots. In some cases, the clots were removed, fixed in 25% glutaraldehyde in cacodylate buffer for scanning electron microscopy.

Fibrin clots led to a decrease in the single platelet count, which was dependent on the concentration of thrombin used to form them. Clots made from 10 NIH $units.ml^{-1}$ thrombin caused a 75% decrease in the number of single platelets after 10 minutes accompanied by the release of 1.5 ± 0.3 (n=7) $ng.ml^{-1}$ TXB_2. Scanning electron microscopy showed this to be due to aggregation of platelets on the surface of the fibrin network. A similar decrease in single platelets was obtained with 0.3 NIH units/ml thrombin in solution. Preincubation of platelets with Argatroban (2 minutes) led to a concentration dependent decrease in clot-induced aggregation with estimated IC_{50}s of 32 ± 4.8nM (n=4) against aggregation and 12 ± 0.7nM against TXB_2 release. The IC_{50} of Argatroban against a concentration of thrombin in solution producing similar aggregation was 14 ± 2.2nM (aggregation), and 9 ± 2nM (TXB_2, n = 4). Heparin in the presence of 20nM antithrombin III had an IC_{50} of 0.03 ± 0.02mUnits.ml^{-1} (n=3) against aggregation induced by thrombin in solution, but at least a thousandfold greater concentrations are required to inhibit clot-induced aggregation (IC_{50} = 80 ± 32 $mUnits.ml^{-1}$, n=4).

These data reinforce the theoretical importance of the clot as a reservoir of active thrombin, and provide a basis for the superiority of direct-acting thrombin inhibitors over heparin as anticoagulant/antithrombotic agents in platelet rich thrombi.

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123P THE ROLE OF NITRIC OXIDE IN A CONSCIOUS MOUSE MODEL OF ENDOTOXIN SHOCK

D.D. Rees, J.E. Monkhouse, A.M. Deakin, D. Cambridge & S. Moncada. Wellcome Foundation Ltd., Beckenham, Kent, BR3 3BS

Septic shock in man following serious bacterial infection, is characterised by hypotension with hyporeactivity to vasoconstrictor agents, inadequate organ perfusion and vascular damage leading to a multiple organ dysfunction and death (Petros et al., 1994). In experimental studies bacterial endotoxin induces a progressive loss of vascular tone with hyporesponsiveness to vasoconstrictor agents which correlates with the expression of a Ca^{2+}-independent nitric oxide (NO)-synthase in the vasculature (Rees et al., 1990, Gray et al., 1991). We have now investigated the role of NO in the haemodynamic profile of endotoxin shock in the conscious mouse.

Female CD-1 mice (25-35 g) were instrumented under gaseous anaesthesia with isofluorane (2%) and connected to a swivel tether system for continuous monitoring of blood pressure and drug administration. After 24 h recovery, endotoxin administration (E. coli, 026:B6, 6-12.5 $mgkg^{-1}$ i.v.) elevated ($P<0.05$) the plasma levels of TNFα (116 fold) and IL-6 (300 fold) at 2 h and 3 h respectively and induced the expression of a Ca^{2+}-independent NO synthase in the mouse heart within 4 h (assayed by the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline). Plasma levels of nitrite/nitrate (NOx) were also elevated from a basal level of 16 ± 4 to 1046 ± 133 μM (n=5-11, $P<0.05$) at 18 h. This was accompanied by a progressive fall in mean arterial blood pressure (MABP) from 94 ± 4 to 67 ± 6 mmHg (n=6, $P<0.05$) at 18 h. The vasopressor effect of noradrenaline (0.5-4 $\mu gkg^{-1}min^{-1}$ i.v.) was significantly attenuated when administered as a continuous infusion, 7 h after endotoxin (n=7, $P<0.05$). The NO synthase inhibitor, N^G-monomethyl-L-arginine hydrochloride (L-NMMA) administered as a continuous infusion (10 $mgkg^{-1}h^{-1}$ i.v.), 4 h after endotoxin prevented the fall in MABP and inhibited the elevation of plasma NOx throughout the 18 h study period (n=5-11, $P<0.05$).

These findings in the mouse support the suggestion that induction of a Ca^{2+}-independent NO synthase is responsible for the hypotension and hyporesponsiveness to pressor agents characteristic of septic shock. Inhibition of NO synthase thus represents an important therapeutic approach to the treatment of septic shock.

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124P AMINOGUANIDINE OR N-ACETYL-5-HT ATTENUATE THE DELAYED HYPOTENSION CAUSED BY LIPOTEICHOIC ACID IN ANAESTHETISED RATS

Sjef J. De Kimpe, Melanie L. Hunter, Christoph Thiemermann & John R. Vane. The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

There is little information regarding the pathophysiology of the circulatory failure in septic shock caused by gram-positive organisms. Lipoteichoic acid (LTA), a wall fragment of *Staphylococcus aureus*, causes a sustained hypotension and induction of nitric oxide synthase (iNOS) in the anaesthetised rat (De Kimpe *et al.*, 1994). Here, we investigate the effects on hypotension and nitrite formation caused by LTA of (i) aminoguanidine, a selective iNOS inhibitor (Corbett *et al.*, 1992), and (ii) N-acetyl-5HT, which attenuates iNOS activity by reducing the availability of the essential co-factor tetrahydrobiopterin (Gross & Levi, 1992).

Male Wistar rats (199 - 268 g) were anaesthetised with sodium thiopentone (120 mg kg^{-1}, i.p.) and the trachea was cannulated to facilitate respiration, the carotid artery to measure blood pressure, and the jugular and femoral veins for the administration of compounds. LTA (10 mg kg^{-1}) was administered intravenously at time 0 and blood pressure was recorded for 300 min (n = 14). At the end of the experiment, plasma was obtained for the measurement of nitrite using the Griess reaction (Gross *et al.*, 1991), in which 0.8 ml of Griess reagent (4% sulphanilamide and 0.2% N-(1-naphthyl)-ethylenediamide in 10% phosphoric acid) was added to 0.2 ml plasma. Statistical comparisons were made by (two-way) ANOVA followed by Bonferroni's test for multiple comparison. Aminoguanidine (15 mg kg^{-1}) was injected 20 min prior to LTA and the injection was repeated every 90 min (n =5). N-acetyl-5HT (25 mg kg^{-1} h^{-1}) was given as a continuous infusion during the experiment starting 20 min before LTA (n = 5). In control rats, this treatment with aminoguanidine or N-acetyl-5HT did not cause a significant rise in blood pressure.

Injection of LTA resulted in a decrease in mean arterial blood pressure (MAP) from 121 ± 4 mmHg (time 0) to 79 ± 4 mmHg at 300 min after LTA ($P < 0.01$). This hypotension was associated with a rise in plasma nitrite from 1.7 ± 0.5 μM in sham-operated rats to 8.1 ± 0.9 μM in LTA treated rats at 300 min ($P < 0.01$). Aminoguanidine attenuated both the delayed fall in MAP (98 ± 8 mmHg) and the rise in plasma nitrite (4.4 ± 0.8 μM) afforded by LTA after 300 min ($P < 0.05$ compared to LTA alone). Similarly, N-acetyl-5HT inhibited the delayed hypotension (102 ± 5 mmHg) and the increase in plasma nitrite (4.8 ± 1.2 μM) elicited by LTA after 300 min ($P < 0.05$, compared to LTA alone).

Thus, an enhanced formation of nitric oxide contributes to the delayed hypotension afforded by LTA. Selective inhibition of iNOS activity with agents such as aminoguanidine or N-acetyl-5HT may be useful in the therapy of gram-positive sepsis.

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125P THE EFFECTS OF PEROXYNITRITE ON HUMAN PLATELETS

M.A. Moro, V.M. Darley-Usmar, A. Brown[1], M.W. Radomski & S. Moncada. Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK and [1]Dept. of Cardiovascular Science, King's College Hospital, London , UK.

Peroxynitrite ($ONOO^-$) is formed from the reaction of nitric oxide (NO) and superoxide anion (O_2^-) and its generation may account for some of the cytotoxic effects of these molecules (Moncada *et al.*, 1991; Crawford & Blankenhorn, 1991). We have previously shown that $ONOO^-$ (20-200 μM) causes concentration-dependent aggregation of human washed platelets and, at high concentrations (200 μM), prevents the inhibition of collagen induced-aggregation induced by NO donors such as S-nitroso-N-acetyl-DL-penicillamine (SNAP). We also demonstrated that this aggregatory action was abolished in the presence of thiols as a result of the generation of S-nitrosothiols and/or NO, which inhibited platelet aggregation (Moro *et al.*, 1994). In order to determine whether reaction with thiols on human washed platelets could result in the formation of NO or an NO donor, we have examined the effect of lower concentrations of $ONOO^-$ on collagen-induced platelet aggregation and the effect of $ONOO^-$ (100-5000 μM) on plasma membrane protein thiols.

Human blood was collected and prostacyclin-washed platelet suspensions (2.5 x 10^8 platelets ml^{-1} in Tyrode's solution) were prepared (Moro *et al.*, 1994). Platelet aggregation was measured in a platelet-ionized calcium lumi-aggregometer. Plasma membrane protein thiols on platelets were measured spectrophotometrically after its reaction with dithionitrobenzoic acid (Ellman, 1959).

We have found that the concentration-response curve for collagen shifted from EC_{50} = 0.38 ± 0.06 μg/ml collagen to EC_{50} = 0.86 ± 0.14 μg/ml (n=3, $p<0.05$) in the presence of 50 μM $ONOO^-$. This inhibitory activity of $ONOO^-$ was reversed by 5 μM oxyhaemoglobin, suggesting that it is an NO-dependent effect. In a parallel series of experiments, plasma membrane protein thiol depletion was observed after treatment of human washed platelets with increasing concentrations of $ONOO^-$ (EC_{50} = 0.9 ± 0.1 mM, n=3) up to 12.3 ± 4.1% of total plasma membrane protein thiols at 5000 μM $ONOO^-$, consistent with the hypothesis that there is a formation of S-nitrosothiols but also thiol depletion with high concentrations of $ONOO^-$. Thus, thiol oxidation state is likely to be key determinant of the biological action of $ONOO^-$ on platelets: in the presence of reduced thiols, the formation of $ONOO^-$ is likely to cause S-nitrosylation. However, thiol depletion may lead to $ONOO^-$-induced platelet activation and thrombosis, conferring a double-edged role for this oxidant in platelet function.

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126P RELATIVE POTENCY AND ARTERIOVENOUS SELECTIVITY OF NITROVASODILATORS ON HUMAN BLOOD VESSELS

R MacAllister, A Calver, J Riezebos, J Collier, P Vallance Department of Pharmacology and Clinical Pharmacology, St George's Hospital Medical School, Cranmer Terrace, London.

Endothelial-derived nitric oxide (NO) modulates vascular tone and blood cell/blood vessel wall interactions. Nitrovasodilators such as glyceryltrinitrate (GTN) have been in clinical use for over 100 years and predominantly cause venous dilatation [1]. The aims of this study were to explore whether venoselectivity is a property of all nitrovasodilators or of NO itself. The arterio-venous potency of sodium nitroprusside (SNP), linsidomine (SIN-1), and S-nitrosoglutathione (GSNO), nitrovasodilators with different mechanisms of biotransformation to NO, was determined in human veins and resistance vessels and compared with GTN. *In vitro* concentration-response curves were constructed to GTN, SNP, GSNO, and SIN-1 (0.001-10 μM) in pre-constricted human saphenous vein and to GTN, GSNO, and SIN-1 (0.001-10 μM) in pre-constricted omental resistance vessels (internal diameter 120±28μM; n=4-6). *In vivo* the dilator responses of the dorsal hand vein and the forearm resistance bed were recorded during local infusions of GTN, SNP, GSNO and SIN-1 (1pmol-160nmol/min; n=4-6). SNP and SIN-1 had similar arterio-venous profiles to GTN *in vitro* and *in vivo* (fig 1). SNP was equipotent with GTN in arterioles and veins but SIN-1 was 10-fold less potent than GTN *in vitro* and 100-fold less potent *in vivo* (fig 1). GSNO was equipotent with GTN in arterioles but at least 10-fold less potent in veins *in vitro* and *in vivo* (fig 1). These results demonstrate that most nitrovasodilators are venoselective *in vivo*, regardless of whether they are metabolised to NO or release NO spontaneously and this finding suggests that NO itself may be venoselective *in vivo*. However, SIN-1, a compound that also generates superoxide, was markedly less potent in all vessels, indicating that co-generation of this radical impairs the response to NO. GSNO was the only compound with a different arterio-venous profile compared with GTN (more arterioselective). This might indicate that endogenous carrier molecules, including glutathione, could alter the vascular profile of NO.

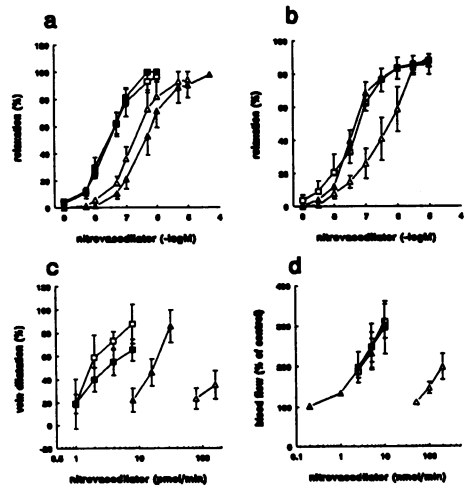


Fig 1. Responses *in vitro* of (a) saphenous vein and (b) small arteries, and *in vivo* of (c) dorsal hand veins and (d) the forearm vascular bed to GTN (■), SNP (□), SIN-1 (△) and GSNO (▲)

1. Collier *et al*; 1978; Br J Clin Pharmacol: 5, 35-44

127P FUNCTIONAL AND STRUCTURAL ALTERATIONS OF CEREBRAL ARTERIOLES IN CHRONIC HYPERTENSION INDUCED BY NITRIC OXIDE SYNTHESIS INHIBITION

J.M. Chillon & G.L. Baumbach (introduced by S.M. Shreeve), Department of Pathology, College of Medicine, University of Iowa, Iowa City, IA 52242, USA.

We have previously reported that, whereas hypertrophy and increased distensibility of cerebral arterioles are observed during chronic hypertension in both spontaneously hypertensive rats (SHR) and renal hypertensive rats (1-kidney, 1-clip), remodeling (defined as a reduction in external diameter) is present only in SHR (Baumbach & Hadju, 1993). The goal of this study was to test the hypothesis that remodeling of cerebral arterioles, in contrast to hypertrophy and increased distensibility, may be limited to genetic forms of chronic hypertension.

To test the hypothesis, we studied another form of nongenetic hypertension induced by chronic inhibition of nitric oxide biosynthesis (Ribeiro et al., 1992). Beginning at four weeks of age, Sprague-Dawley rats were treated for twelve weeks with L^G-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg/day in the drinking water) an inhibitor of nitric oxide synthase. Pressure (servonull) and internal diameter of cerebral arterioles were measured in maximally dilated (EDTA) pial arterioles in anesthetised rats. Cross-sectional area of the vessel wall (CSA) was measured histologically. External diameter and slope of tangential elastic modulus versus stress (EM/ST) were calculed from values of pial arteriolar pressure, internal diameter and CSA.

Hypertension induced by treatment with L-NAME resulted in an increase in internal and external pial arteriolar diameter and CSA but did not significantly alter EM/ST (Table). Thus, L-NAME induced hypertension was associated with hypertrophy of cerebral arterioles, but not increased distensibility or remodeling. If anything, the increase in external diameter suggests that inhibition of nitric oxide synthase resulted in " reverse remodeling ". The findings also support the hypothesis that remodeling of cerebral arterioles may be limited to genetic forms of chronic hypertension.

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Table. Intravascular mean and pulse pressure (MAP and PAP), internal (ID) and external (ED) diameter (measured at a MAP of 30 mm Hg) cross-sectional area of vessel wall (CSA) and slope of tangential elastic modulus versus stress (EM/ST) in maximally dilated pial arterioles of rats treated with L-NAME (n = 9) or untreated (n = 6).

	MAP (mm Hg)	PAP (mm Hg)	ID (μm)	ED (μm)	CSA (μm^2)	EM/ST
Untreated	42 ± 2	13 ± 1.5	80 ± 2	87 ± 3	1044 ± 63	5.04 ± 0.59
L-NAME	85 ± 6*	25 ± 1.7*	93 ± 6*	105 ± 5*	1838 ± 70*	5.27 ± 0.42

Results are mean ± sem; * $p<0.05$ versus untreated, unpaired t test.

128P ATRIAL NATRIURETIC FACTOR INHIBITS CYTOKINE-STIMULATED NITRIC OXIDE PRODUCTION BY HUMAN PROXIMAL TUBULAR CELLS: EVIDENCE FOR AN ANF-C RECEPTOR-MEDIATED RESPONSE

PK Chatterjee, AG Jardine*, NG McKay, GM Hawksworth and JS McLay
Department of Medicine & Therapeutics, University of Aberdeen, *Department of Medicine, Western Infirmary, Glasgow.

Renal proximal tubular cells (PTC) are believed to have an immunological function being able to process and present antigens and produce cytokines (Kelley & Singer,1993). Nitric oxide (NO) may play an important role in the pathogenesis of immune mediated renal diseases such as glomerulonephritis and allograft rejection (Cattell & Cook,1993). Recent evidence suggests that atrial natriuretic factor (ANF) may also have an immunological function (Vollmar & Schulz,1994). We have previously demonstrated that the combination of cytokines IL-1β, TNF-α and IFN-γ can induce nitric oxide synthase (iNOS) in human PTC (McLay *et al*,1994), and that this response can be significantly reduced following pre or co-incubation of the HPT cells with ANF (Chatterjee *et al*,1994). Furthermore we suggested that this response may be mediated by the ANF-C receptor. The evidence that ANF may have an immunomodulatory function mediated via the ANF-C receptor prompted us to examine the effect of ANF and the ANF-C receptor specific ligand $c_{(4-23)}$ANF upon cytokine-stimulated NO production.

Human PTC were isolated from nephrectomy samples. $1x10^5$ cells were plated onto 24 well plates and allowed to reach confluence. Cells were either preincubated with ANF or $c_{(4-23)}$ANF and then with the combination of cytokines IL-1β (10u/ml), TNF-α (10ng/ml) and IFN-γ (100u/ml) for 24 hours. NO, cGMP and protein were determined using the Griess assay, radioimmunoassay and Lowry assays respectively. Using degenerate primers for NOS the mRNA obtained from human PTC incubated with cytokines and ANF was amplified using PCR. A predicted 336bp product was sequenced directly and compared to the sequence for human hepatocyte iNOS.

Cytokine-stimulated nitrite production by HPT cells following preincubation with ANF or cANF

	ANF	C-ANF
	Nitrite production(nmol/mg protein)	
Control	2.7±0.8	2.4±1.1
Cytokines only	42.9±2.6	44.5±3.2
1hr preincubation	31.5±4.2*	28.3±4.9*
4hr preincubation	32.5±3.2*	25.1±2.1*
12hr preincubation	22.5±2.2*	15.8±6.2*
24hr preincubation	16.6±3.5*	14.2±3.3*

n=6, * $p<0.01$ vs. cytokines only

PCR amplification not only revealed a 336bp product in those cells stimulated directly by cytokines but also in those in which NO production had been inhibited by ANF. These results therefore suggest that ANF is a potent inhibitor of NO production and that this response is mediated via the ANF-C receptor. The PCR results confirm cytokine induction of iNOS. The inhibition of NO by ANF was not accompanied by a reduction in the transcription of iNOS suggesting that ANF inhibits translation or post translational modification of iNOS via the ANF-C receptor.

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129P DOES AMINOGUANIDINE PROMOTE ACUTE LEUKOCYTE ADHERENCE IN RAT MESENTERIC VENULES THROUGH INHIBITION OF CONSTITUTIVE NITRIC OXIDE SYNTHASE?

J. Lopez-Belmonte, B.J.R. Whittle & S. Moncada. Wellcome Foundation Ltd, Beckenham, Kent. BR3 3BS

Inhibition of constitutive nitric oxide (NO) synthase increases neutrophil adherence to mesenteric venules (Kubes et al, 1991; Arndt et al, 1993). Aminoguanidine has been proposed to inhibit selectively the inducible isoform of NO synthase (Misko et al, 1993). We have now compared the actions of aminoguanidine to those of the potent inhibitor of constitutive NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME) on leukocyte rolling and adherence in the venules and on the diameter of arterioles of the rat mesenteric vascular bed.

Rats were anaesthetised with pentobarbitone (60 mg kg^{-1} i.p.) and following a midline incision, a segment of the ileocaecal area of the mesentery was exteriorized, placed over a viewing chamber for microscopic observation and superfused with Krebs' solution (37°C). After a 30 min stabilisation period, leukocyte rolling and adherence in the venule and arteriole diameter was determined. Administration of L-NAME (1-10mg kg^{-1} i.v.) caused a time- and dose-dependent increase in leukocyte adherence, from a resting control value of 2±0.4 cells 100 μm^{-1} of venule to 6±1, 9±1 and 11±1 cells 100 μm^{-1} ($P<0.01$ for each, n=6) at 10, 30 and 60 min after L-NAME (5 mg kg^{-1}), whereas saline administration had no effect over this time period. At the same dose, L-NAME decreased leukocyte rolling velocity from 37±2μm sec^{-1} to 16±2, 14±4 and 13±4 μm sec^{-1} ($P<0.001$) at these time intervals following administration. Likewise, aminoguanidine (25-100mg kg^{-1}) dose-dependently increased leukocyte adherence in the venules; aminoguanidine (50mg kg^{-1} i.v.) increased adherence to 9±1, 16±3 and 19±3 cells 100 μm^{-1} ($P<0.01$, n=6) after 10, 30 and 60 min and decreased leukocyte rolling velocity to 19±3, 14±3 and 14±2 μm sec^{-1} at these times. L-NAME (5mg kg^{-1}) also reduced arteriole diameter from a resting value of 26±4 to 21±4 μm, ($P<0.05$; ANOVA), determined after 30 min, while aminoguanidine (50 mg kg^{-1}) reduced arteriole diameter to 17±1 μm ($P<0.01$). Pretreatment (5 min) with L-arginine (300 mg kg^{-1} i.v.) abolished the effects of aminoguanidine on arteriolar constriction, and reversed its effects on leukocyte rolling and adherence, by 50 and 66% respectively (n=4, $P<0.05$), determined after 30 min.

These findings indicate that like L-NAME, aminoguanidine can rapidly promote leukocyte adherence to the venular wall as well as reduce arteriole diameter in the mesentery. Since these acute effects are likely to involve actions on constitutive NO synthase, the use of aminoguanidine as a selective inhibitor of the inducible isoform *in vivo* should be regarded with caution.

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130P ECONAZOLE INHIBITS THE INDUCTION OF NITRIC OXIDE SYNTHESIS IN LIPOPOLYSACCHARIDE-TREATED RAT AORTA

R.G.Bogle & P.Vallance, Clinical Pharmacology Unit, Department of Pharmacology & Clinical Pharmacology, St. George's Hospital Medical School, London SW17 0RE. UK

Induction of the macrophage-type isoform of nitric oxide synthase (macNOS) in blood vessels following cytokine- or bacterial lipopolysaccharide-treatment is though to be an important inflammatory response which may be fundamental in the pathogenesis of septic shock (Vallance & Moncada, 1993). Drugs which modulate this pathway may be useful experimental tools and have potential therapeutic applications. We have previously reported that anti-fungal imidazole drugs such as clotrimazole and econazole inhibit the induction of macNOS in the murine macrophage cell line J774 (Bogle *et al.*, 1994). In the present study we have investigated the effects of econazole on induction of macNOS in isolated rat aorta exposed to lipopolysaccharide (LPS).

Aortae were obtained from male Wistar rats (250-300g), cut into 2 mm rings and mounted to an tension of 1g in organ baths at 37°C. Preparations were suspended in physiological salt solution (mmol/L: NaCl 118, KCl 5.3, $CaCl_2$ 2.5, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 25, glucose 5.5, pH 7.4) and gassed with 95% O_2/5% CO_2. Isometric contraction was measured via force transducers. Rings allowed to equilibrate for 60min and then pre-constricted with 10^{-6}M phenylephrine. Once stable contractions were obtained lipopolysaccharide (LPS, 5$\mu g ml^{-1}$, *E.Coli.*, serotype 055:B5) was added to the rings and the tension observed.

LPS resulted in a time-dependent relaxation of aortic rings such that after 8h tone was 20±8% (mean±SEM, n=4) of the initial value. This loss of tone was reversed (86±4%) by addition of N^Gmonomethyl-L-arginine (3×10^{-4}M, n=3). Addition of L- but not D-arginine (3×10^{-4}M) to rings which had been exposed to LPS for 4h resulted in a rapid relaxation. These results suggest that incubation with LPS induces nitric oxide synthase within these blood vessels. Co-incubation of rings with LPS (5 $\mu g ml^{-1}$) and econazole (10^{-5}M) resulted in a significant inhibition ($p<0.05$, unpaired *t*-test) of the relaxation observed during 8h incubation with LPS. When concentration-response curves to phenylephrine were constructed and EC_{50} values determined it was found that phenylephrine caused a concentration-dependent contraction in aortic rings incubated for 8h in PSS (EC_{50} 1.9×10^{-8}M). In aortic rings treated with LPS (5$\mu g ml^{-1}$) there was a 16-fold right shift in the concentration-response curve to phenylephrine (EC_{50} 3.1×10^{-7}M) which was accompanied by a depression in the maximum contraction. In rings incubated with LPS and econazole (10^{-5}M) the development of hyporesponsiveness to phenylephrine was significantly ($P<0.05$) attenuated (EC_{50} 6.5×10^{-8}M).

These results demonstrate that LPS results in the induction of nitric oxide synthase activity which is prevented in the presence of econazole. The ability of econazole to prevent the functional consequences of NO synthase induction suggest that it may be a potentially useful experimental, and possible therapeutic tool.

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131P INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN J774 CELLS BY THE NOVEL NONSTEROIDAL ANTI-INFLAMMATORY COMPOUND, FLURBIPROFEN-NITROXYBUTYLESTER

G. Cirino, A.W. Tigley[+], F.D'Acquisto[+], J.L. Wallace[+] & A.R. Baydoun[++], Department of Experimental Pharmacology, University of Naples, Via Domenico Montesano 49, 80131 Naples, Italy. [+]Department of Pharmacology and Therapeutics, 3330 Hospital Drive NW Calgary, Alberta, T2N 4N1 Canada, [++]Vascular Biology Research Centre, Biomedical Sciences Division, King's College, Campden Hill Road, London W8 7AH, U.K.

Flurbiprofen-nitroxybutylester (FNBE) is a novel nonsteroidal anti-inflammatory drug, which has been shown to elevate plasma nitrite levels *in vivo* (Wallace et al., 1994) and increase cGMP levels in human cultured umbilical vein endothelial cells (Baydoun *et al.*, 1994). These effects although indicative of nitric oxide (NO) generation do not appear to involve activation of the constitutive NO pathway (Baydoun *et al.*, 1994) and the precise mechanism by which these effects are mediated still remains to be established. In the interim we have examined the effects of FNBE on the inducible NO pathway by investigating whether FNBE modulates NO biosynthesis in endotoxin activated macrophages using the murine macrophage cell line J774.

J774 cells, maintained in continuous culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal calf serum, were plated at a density of 10^5 cells per well in 96-well microtiter plates and allowed to adhere for 2-4 h. Thereafter cells were incubated with either fresh DMEM or DMEM containing *E. Coli* lipopolysaccharide (LPS; 1 μg/ml) alone or in combination with FNBE (0.1-100 μg/ml) or flurbiprofen (0.1-100 μg/ml). In another series of experiments, FNBE (100 μg/ml) was added to wells at various time points after exposure of cells to LPS (1 μg/ml). Nitric oxide production was assayed after 24 h exposure to LPS by measuring the accumulation of nitrite in the culture medium using the Griess reaction. Expression of inducible nitric oxide synthase (iNOS) was analyzed by immunoblotting with anti-iNOS antibody.

Exposure of J774 cells to LPS elevated accumulated nitrite levels from a basal value of 0.02 ± 0.003 to 0.75 ± 0.01 nmoles/μg protein/24 h (n=3). This increase in NO production was blocked in a concentration-dependent manner by FNBE which at 100 μg/ml decreased nitrite levels by 86 ± 0.2 %. The parent compound, flurbiprofen had only a marginal effect (24 ± 0.9 % inhibition) at the maximum concentration used (100 μg/ml). Time course analysis revealed that FNBE reduced LPS-induced NO production even when added 4 h after exposure to LPS. Thereafter, very little or no inhibition was observed suggesting that FNBE had no direct action on iNOS activity once the enzyme was induced. The reduction in NO production observed in our studies may therefore result from the inhibition of iNOS expression by FNBE. In agreement with this hypothesis, Western blot analysis revealed that iNOS protein levels were markedly attenuated in J774 cells after incubation with FNBE (100 μg/ml; 24 h). In conclusion, our data suggest that FNBE, in addition to its inhibitory effects on cyclooxygenase, is a potent inhibitor of NO production in LPS-activated J774 cells. This action may contribute to the anti-inflammatory properties of this compound, as well as having therapeutic implications in conditions such as septic shock.

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132P ISOTHIOUREAS ARE COMPETITIVE INHIBITORS OF INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY IN J774.2 MACROPHAGES

G. J. Southan, Cs. Szabó, C. Thiemermann & J. R. Vane.
The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ, United Kingdom

The induction of a calcium-independent isoform of nitric oxide (NO) synthase (iNOS) and a subsequent enhanced formation of NO has been implicated in the pathophysiology of a variety of diseases including inflammation and circulatory shock. We studied the effects of S-substituted isothioureas (TU) on nitrite production by endotoxin-stimulated J774.2 macrophages *in vitro*. Nitrite production in the supernatant of cultured J774.2 cells activated by LPS (1 μg/ml) or IFN (50 U/ml) was measured at 24h by the Griess reaction (Szabó *et al.*, 1993).

S-methyl-isothiourea sulphate (SMT) potently inhibited nitrite accumulation with an EC_{50} of 6 μM (Table 1). SMT does not interfere with the induction of iNOS, as its inhibitory effect on nitrite formation did not change when given 6h after the LPS (Table 1). The inhibition of iNOS activity was dose-dependently prevented by excess of L-arginine (L-Arg), but not by excess tetrahydrobiopterin (BH_4) (Table 1), suggesting that SMT is a competitive inhibitor of iNOS at the L-arginine binding site. SMT is a potential methylating agent and guanylating agent of free amines. However, the potency of SMT on nitrite production was not altered by excess albumin or lysine, but was attenuated by replacing the medium with SMT free medium or the addition of L-arg 8 h after induction of the macrophages.

The inhibitory activity of S-substituted isothioureas on iNOS activity declines sharply when the side chain exceeds 2 carbon atoms in length (Table 1). Similarly, substitution of the ethylene side chain of ethyl-TU results in a diminished potency. Substitution of either one or both nitrogens of SMT with either amino or alkyl groups also reduces its inhibitory potency.

Thus, isothioureas represent a new class of NOS inhibitors, some being the most potent inhibitors of iNOS activity yet reported. These will be useful tools for studying the role of iNOS in disease models and may be useful in the therapy of diseases associated with an enhanced formation of NO due to iNOS induction, such as inflammation and circulatory shock.

Table 1. Inhibitory effect of substituted isothioureas and reference compounds on nitrite production by activated macrophages.

Compound	EC_{50} (μM)	Compound	EC_{50} (μM)
N^G-Methyl-L-Arg	48	Cetyl-TU	>1000
N^G-Nitro-L-Arg	400	Benzyl-TU	100
L-N^G-aminoarginine	90	Chloroethyl-TU	28
Aminoguanidine	130	Carboxymethyl-TU	>1000
Thiourea	>1000	Cyanomethyl-TU	>1000
SMT	6	Acetamido-TU	>1000
SMT (+6h)	6		
SMT + L-Arg (30mM)	230	Aminoethyl-TU	3
SMT +BH_4 (1 mM)	6	Methoxy-ethyl-TU	68
SMT+ albumin (1 mg/ml)	5	Methylthioethyl-TU	160
SMT + lysine (1 mM)	5	S,S-propandiyl bis-TU	165
Ethyl-TU	2	S,S,-ethylene di-TU	84
iso-Propyl-TU	2	2-aminothiazoline	38
n-Propyl-TU	78	2-Aminothiazole	470
n-Butyl-TU	280	N-methyl-SMT	350
n-Pentyl-TU	>1000	N,N'-dimethyl-SMT	>1000

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133P MODULATION OF THE INDUCIBLE NITRIC OXIDE SYNTHETASE (iNOS) IN THE HUMAN COLONIC EPITHELIAL CELL LINE HT-29

Kolios, G., Robson, R. L., Brown, Z., Robertson, D. A. F. and Westwick, J.
Department of Pharmacology, University of Bath, and Royal United Hospital, Bath, Avon, UK.

A major source of NO is via the expression and activation of inducible nitric oxide synthase (iNOS) (Moncada & Higgs, 1993). The human form of this enzyme was recently cloned and its expression was induced in hepatocytes and smooth muscle cells (Geller *et al,* 1993). We have used the fluorescent substrate 2,3-diaminonaphthalene (Misko *et al.*, 1993) to detect nitrite production and oligonucleotide probes specific for human iNOS mRNA to examine the activity and expression in the human colonic epithelial cell line HT-29.

The cells were cultured in McCoys medium plus 10% FCS in six well Limbro plates until confluent, then in the absence of FCS for 24h prior to and up to 72h following the addition of cytokines. The human recombinant cytokines IL-1α (sp. act. $5x10^7$ U/mg), TNFα (sp. act. $6x10^7$ U/mg), IFN-γ (sp. act $>2x10^7$ U/mg), IL-4 and IL-13 over a 3 log order concentration range were added alone or in combination at 0h and the levels of nitrite were determined at 0, 12, 24, 48 and 72h. None of the cytokines alone induced a significant increase in nitrite production. However the combination of IL-1α (10 ng/ml)/IFN (300 u/ml) or the combination of IL-1α (10 ng/ml)/IFN (300 u/ml)/TNFα (100 ng/ml) produced a highly significant ($p<0.0001$) increase in nitrite production of 276 ± 5, and 408 ± 7 nM/10^6 cells (n=3) at 48h, compared to basal values of 73±2. Pre-treatment of HT-29 cells with cycloheximide (5 μg/ml) prior to IL-1α/IFN stimulation reduced nitrite levels to 76 ± 2 nM/10^6 cells, not significantly different to basal nitrite production. Time course studies with IL-1α (3 ng/ml) IFN(300 u/ml) combinations revealed significant increases in nitrite production at 24h (144 ± 3), 48h (276 ± 9) and 72h (406 ± 8) compared to basal values of 50 ± 1, 73 ± 2 and 111 ± 4 nM/10^6 cells respectively (n = 4). IFN (0, 10, 30, 100, 300u/ml) in the presence of IL-1α (10ng/ml) produced a dose related generation of nitrite of 86 ± 5, 81 ± 3, 111 ± 7, 163 ± 5, and 262 ± 14 nM/10^6 cells at the 48h respectively. Similarly IL-1α (0.3, 1, 3, 10 ng/ml) in the presence of IFN (300 u/ml) produced a dose related generation of nitrite of 75 ± 4, 142 ± 1, 217 ± 4, 284 ± 15 nm/10^6 cells respectively compared to basal value of 73 ± 17. To determine whether the inducible generation of nitrite was due to the induction of iNOS, the iNOS mRNA expression was determined by northern analysis. Expression of iNOS mRNA in IL-1α/IFN stimulated cells was absent until 6h, peaked at 24h and was undetectable by 72h. These results suggest that HT-29 cells are a useful model to examine the modulation of human iNOS expression and colonic epithelial cells in inflammatory bowel disease may be a major source of NO production.

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134P CHARACTERISATION OF NITRERGIC NEUROTRANSMISSION DURING SHORT- AND LONG-TERM ELECTRICAL STIMULATION OF THE RABBIT ANOCOCCYGEUS MUSCLE

S. Cellek*, L. Kasakov**, S. Moncada*
*Wellcome Research Laboratories, Beckenham, Kent, UK & ** Bulgarian Academy of Sciences, Sofia, Bulgaria

Nitric oxide (NO) acts as a neurotransmitter in the peripheral nervous system (Sanders & Ward, 1992) and is continuously released from the nerve terminals to maintain a nitrergic relaxant tone (Ward et al., 1992; Wiklund et al., 1993). We have investigated the effect of long-term electrical field stimulation (EFS) of the rabbit anococcygeus muscle in order to understand the nature of this continuous release.
Short- (5 s) and long- (10 min-2 hr) term EFS (50 V, 0.3 ms duration of square pulses, 0.5-50 Hz) elicited a contractile response in the rabbit anococcygeus muscle which was enhanced by NO synthase inhibitors, showing that sympathetic transmission is modulated by endogenous NO. Following inhibition of the adrenergic and cholinergic components of neuronal innervation with guanethidine (10 μM) and scopolamine (10 μM), and elevation of the tone of the tissue with histamine (0.5 μM), long-term EFS elicited relaxation and release of NO (measured as its oxidation products nitrite and nitrate by chemiluminescence) which both persisted for the duration of the stimulation (up to 2 hours). This relaxation was inhibited by NO synthase inhibitors (IC_{50} for L-NOARG, 56.2±6.2 μM), oxyhemoglobin (1-15 μM), ω-conotoxin (0.5 μM) and tetrodotoxin (2 μM). Both NO synthase inhibitor (L-NIO, 1 mM) and tetrodotoxin (2 μM) reduced the basal and stimulated release of NO. Inhibitors of NO synthase also increased the basal tone of the tissue. Long-term EFS which induces ~50% of the maximum relaxation could be enhanced by addition of L- , but not D-, arginine to the perfusion medium.
These data show that there is a continuous basal release of NO from nitrergic nerve terminals which maintains a relaxant tone in the rabbit anococcygeus muscle. In addition, NO is released during short- and long-term EFS which further relaxes the preparation and modulates sympathetic transmission. Activation of L-arginine:NO pathway for periods up to 2 hours does not exhaust nitrergic transmission in any appreciable way.

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135P EFFECTS OF 7-NITRO INDAZOLE ON VENTRICULAR FIBRILLATION, HAEMODYNAMICS AND NITRIC OXIDE LEVELS IN THE ISOLATED RAT HEART

Ravinder Pabla & Michael J. Curtis, Cardiovascular Research Laboratories, Vascular Biology Research Centre, Pharmacology Group, Division of Biomedical Sciences, King's College, Manresa Road, London SW3 6LX.

The compound 7-nitro indazole (7-NI) is a neuronal-selective nitric oxide (NO) synthase blocker with no effect on blood pressure (Moore et al, 1993) unlike other potent blockers of NO synthase. Based on this observation it has been proposed that 7-NI does not inhibit vascular endothelial NO synthase at a concentration which inhibits the neuronal form. In the present study we used this compound to dissect out the source of NO involved in cardioprotection by investigating the effects of 7-NI and its inactive analogue, 6-amino indazole (6-AI) on ventricular fibrillation (VF) incidence in the setting of myocardial reperfusion in the isolated rat heart. Male Wistar rats (240-260g) were anaesthetised with pentobarbitone (60 mg kg^{-1}, i.p.) and given 250 iu sodium heparin (i.p.). Hearts were excised and perfused (Langendorff mode) with solution containing (in mM) NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.0, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.4 and glucose 11.1, pH 7.4, 37°C. Hearts were randomised to control (vehicle: 0.1% ethanol), 1 μM 7-NI, 10 μM 7-NI, 10 μM 7-NI plus 1 mM L-arginine (L-arg), 1 mM L-arg plus 10 μM 6-AI or 10 μM 6-AI alone (n=10/group). Following 60 min left regional ischaemia, hearts were reperfused for 10 min, whereupon reperfusion-induced VF was detected using a unipolar electrogram. Heart rate (HR), coronary flow (CF) and recovery of flow (RF) were measured, the latter by timed collection of coronary effluent (Curtis & Hearse, 1989). NO levels were determined by collecting coronary effluent during the first min of reperfusion into eppendorff tubes which were immediately frozen in liquid nitrogen for subsequent chemiluminescence analysis (Menon et al, 1991). Data were recorded and analysed by ANOVA plus Dunnett's test for mean±s.e.mean data or Chi^2 for % incidence data as described previously (Curtis & Hearse, 1989).

Table 1 *P <0.05 vs control

Group	%VF	HR (beats/min)	CF (ml/min/g)	RF (ml/min/g)	NO (pmol/min/g)
control	0	339±16	11.4±1.3	8.5±0.9	1109±407
1 μM 7-NI	60*	280±14*	10.5±0.9	8.3±1.8	< 1*
10 μM 7-NI	60*	284±10*	9.1±0.6	6.2±0.8	< 1*
10 μM 7-NI + L-arg	20	335±13	12.4±1.0	11.0±1.0	1826±437*
L-arg + 6-AI	10	332±9	14.0±1.3*	12.9±1.5*	2925±571*
6-AI	0	349±8	10.4±0.4	9.0±1.1	1091±432

7-NI was pro-fibrillatory (Table 1) and slowed heart rate without affecting coronary flow. 7-NI reduced NO levels to below the limit of detection. L-arg overcame all the effects of 7-NI. 6-AI was inactive. In conclusion this study implicates a major role for neuronal NO as an endogenous cardioprotectant against reperfusion-induced VF.

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136P CHRONIC ETHANOL PROMOTES NEURONAL DIFFERENTIATION OF NG108-15 CELLS

P.K. Harrison, M. Connor, G. Henderson & E. Kelly, Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD

Recent studies suggest that chronic ethanol can enhance the ability of differentiating agents to modify neuronal growth and development, whereas chronic ethanol alone is reported not to induce neuronal differentiation (eg. Wooten & Ewald, 1991). However, here we present morphological, biochemical and electrophysiological evidence that chronic ethanol alone can induce neuronal differentiation of NG108-15 cells.

NG108-15 cells were grown in DMEM supplemented with 6% fetal calf serum. Ethanol was added directly to the medium and control and ethanol treated cells grown in parallel. Chronic ethanol (10-200mM for up to 3 days) concentration-dependently inhibited NG108-15 cell proliferation and increased cell protein content. Chronic ethanol (200mM, 3days) reduced cell number from 2.03 ± 0.31 $x10^6$ to 0.63 ± 0.1 $x10^6$ cells/25cm^2 flask (n=5 for each, $p<0.05$, Student's t test), and increased cell protein content from 0.87 ± 0.07 $x10^{-6}$ to 1.77 ± 0.34 $x10^{-6}$ mg protein/cell (n=5 for each, $p<0.05$, Student's t test). Chronic ethanol also produced a clear increase in neurite outgrowth from NG108-15 cells and concentration-dependently increased acetylcholinesterase (AChE) activity (after 200mM ethanol for 3 days, AChE was 37.8 ± 2.4 in treated cells, n=8, and 20.1 ± 2.5 nmoles/min/mg protein in control cells, n=10, $p<0.05$, Student's t test). Addition of the standard differentiating agents forskolin (1-50μM) or sodium butyrate (0.1-1mM) to NG108-15 cells for 3 days also enhanced neurite outgrowth, reduced cell proliferation and enhanced cell protein and AChE activity similar to ethanol.

Differentiation of NG108-15 cells is reported to increase membrane Ca^{2+} currents (I_{Ca}). To assess the effects of chronic ethanol on I_{Ca} in NG108-15 cells, whole cell patch-clamp recordings were made from control and chronic ethanol treated cells in ethanol free medium. I/V relationships were determined for each cell, and I_{Ca} measured at the beginning and end of a 200ms pulse. In cells treated with 200mM ethanol for 2 days the maximum transient I_{Ca} was increased from 4.4 ± 0.5 in control to 8.4 ± 1.6 pA/pF (n=15 for each, $p<0.05$, Student's t test), and the sustained I_{Ca} from 1.5 ± 0.3 in control to 4.4 ± 1.0 pA/pF (n=15 for each, $p<0.05$, Student's t test).

In conclusion, these experiments demonstrate that in the absence of other differentiating agents, ethanol alone is able to promote the neuronal differentiation of NG108-15 cells. This may have relevance to the morphological changes that occur to neurones in alcoholism or the Fetal Alcohol Syndrome.

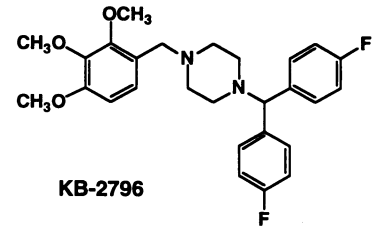
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137P EFFECTS OF THE NEUROPROTECTIVE AGENT, KB-2796, ON THE VOLTAGE-DEPENDENT SODIUM CURRENT IN MOUSE NEUROBLASTOMA, N1E-115

J.G. McGivern, L. Patmore and R.D. Sheridan, Department of Pharmacology, Syntex Research Centre, Edinburgh EH14 4AP, U.K.

The trimetazidine analogue, KB-2796 (1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride), is neuroprotective in animal models of cerebral ischaemia (Handa *et al.*, 1990). Studies on the mechanism of action of KB-2796 have concentrated largely on its calcium antagonist properties: it potently displaces [^{3}H]-nitrendipine from guinea-pig cerebrocortical synaptosomes (IC_{50} = 86 nM; Iwamoto *et al.*, 1988) and inhibits L- and T-type calcium currents in voltage clamped rat hippocampal CA1 neurones with IC_{50}s of 4 and 1.9 μM, respectively (Akaike *et al.*, 1993). Although the effect of KB-2796 on voltage-dependent sodium currents is unknown, the compound is a potent displacer of the sodium channel ligand, [^{3}H]-batrachotoxin, from rat brain synaptosomes (IC_{50} = 26 nM; C.M. Brown *et al.*, Syntex Research Centre, unpublished data). The present study was therefore undertaken in order to establish whether the sodium channel interaction highlighted by the binding data translated into functional effects on sodium currents.

Sodium currents were recorded from voltage clamped N1E-115 cells (at room temperature) using conventional whole-cell recording techniques. The effect of KB-2796 was examined at 3 membrane holding potentials: -100, -80 and -70 mV. Current/voltage relationships were obtained for each cell before and 10 min after application of KB-2796 and the effect on peak sodium current measured. The IC_{50} concentrations of KB-2796 at the 3 holding potentials were: 15.8, 3.0 and 0.18 μM, respectively. This increase in potency at less negative holding potentials strongly suggested that KB-2796 interacted with the inactivated state of the channel (which predominates at more depolarized potentials), and this interaction was studied in more detail by examining the effects of the compound directly on steady-state inactivation. Steady-state inactivation curves were constructed before and 10 min after application of 1 μM KB-2796 by clamping the cells for 1 min at each of the pre-pulse potentials (-100 to -20 mV in 10 mV steps) and stepping to a test potential of 0 mV. KB-2796 induced a 14.6 ± 1.9 mV leftward shift of the steady-state inactivation curve (n = 3) with no significant change in slope. Finally, the interaction of KB-2796 with open channels was examined by studying the effects of the compound on chloramine-T-modified currents. (Modified currents last hundreds of milliseconds as a result of a greatly increased channel open time (Niemann *et al.*, 1991); drugs which block the open state produce an acceleration of the decay of the modified current.) When studied under these conditions, KB-2796 (1 μM) produced a slight acceleration of the decay of the chloramine-T modified current (n = 5).



KB-2796

The present results demonstrate that KB-2796 potently inhibits sodium currents in N1E-115 cells. Although, the compound interacts potently with the inactivated state of the sodium channel, interactions with open and resting states are also evident.

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138P THE EFFECTS OF THE κ-OPIOID RECEPTOR AGONIST U-50,488H UPON KAINIC ACID-INDUCED ALTERATIONS IN GLUCOSE UTILIZATION IN THE RAT HIPPOCAMPUS

A. Ableitner, Institute of Pharmacology, Toxicology and Pharmacy, Königinstraße 16, 80539 München , Germany

There exist an abundance of evidence that opioids are implicated in the regulation of hippocampal excitability. μ- and δ-opioid receptor agonists have been found to increase the excitability of hippocampal pyramidal cells, in both CA1 and CA3, via an inhibition of inhibitory interneurons (Lupica *et al.*, 1992). In contrast, for κ-opioid receptor agonists a lack of effect as well as excitatory and inhibitory actions have been described (Chavkin *et al.*, 1988).

This issue has been further investigated in the present study. Kainic acid was systemically applied in order to excite hippocampal pyramidal neurons and possible modulatory effects of the nonpeptide κ-opioid receptor agonist U-50,488H (VonVoigtlander *et al.*, 1983) were evaluated in the awake rat by use of the quantitative autoradiographic 2-deoxyglucose technique (Sokoloff *et al.*, 1977). The number of animals per treatment group was n=5-7. The Newman Keul's multiple range test was used for statistical comparisons. Kainic acid (12mg/kg, i.v.) caused statistically significant increases in glucose utilization in the hippocampus ($p<0.01$). The ventral hippocampus showed greater increases in glucose utilization (up to 400%) than the dorsal hippocampus (up to 250%) and changes in CA3 (150%-400%) were greater than those in CA1 (100%-250%).
U-50,488H (0.5-5mg/kg, i.v.) did not significantly alter glucose utilization in the hippocampal formation.

However, kainic acid induced-changes in this region were modulated by pretreatment with U-50,488H (5mg/kg, i.v.). In field CA3 the effects of kainic acid were generally enhanced by U-50,488H ($p<0.01$). In the stratum lacunosum moleculare of field CA1, however, the increases were reduced by 20% ($p<0.05$). All modulatory effects of U-50,488H on kainic acid-induced changes were antagonized by the opioid receptor antagonist naloxone (10mg/kg, i.v.; $p<0.05$).

Taken together, the pesent study provides *in vivo* evidence that hippocampal excitability is enhanced by the κ-opioid receptor agonist U-50,488H, most notably in CA3. In addition, an inhibitory action of the κ-opioid receptor agonist in the stratum lacunosum moleculare of CA1 is indicated.

Acknowledgement: This study was supported by the Deutsche Forschungsgemeinschaft

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139P EFFECT OF DEPOLARISATION ON STRIATAL EXCITATORY AMINO ACID AND AMYLOID PRECURSOR PROTEIN RELEASE *IN VIVO*

S.N. Dijk*, P.T. Francis, M.T. Webster, G.C. Stratmann and D.M. Bowen, Institute of Neurology, Miriam Marks Department of Neurochemistry, 1 Wakefield Street, London WC1N 1PJ

Hypoactivity of cortical glutamatergic neurones may underlie the histopathological and cognitive changes found in Alzheimer's disease. Acetylcholine M_1 receptors are enriched on these neurones (Bowen et al., 1994). This study tests the functional significance of this enrichment *in vivo* using slight modifications of the method of Palmer et al., (1989). Male Sprague Dawley rats (200-250 g.) were anaesthetised with halothane/O_2/N_2O. A microdialysis probe or push pull cannula was implanted in the striatum (coordinates with bregma as reference: AP 0 mm; L 2.5 mm; DV 7 mm from skull). To be able to stimulate pyramidal neurone bodies in the cortex, the cranium was opened over the ipsilateral frontal cortex and the dura carefully removed. Starting 1 hour after implantation, 15 (10 minute, flow rate 1 µl/min) samples were collected. At the beginning of each sampling period the craniotomy was filled with phosphate buffered saline (PBS; 10 µl). At the beginning of the seventh collection period either PBS or drug dissolved in PBS was applied. Samples were frozen at -70 °C until assayed by HPLC with fluorescent detection following 0-phthaldialdehyde/2-mercaptoethanol derivatisation. The M_1 agonist, 1-azabicyclo[2.2.1]heptan-3-one, o-[3-93-methoxyphenyl)-2-propynyl]oxime, (z)-(=+/)-, ethanedioate (1:1) (salt) (PD 142505-0028, 10 µM, n=6) increased basal glutamate (but not aspartate) release (3.14 ± 0.51 pmoles/10µl, n=10) by 166% (maximum) from the seventh until the ninth sample (unbalenced repeated measures ANOVA, followed by Student's t test; $P<0.05$). Physostigmine (PHYS, 0.3 mg/kg given i.m. at the beginning of the second collection period; n=7) also significantly increased basal glutamate (but not aspartate) release (3.12 ± 0.22 pmoles/10µl, n=5) by 163% (maximum) during sample numbers 4-14 (repeated measures ANOVA, followed by oneway ANOVA and the LSD test , $P<0.05$). PHYS perfused through the probe had no effect, nor had vehicle injection.

To investigate whether depolarisation of the striatum increases amyloid precursor protein release, a push pull cannula was implanted in the striatum and 100 mM K^+ perfused through the cannula.Using Western blotting and the antibody 22C11 (Webster et al., 1994), in 11 out of 13 animals an obvious increase in the protein signal was found, typically two hours after the start of the potassium challenge.

Our results suggest that the M_1 agonist PD142505-0028 can depolarise cortical pyramidal neurones *in vivo* and that depolarisation stimulates secretion of amyloid precursor protein, which may reduce the extracellular amyloid load.

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140P METABOTROPIC GLUTAMATE RECEPTOR ACTIVATION INDUCES HIGH FREQUENCY (β-EEG) SYNCHRONIZATION IN HIPPOCAMPAL CA1 SUBSLICES OF THE RAT

P.H. Boeijinga & H.W.G.M. Boddeke, Sandoz Pharma Ltd., Preclinical Reasearch, Bld. 360/605, CH-4002 Basle, Switzerland

Agonists of metabotropic glutamate receptors (mGluR) increase excitability of neurons mainly by reducing K^+-conductances, leading to depolarization and removing spike-accomodation. There is a strong similarity with the effects of muscarine acetylcholine receptor agonists (Bashir et al., 1993). Muscarine receptors are known to be involved in the generation of theta rhythm, as has been shown in vitro (Konopacki et al., 1987). We have investigated rhythmic activity, induced by the metabotropic glutamate receptor agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) in slices of the CA1 area of the rat hippocampus.

In freshly prepared transversal slices of the hippocampus, the fascia dentata and CA3 subfields were removed by two transections. The isolated CA1 subslices were perfused in an interface chamber with arteficial cerbro-spinal fluid at 34 °C. Extracellular recordings were made from the pyramidal cell-layer, the signals were filtered between 1 and 100 Hz, and FFT-spectra were computed using epochs of 16 s. Mean power (μV^2/Hz) was calculated in the band from 13 to 40 Hz. Student's t-test was used for statistical comparisons. Averages are expressed as mean ± s.e.mean.

1S,3R-ACPD, in a concentration of 100-200 µM, evoked rhythmic activity with a dominant frequency in the beta (15-25 Hz) range. The ratio of mean power in the beta range of agonist-treated and untreated slices was 14.8 ± 4.1 (n=10). The effect of 1S,3R-ACPD was inhibited by the selective mGluR antagonist (+)α-methylcarboxyphenylglycine ((+)αMCPG, 600 µM) to 28 ± 8 % (n=4, $p<0.05$) of the pre-incubation power values. Thapsigargin (10 µM), a depletor of intracellular calcium stores and staurosporin (20 µM), a protein kinase-inhibitor, did not affect the response to 1S,3R-ACPD. The potassium channel blocker tetraethylammonium (20 mM) suppressed the synchronizing effect of 1S,3R-ACPD by 60 ± 15 % (n=3, $p<0.05$), suggesting that coupling to a K^+-conductance plays the major role in beta-oscillations of the neuronal population.
The synchronization of neuronal activity into beta-wave activity by activation of mGluRs may constitute an important role in functional integration by glutamatergic neurotransmission.

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141P INVOLVEMENT OF PHOSPOLIPASE C AND K^+-CONDUCTANCES IN CARBACHOL-INDUCED THETA RHYTHM IN CA1 SUBSLICES OF THE RAT HIPPOCAMPUS

H.W.G.M. Boddeke & P.H. Boeijinga, Sandoz Pharma Ltd., Preclinical Reasearch, Bld. 360/605, CH-4002, Basle, Switzerland

Muscarine M1-receptors in the hippocampal formation play an important role in the generation of theta rhythmic activity (Golebiewsky et al. 1993). The role of second messengers in muscarine receptor mediated modulation of K^+-conductances, but not of oscillatory properties, has been investigated (Nicoll et al. 1990). We have investigated the involvement of phospholipase C coupled mechanisms in rhythmic activity, induced by the muscarine receptor agonist carbachol, in vitro.

In freshly prepared transversal slices of the rat hippocampus, the fascia dentata and CA3 subfields were removed by two subsequent transections. The isolated CA1 subslices were perfused in an interface chamber with arteficial cerebro-spinal fluid at 34 °C. Filtered (1-100 Hz) field potentials were recorded in CA1 stratum pyramidale/oriens, and fast-Fourier-transform spectra were computed using epochs of 16 sec. Mean power (μV^2/Hz) was calculated in the frequency band from 4-12 Hz. Student's t-test was used for statistical comparisons. Data are expressed as mean ± s.e.mean.

Carbachol (10-50 μM) induced theta activity with a dominant frequency of 9.1 ± 0.5 Hz (n = 26). Compared to baseline (μV^2/Hz) the mean power increased 6.3 ± 1.0 (n=11) fold. The effect of carbachol was attenuated by the muscarine receptor antagonist scopolamine (5 μM) by 61 ± 13 % (n=4, $p<0.05$). Depletion of intracellular calcium stores by the calcium pump inhibitor thapsigargin (10 μM) suppressed the response to carbachol by 70 ± 6 % (n=3, $p<0.05$). Inhibition of protein kinase activity with staurosporin (20μM) also attenuated the response to carbachol by 63 ± 5 % (n=4, $p<0.05$). The potassium channel blocker tetraethylammonium (20 mM) completely abolished the synchronizing effect of carbachol.
We conclude that induction of theta rhythm by muscarine receptor activation is mediated by phospholipase C. Potassium channels may be involved in this effect, although, at the concentration used, TEA may also interfere with muscarine receptors.

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142P IMAGING AND QUANTIFICATION OF MUSCARINIC RECEPTORS IN THE HUMAN HEART AND BRAIN *IN VIVO*

E.A. Dubois, R.J. Vermeulen, A.F.M. Janssen, M. Pfaffendorf, E.A. van Royen and P.A. van Zwieten, Departments of Pharmacotherapy and Nuclear Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Muscarinic receptors are believed to play a pivotal role in both cardiac and cerebral diseases. The present study was undertaken to visualize these receptors in vivo in humans. The kinetic profile of a newly synthesized compound, [^{123}I]4-Iododexetimide (IDEX), was studied after intravenous injection of 185 MBq IDEX (specific activity > $2.22*10^8$ MBq/mmol) in five healthy volunteers, using SPECT (Single Photon Emission Computerized Tomography). Tomographic images of the heart using a three-headed gammacamera (1 and 4 h post injection (p.i.)) and brain using a multi-detector system (Strichman Medical Equipment (3,10,24 h p.i.) were made, reconstructed and subsequently quantified. In order to establish radioactivity in the heart, standardized, concentric, elliptic regions of interest of myocardium (MYO) and left ventricular cavity (C) of all short-axis slices were quantified using the CASPAN program (Cardiac SPECT analysis). Radioactivity in C was used as a reference since it can be quantified by measuring radioactivity in a blood sample and systematic errors in reconstruction are similar to those in MYO. The method proved to be accurate and reproducible, yielding a mean MYO/C ratio of 5.15 ± 0.79 (n=5) at 1 h p.i.. Four hours p.i., myocardial uptake could no longer be quantified because of radioactivity accumulating in the lungs. Radioactivity in different areas in the brain was quantified by using a stereotactic atlas of the brain (Matsui et al.,1978;Schaltenbrand et al.,1977), where regions were drawn on different areas of the brain on the transverse slice where highest radioactivity was observed in striatal region. The pixel size as well as the amount of radioactivity in the specific areas was measured at the time points mentioned above. In Table 1 the respective values for mean ± s.e.mean radioactivity expressed as Bq/ml) in the frontal cortex (FRC), striatum (ST), occipital cortex(OC), thalamus (TH), anterior cingulate (AC), posterior cingulate (PC), posterior temporal (PT) and the hemisphere (HS) are presented.

Table 1. Radioactivity expressed as means (Bq/ml *100) ± s.e.mean (n=5) in different areas of the brain at 3, 10 and 24 h p.i..

	3h	10h	24h
FRC	47.84±3.77	58.25±7.06	38.41±2.73
ST	59.22±4.94	67.02±6.56	47.82±3.16
OC	49.36±4.61	57.09±3.80	38.51±1.21
TH	36.99±2.69	37.23±3.50	25.31±0.17
AC	58.35±5.76	66.04±8.57	49.37±4.50
PC	51.81±3.01	58.78±5.68	37.79±1.78
PT	52.19±5.07	61.61±5.11	43.27±3.16
HS	47.56±3.44	54.28±4.59	38.68±1.45

From these in vivo studies we conclude that it is feasible to image and subsequently quantify muscarinic receptors in the heart (1h p.i.) and brain (10h p.i.), in vivo in humans.

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143P MUSCARINIC m1 RECEPTORS ARE PHOSPHORYLATED IN CHO CELLS FOLLOWING AGONIST STIMULATION

M.G. Waugh, N.T. Burford, S.R.Nahorski. and A. B. Tobin. Dept. of Cell Physiology and Pharmacology, University of Leicester, University Road, Leicester, U.K., LE1 9HN.

Five types of muscarinic receptor have been cloned so far (m1-m5) and each type has a distinct pharmacological and tissue distribution profile. This study used Chinese hamster ovary (CHO) cells that have been stably transfected with cDNA encoding an individual receptor subtype.

CHO-m1 cells (βmax 1108+/-44.5 fmol/mg protein, determined by [^{3}H]-N-methyl scopolamine binding) were stimulated with agonist and inositol 1,4,5-trisphosphate (IP_3) mass levels measured (Challiss et al. 1988). Addition of carbachol resulted in a peak and plateau pattern of IP_3 production. At 10 seconds IP_3 mass increased from a basal level of 54=/-12 ρmol/mg protein to a peak level of 593=/-84 ρmol/mg protein. IP_3 production then fell but still remained above basal for at least 10 minutes after agonist application (215+/-50 ρmol/mg) protein. Similar peak and plateau patterns of IP_3 were seen if the agonist applied was methacholine or carbachol and when stimulations were carried out on plated-down cells or cells in suspension.

The presence of this apparently desensitising peak of IP_3 production in CHO-m1 cell is very similar to that reported for CHO-m3 cells (Tobin et al 1992). Both m1 and m3 receptors are believed to couple to PLC-β1 via Gq/G11 G-proteins. In addition the m3 receptor has been shown to be rapidly phosphorylated in response to agonist exposure (Tobin and Nahorski 1993) and it is possible that this rapid phosphorylation event might be involved in desensitisation.

In order to investigate m1 receptor phosphorylation, an antiserum (Mc-1) was raised against the C-terminal domain of the m1 receptor. Western blotting studies on transfected CHO cell membrane proteins identified the m1 receptor as a band at approximately 80 kilodaltons. The antiserum did not cross-react with any other muscarinic receptor subtype. *In vivo* phosphorylation studies were carried out as follows. CHO-m1 cells were resuspended in Krebs/HEPES buffer pH 7.4. The intracellular pools of ATP were then labelled by incubation with ^{32}P-orthophosphate and then treated with various reagents. Following solubilisation, immunoprecipitation and separation by polyacrylamide gel electrophoresis, phosphorylated proteins were visualised by autoradiography. The results show that the m1 receptor is phosphorylated under basal conditions and that the addition of methacholine resulted in an increase in receptor phosphorylation. This effect was blocked by the muscarinic antagonist atropine. Stimulation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (10nM) also resulted in an increase in receptor phosphorylation. Ionomycin (1μM) and forskolin (1μM) did not increase m1 receptor phosphorylation probably ruling out the calcium/calmodulin dependent kinase and protein kinase A respectively as the agonist stimulated receptor kinase. Further experiments are needed to characterise this kinase and also to elucidate a possible functional role of agonist-induced m1 receptor phosphorylation.

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144P AGONIST-STIMULATED CYCLIC AMP ACCUMULATION IN CHO CELLS EXPRESSING RECOMBINANT m1, m3 OR m5 MUSCARINIC RECEPTORS

N. T. Burford and S. R. Nahorski. Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, LE1 9HN.

The muscarinic receptor subtypes m1, m3 and m5 are thought to couple to the activation of phospholipase C via the pertussis toxin (PTX)-insensitive G proteins of the $G_{q/11}$ family (Caulfield, 1993). However, agonist stimulation of m1 and m3 muscarinic receptors has also been shown to produce large increases in adenosine 3',5'-cyclic monophosphate (cAMP) accumulation in some cell lines (Caulfield, 1993). In order to investigate this further we have used Chinese hamster ovary (CHO) cells expressing recombinant human m1 (CHO-m1), m3 (CHO-m3) or m5 (CHO-m5) muscarinic receptors (Buckley *et al.*, 1989). These clones were characterised by [^{3}H]N-methyl scopolamine (NMS) saturation binding and shown to express these receptor subtypes at levels of 1144 ± 192, 930 ± 73, and 334 ± 43 fmol / mg protein, respectively (mean ± s.e of 3 experiments). Accumulation of cAMP in CHO cell clone suspensions was measured using a competitive binding assay (Brown *et al.*, 1971). Maximal cAMP accumulation occurred 5 min after incubation of CHO-m1 or CHO-m3 cells, in Krebs-HEPES buffer, pH 7.4, with 1 mM carbachol, at 37 °C. Carbachol dose-response curves, after 5 min incubation, produced maximal responses (in pmol / mg protein) of 307 ± 2 and 447 ± 67, from basal levels of 13 ± 2 and 16 ± 4, with EC_{50} values (-log. M) of 3.93 ± 0.07 and 3.75 ± 0.17 (mean ± s.e. of 3 experiments), in CHO-m1 and CHO-m3 cells, respectively. Carbachol did not stimulate any cAMP accumulation above basal levels in CHO-m5 cells. The cAMP responses in CHO-m1 and CHO-m3 cells were not attenuated by PTX pretreatment of the cells (100 ng / ml for 20 h). Carbachol stimulation of a CHO cell clone expressing m3 muscarinic receptors at a level of 300 - 400 fmol / mg protein (CHO-vt9) did not produce any accumulation of cAMP above basal levels. Further studies showed that a 5 min preincubation of CHO-m1 cells with phorbol dibutyrate (1 μM) did not enhance either basal or carbachol-stimulated cAMP accumulation. Carbachol (1 mM) stimulation of CHO-m1 cell membranes in a cytosol-like buffer (containing 2 mM ATP, with the free [Ca^{2+}] buffered to approximately 100 nM with EGTA) (Wojcikiewicz *et al.*, 1990), resulted in a cAMP accumulation of 262 ± 18 pmol / mg protein from basal levels of 62 ± 11 pmol / mg protein (mean ± s.e. of 3 experiments), after 20 min incubation, at 37 °C .

These results suggest that the cAMP response (particularly in CHO-m3 cells) appears to be largely dependent on the expression level of the muscarinic receptor subtype and, therefore, the lack of cAMP response in CHO-m5 cells may not necessarily mean that m5 muscarinic receptors could not couple to the stimulation of adenylyl cyclase (AC) when expressed at a higher level in CHO cells. However, m1 muscarinic receptors produced a larger cAMP accumulation compared with m3 muscarinic receptors when expressed at identical levels in CHO cells. The studies on CHO-m1 cells also suggest that carbachol-stimulated cAMP accumulation was not mediated via protein kinase C activation or via Ca^{2+} activation of AC. The mechanism of activation of AC in this system, therefore, probably involves either a direct coupling of m1 muscarinic receptors with G_s, or G protein βγ activation of AC via PTX-insensitive G proteins.

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145P EFFECTS OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR DOWNREGULATION BY CHRONIC MUSCARINIC RECEPTOR ACTIVATION ON Ca^{2+} SIGNALLING IN CULTURED CEREBELLAR GRANULE CELLS

P.B. Simpson, S.R. Nahorski and R.A.J. Challiss, Dept. of Cell Physiology and Pharmacology, University of Leicester, Leicester.

We have recently reported that chronic incubation of cerebellar granule cells with carbachol (CCh) causes a marked downregulation of inositol 1,4,5-trisphosphate receptor ($InsP_3R$) expression (Simpson et al., 1994b). Maintained CCh treatment also decreases muscarinic receptor expression and M_3-mediated inositol phosphate production, but is without effect on the inositol phosphate responses mediated by other phosphoinositide (PI)-linked receptors in these neurones (Dillon-Carter & Chuang, 1989). Here we report the effects of CCh pretreatment on $[Ca^{2+}]_i$ responses to a range of agents in cerebellar granule cells.

Cerebella were obtained from neonatal Wistar rats, and granule cells cultured as described previously (Whitham et al., 1991). Cells were pre-treated with CCh (1 mM) in culture medium for 24 h, then loaded with 2 μM fura 2-AM in the absence of CCh. After a further wash, cells were left for ≥30 min in CCh-free buffer. $[Ca^{2+}]_i$ experiments were performed on groups of 5-8 granule cells using epifluorescence microscopy as described previously (Simpson et al., 1993).

The basal 340/380 nm ratio in CCh-pretreated cerebellar granule cells was not significantly different to that in control cells (control ratio = 0.96 ± 0.07, CCh-pretreated ratio = 1.06 ± 0.11). 24 h pretreatment with CCh reduces $[^3H]InsP_3$ binding in cerebellar granule cell membranes to 36 ± 6% of control (Simpson et al., 1994b). CCh (1 mM) evoked a much smaller rise in $[Ca^{2+}]_i$ in pretreated cells than in control cells from the same preparation (23 ± 14% of control). Responses to 1-aminocyclopentane-1S,3R-dicarboxylic acid (1S,3R-ACPD)(200 μM) were also greatly reduced compared to control cells (27 ± 1% of control). CCh-evoked $[Ca^{2+}]_i$ responses appear to involve activation of ryanodine-sensitive Ca^{2+} release channels, probably consequent to Ca^{2+} release via $InsP_3Rs$ (Simpson et al., 1994a). However, $[Ca^{2+}]_i$ responses to caffeine (50 mM) were unaffected by chronic CCh pretreatment (110 ± 13% of control). *N*-Methyl-D-aspartate (NMDA)-evoked responses in cerebellar granule cells involve a significant component of Ca^{2+}-induced Ca^{2+} release (CICR) (Simpson et al., 1993). The NMDA response is partially ryanodine-sensitive, but as $InsP_3Rs$ are sensitive to cytoplasmic $[Ca^{2+}]$, it was possible that NMDA-evoked CICR could also occur via $InsP_3Rs$. The response to NMDA (200 μM) after CCh-evoked $InsP_3R$ downregulation was not significantly different to that in control cells, in terms of either peak or plateau response size (peak = 97 ± 8% of control, plateau = 97 ± 13% of control).

Thus, chronic CCh treatment appears not to affect ryanodine receptor function, and activation of $InsP_3Rs$ may not contribute significantly to NMDA-evoked elevation of $[Ca^{2+}]_i$ in cerebellar granule cells. It has previously been shown that CCh only directly causes homologous, not heterologous, desensitization in granule cells (Dillon-Carter & Chuang, 1989). However, the present study demonstrates that via an action not on receptor-effector coupling but rather downstream of PI turnover, by downregulating $InsP_3R$ expression, CCh pretreatment markedly inhibits $[Ca^{2+}]_i$ responses evoked via another PI-linked receptor.

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146P MUSCARINIC AGONISTS AND PHORBOL 12,13-DIBUTYRATE (PhB) AUGMENT ADENOSINE-EVOKED HYPERPOLARISATION OF RAT SUPERIOR CERVICAL GANGLIA (SCG)

G. P. Connolly & Abbott, N.J. Physiology Group, King's College London, Strand, London. WC2R 2LS.

Adenosine (AD) selectively depressed depolarisations of rat SCG evoked by muscarine (MUS) but not those evoked by gamma-aminobutyric acid (Connolly & Stone, 1988). To examine this phenomenon further, the ability of non-muscarinic agents (including PhB (an activator of protein kinase-C (PKC)) and 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine) (H7) (an inhibitor of PKC) to alter AD-evoked hyperpolarisation of SCG were compared to the effects of MUS, N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)acetamide (BM5) pilocarpine (PILO). The effects of H7 (50μM, 10 & 36 min incubation) on depolarisations evoked by MUS or AD-evoked depression of MUS depolarisation were studied. SCG from male Wistar rats (200-350g) were desheathed and placed in a chamber where the d.c. potential between the body of the ganglion and the postganglionic internal carotid nerve was recorded across a 'grease-gap' (for methods see Connolly & Stone, 1988; 1993). Responses evoked by MUS, 2-methyl-5-hydoxytryptamine (2Me5HT), BM5, nicotine (NIC) applied for 1 min, PILO, isoprenaline (ISO)) (2 min) or continuous application (PhB, K^+, H7(22 min)) and the responses evoked by AD (100μM, 2min) before (control) and during responses evoked by these agents are summarised in Table 1. Statistically significant differences (paired t-test) between responses evoked by AD are denoted by * = $P<0.05$ and ** = $P<0.01$. AD-evoked hyperpolarisations were significantly enhanced by MUS, PILO, BM5, PhB, unaltered by 2Me5HT, ISO, K^+ or H7 and reduced by NIC (Table 1). Both the depression of muscarine (0.1μM)-evoked depolarisation by AD (100μM) (37 ± 3%, c.f. in H7 40 ± 5%), and responses (μV ± s.e. mean) evoked by MUS (0.1μM, 1 min) and PhB (0.02μM, 5 min) were unaltered by H7 (247 ± 42 and 90 ± 6 c.f. in H7 (50μM) 187 ± 34 and 113 ± 18, n=3, respectively).

The ability of muscarinic agonists but not non-muscarinic agonists to augment AD-evoked hyperpolarisations, supports the idea that there is a selective interaction between these compounds. The inability of ISO and H7 to alter AD-evoked hyperpolarisations indicates that this response may be independent of the activation of cyclic-AMP. The inactivity of H7 on the depolarisations evoked by muscarine or PhB was in part unexpected as it has been reported that staurosporine (an inhibitor of PKC) depressed depolarisations of the rat SCG evoked by PhB but not muscarine (Grove *et al.*, 1990). Our results are, however, consistent with a lack of effect of H7 on phorbol ester-evoked depolarisation of bullfrog ganglia (Chen *et al.*, 1994). Thus the biochemical mechanisms by which AD hyperpolarises and depresses muscarinic agonist-evoked depolarisations remain unknown but may not involve the activation of PKC via a H-7 insensitive mechanism.

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Table 1. Effect of depolarising agents and H7 on the adenosine (100μM)-evoked hyperpolarisation of the rat SCG (n = 3 to 8 ganglia).

Response to (Con^C μM)	MUS (0.1)	PILO (1)	BM5 (0.1)	PhB (0.2)	2Me5HT (30)	ISO (1)	K^+(8000)	NIC (0.1)	H7 (50)
Agent (μV ± s.e.mean)	140 ± 10	360 ± 63	171 ± 24	323 ± 82	381 ± 60	193 ± 28	277 ± 28	203 ± 59	-65 ± 13
AD as % control	146 ± 13*	218 ± 6*	167 ± 15*	334 ± 113**	103 ± 2	113 ± 10	111 ± 6	-59 ± 20**	127 ± 4

147P CHARACTERIZATION OF NEURONAL MUSCARINIC RECEPTORS REGULATING [^{3}H]ACETYLCHOLINE RELEASE IN HUMAN ISOLATED BRONCHI

I. Wessler, P. Härle, K.D. Höhle[1], M. Pintz & K. Racké[2]. Dept. Pharmacol., Univ. Mainz, Obere Zahlbacher Str. 67, [1]St. Hildegardis Hospital, D-55101 Mainz and [2]Dept. Pharmacol., Univ. Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt/Main, F.R.G.

Several types of muscarinic receptor subtypes are expressed in human airways; high affinity pirenzepine-sensitive sites have been found to contribute substantially (70%) to the total binding at muscarinic receptors (for literature see Barnes, 1993). The functional role of the different subtypes, however, is less clear. In the airways of different species (guinea-pig, rat, dog, rabbit) evidence has been found that M_1-receptors may facilitate ganglionic transmission, whereas M_2- or M_4-receptors are proposed to be involved in muscarinic inhibition of acetylcholine release. In human airways the subtypes of neuronal receptors modulating acetylcholine release remain to be characterized. In the present study release of newly-synthesized [^{3}H]acetylcholine was measured in human isolated bronchi (Wessler et al., 1994), to characterize neuronal muscarinic receptors. Human tissue was obtained at thoracotomy from patients with lung cancer. In hospital, macroscopically normal bronchi (diameter of 3 - 6 mm) were dissected and placed in oxygenated salt solution within 5 to 20 min after surgical resection. Airway mucosa was removed mechanically at the start of the experiments. Bronchi were set up in an organ bath, incubated with [^{3}H]choline (30 min) and after a subsequent washout period tritium efflux was measured in 3 min periods. Release of [^{3}H]acetylcholine was elicited by four periods (S_1-S_4) of transmural electrical stimulation (four 20 s trains at 15 Hz during the individual stimulation periods). Increasing concentrations of oxotremorine were added 18 min before S_2, S_3 and S_4. Propranolol (1 μM) and indomethacin (3 μM) were present throughout. In most experiments (+)-tubocurarine (100 μM) was also present.

Transmural electrical stimulation (S_1) caused an evoked tritium outflow of 1200 ± 260 d.p.m. 100 mg^{-1} (n = 11) reflecting evoked release of [^{3}H]acetylcholine. During the subsequent three stimulations [^{3}H]acetylcholine release declined progressively resulting in ratios S_2/S_1, S_3/S_1 and S_4/S_1 of 0.83 ± 0.04, 0.59 ± 0.06 and 0.48 ± 0.04, respectively (n = 11). Oxotremorine inhibited evoked tritium outflow in a concentration-related manner, tritium outflow evoked in the presence of 0.01, 0.1 and 1 μM oxotremorine amounted to 90 ± 2%, 56 ± 6% and 22 ± 6% (n = 12) of the control. Atropine (1, 3, 10 and 30 nM) shifted the concentration-response curve of oxotremorine to the right in a manner suggesting competitive interaction (pA_2 value of 8.80; slope 1.10). Atropine alone enhanced evoked tritium efflux, the effect was maximal at a concentration of 10 nM (increase by 37 ± 3 %; n = 4). Also different concentrations of the M_1-selective antagonist pirenzepine antagonized the inhibitory effect of oxotremorine but an erratic Schild plot was obtained. However, in the presence of tubocurarine pirenzepine shifted the oxotremorine curve to the right in a manner suggesting competitive interaction (pA_2 value of 6.60; slope 0.85). Pirenzepine alone did not significantly modify evoked [^{3}H]acetylcholine release but a low concentration (0.1 μM) tended to reduce evoked [^{3}H]acetylcholine release. A subtype-specific concentration (0.1 μM) of the M_2-selective antagonist AF-DX 116 or of the M_3-selective antagonist *p*-fluoro-hexahydrosiladifenidol did not antagonize the inhibitory effect of 0.1 or 1 μM oxotremorine.

The present experiments suggest that in human airways the release of acetylcholine is regulated by inhibitory and facilitatory muscarinic receptors. Muscarinic receptors exhibiting a high affinity for pirenzepine are functionally inactivated by (+)-tubocurarine. The subtype of the prejunctional inhibitory muscarinic receptors remains to be characterized, but a M_2-, M_3- or M_4-subtype appears unlikely.

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148P DESENSITISATION OF THE CARBACHOL-INDUCED PHOSPHOINOSITIDE RESPONSE IN HUMAN CULTURED DETRUSOR SMOOTH MUSCLE CELLS

K.A. Marsh, D.R. Harriss* and S.J.Hill. Dept of Physiology & Pharmacology, Queen's Medical Centre, Nottingham NG7 2UH; *Dept. Urology, City Hospital Nottingham NG5 1PB.

We have previously demonstrated the successful culture of human detrusor smooth muscle cells which appear to be coupled to phosphoinositide hydrolysis via the muscarinic M_3 receptor (Harriss *et al.*, 1994). Desensitisation of the muscarinic receptors of the detrusor could have major implications in the regulation of bladder function and associated disorders. It has recently been demonstrated that muscarinic M_3 receptors which have been transfected into CHO-K_1 cells undergo a rapid desensitisation and subsequent reduction in the carbachol (CCh)-induced increase in inositol trisphosphate (Tobin *et al.*, 1992). This study demonstrates desensitisation of phosphoinositidase C (PIC)-linked M_3 receptors in non-transfected primary cultures of human detrusor smooth muscle cells.

Cell cultures were prepared from cold cup pinch biopsies of the human detrusor muscle using explant culture methods. All experiments were performed on cells between passage 5 and 10. Accumulation of ^{3}H-inositol phosphates was measured on confluent monolayers as described previously in detail (Marsh & Hill, 1992). Desensitisation was achieved by pre-incubation with CCh for 5-30min prior to the incubation with 20mM LiCl for 20min. Naive or CCh-pretreated cells were then stimulated with 0.1mM CCh for a further 40min in the continued presence of 20mM LiCl. Stimulation was stopped by the addition of ice cold methanol/0.12M HCl (1:1 v/v) and total inositol phosphates separated by ion exchange chromatography (Hall & Hill, 1988).

In this study CCh induced a concentration-dependent increase in phosphoinositide turnover revealing, in naive cells, an EC_{50} value of 14 ± 1μM (n=4) and a CCh-induced (0.1mM; 40min) increase over basal levels of 12.8 ± 1.2 fold (n=43). The response to 0.1mM CCh was evident after 30s exposure, however the rate of phosphoinositide stimulation appeared to decrease at agonist exposure times over 60s duration. Preincubation of the cells with CCh (10nM -1mM; 30min) produced a concentration-dependent decrease in the inositol phosphate response to the second challenge with 0.1mM CCh (40min). The IC_{50} value for this inhibition was found to be 7±1μM (n=4). Pre-exposure to CCh (0.1mM) for 5min prior to rechallenge reduced the mean size of response of the second stimulation to 49% of that observed in naive cells (n=4) and to 22% and 20% of the control response after 20min and 30min respectively (n=4).

The results of this study demonstrate that the muscarinic receptors of cultured detrusor smooth muscle are susceptible to desensitisation with respect to their inositol phosphate response. This desensitisation is rapid, apparent within 5min, and is evident following the 20min incubation time with LiCl which occurs between challenges. These cells provide an ideal system for the further study of the mechanisms of desensitisation of the PIC-linked muscarinic receptors of the human bladder.

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149P 5-HT_{1A} RECEPTOR BLOCKADE PREVENTS THE INHIBITORY EFFECT OF PAROXETINE ON 5-HT CELL FIRING: RELATIONSHIP TO CHANGES IN BRAIN EXTRACELLULAR 5-HT

S. E. Gartside, V. Umbers, M. Hajós & T. Sharp, Oxford University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE.

Combined treatment with a selective 5-HT reuptake inhibitor (SSRI) and a non-selective 5-HT_1 antagonist is reported to produce a rapid relief of treatment-resistant depression (Artigas *et al.*, 1994). In the rat, the ability of SSRIs to elevate extracellular 5-HT in the forebrain has been reported to be potentiated by co-administration of a 5-HT_{1A} antagonist (Hjorth, 1993). Although the mechanism underlying this effect is unclear, it is established that SSRIs acutely inhibit 5-HT cell firing. If 5-HT_{1A} receptor antagonists prevent the SSRI-induced inhibition of cell firing, this may enhance the effect of SSRIs on extracellular 5-HT in the forebrain. Here we have used *in vivo* electrophysiological recording and microdialysis to examine the effect of the SSRI paroxetine, alone and in combination with the 5-HT_{1A} receptor antagonist WAY 100635 (Fletcher *et al.*, 1994), on 5-HT cell firing and brain extracellular 5-HT.

Male Sprague-Dawley rats (270-310 g) were anaesthetised with chloral hydrate (400 mg/kg i.p.), mounted in a stereotaxic frame, and a lateral tail vein was cannulated for drug administration. Extracellular recordings were made from 5-HT cells (1 cell per rat) in the dorsal raphe nucleus (DRN) using glass microelectrodes (*in vitro* impedance 3-8 MΩ). In separate experiments, microdialysis probes were implanted in either the DRN or frontal cortex (FCx), and perfused (2 µl/min) with artificial CSF. Dialysates (20 min samples) were analysed for 5-HT by HPLC with electrochemical detection. Data are expressed as the mean ± s.e.mean (n=number of rats).

Paroxetine dose-dependently (0.1-1.6 mg/kg i.v.) inhibited the firing of 5-HT neurones in the DRN (ED_{50}:-0.281 ± 0.073 mg/kg i.v. (n=7); estimated maximally effective dose 0.8 mg/kg i.v.). This inhibitory effect was reversed by subsequent administration of WAY 100635 (0.1 mg/kg i.v.) in all 5 cells tested. Furthermore, in animals pretreated with WAY 100635 (0.1 mg/kg i.v.), the inhibitory effect of paroxetine (0.2-1.6 mg/kg i.v.) was almost completely abolished (% inhibition by 0.8 mg/kg:- 7.2 ± 6.1 % (n=5)). WAY 100635 (0.1 mg/kg i.v.) alone had small and inconsistent effects on 5-HT cell firing.

In microdialysis studies, 0.8 mg/kg i.v. paroxetine increased levels of 5-HT in DRN dialysates approximately two-fold (from 12.6 ± 3 fmol/sample at t=0 to 22.9 ± 1 fmol/sample at t=40 (n=4)), but did not alter 5-HT in FCx dialysates during the following 2 h (n=5). However, in animals pretreated with WAY 100635 at a dose (0.1 mg/kg i.v.) which blocked the paroxetine-induced inhibition of 5-HT cell firing, paroxetine (0.8 mg/kg i.v.) caused an approximate trebling of 5-HT in FCx dialysates (from 6.6±1.1 fmol/sample at t=0 to 19.5 ± 2.4 fmol/sample at t=40 (n=7)). WAY 100635 alone (0.1 mg/kg i.v.) had no effect on 5-HT in FCx dialysates (n=5).

Our data show that in the rat, administration of a selective 5-HT_{1A} receptor antagonist blocks the inhibitory effect of an SSRI on DRN cell firing and, at the same time, potentiates the effect of an SSRI on levels of extracellular 5-HT in the forebrain. Similar changes in man may explain the observed enhancement of the antidepressant effects of SSRIs by co-administration of a 5-HT_1 antagonist.

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150P ROLE OF THE 5-HT_{1A} RECEPTOR ON *IN VIVO* ACETYLCHOLINE RELEASE IN RAT HIPPOCAMPUS

K.J. Parkins, C.H.K. Cheng, B. Costall and R.J. Naylor Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP, U.K.

5-HT_{1A} receptor agonists have previously been demonstrated to increase *in vivo* acetylcholine (Ach) release from the cerebral cortex of guinea pigs (Bianchi *et al.*, 1990). The present study examines the role of 5-hydroxytryptamine (5-HT) and 5-HT_1 receptor ligands on *in vivo* Ach release from the rat hippocampus.

Microdialysis probes were implanted into chronically indwelling cannulae located in the right dorsal hippocampus (Ant. +2.0, Vert. –8.0 and Lat. -4.8) of male Lister Hooded rats (375-500g) 16 hours prior to the experiment. Probes were perfused with artificial cerebrospinal fluid containing 2 µM neostigmine at a rate of 2.0 µl min^{-1}. After 100 min stabilisation, 20 min samples (additional 10 min samples in the first 20 min of agonist administration) were collected and immediately analysed for Ach by HPLC-ECD (Mark *et al.*, 1992). After the determination of four basal levels, drugs were administered centrally via the probe for an 80 min (antagonist, at t=0) or 60 min (agonist, at t=20) period.

5-HT (10 and 100 µM), the 5-HT reuptake inhibitor, fluoxetine (10 µM) and the 5-HT_{1B} agonist, CGS 12066B (100 µM) all failed to significantly modify basal extracellular Ach levels (139.4±7.8 pg 40 $µl^{-1}$). The 5-HT_{1A} agonist, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (1, 10, and 100 µM) caused a dose related increase of basal Ach release (Table 1). Treatment with the selective 5-HT_{1A} receptor antagonist, [N-tert-butyl 3-4-(2-methoxyphenyl)piperazin-1-yl-2-phenyl-propanamide dihydrochloride] (WAY 100135) (200 µM) prevented the 8–OH–DPAT (10 µM) induced increase of basal Ach release (Table 1). WAY 100135 (200 µM) alone had no significant effect on hippocampal extracellular Ach levels (n=6, P>0.05).

The results support the recent findings of Izumi *et al.* (1994) that activation of the 5-HT_{1A} receptor facilitates Ach release in the hippocampus.

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Table 1. The effect of 8-OH-DPAT and WAY100135 on basal hippocampal acetylcholine release.

Time (min)	0	20	30	40	60	80	100	120	140
Control	90±10	105±15	-	109±17	107±12	80±11	86±19	78±15	71±10
8-OH-DPAT 1 µM	83±7	111±15	112±19	122±16	107±19	103±10	90±5	97±14	84±11
10 µM	81±7	88±7	92±15	103±17	134±14*	157±17**	121±16	102±14	100±10
100 µM	100±7	121±9	152±36	232±43**	214±37**	201±34*	148±17	114±15	99±14
8-OH-DPAT(10 µM) + WAY100135 (200 µM)	100±12	108±12	131±23	124±17	102±14	99±14†	101±24	102±11	107±17

Data represent the mean±SEM (% of basal levels), n=6-9. Significant differences compared to the basal levels and differences between 8–OH-DPAT (10 µM) and 8-OH-DPAT + WAY 100135 treated groups are indicated as *P<0.05, **P<0.01 and †P<0.05 respectively (one way ANOVA followed by Duncan's t test).

151P ANTAGONISM BY WAY-100635 OF THE HAEMODYNAMIC EFFECTS OF ACTIVATION OF FOREBRAIN 5-HT_{1A} RECEPTORS WITH DP-5-CT AND SUMATRIPTAN IN ANAESTHETISED RATS

M. Gallacher & A. G. Ramage, Department of Pharmacology, Royal Free Hospital School of Medicine, Hampstead, London NW3 2PF.

Activation of forebrain 5-HT_{1A} receptors in anaesthetized rats causes sympathoexcitation and a rise in blood pressure while those located in the hindbrain produce sympathoinhibition and a fall in blood pressure (Anderson et al., 1992). The present study investigates the regional haemodynamic effect of activation of these receptors by i.c.v. administration of N,N-di-n-propyl-5-carboxamidotryptamine (DP-5-CT) and the 5-$HT_{1\text{-like}}$ agonist sumatriptan. Further, the effect of the 5-HT_{1A} antagonist WAY-100635 (Fletcher et al., 1994) on these responses was investigated.

In male Sprague Dawley rats (250-350g) anaesthesia was induced with halothane and maintained with α-chloralose (80 mg kg^{-1}). The carotid artery and jugular vein were cannulated for blood pressure (MAP) and heart rate (HR) recordings and i.v. administration of drugs. Rats were artificially ventilated following neuromuscular blockade with decamethonium (3 mg kg^{-1}) and placed in a stereotaxic frame for i.c.v. injections (5 µl over 20s). Pulsed Doppler flow probes were placed, using a retroperitoneal approach, around the mesenteric (M) and renal (R) arteries and the abdominal aorta (H) (a measure of hindquarters flow). Results are expressed as mean ± s.e.mean. Drug induced changes were compared by two-way ANOVA with time-matched saline controls. All changes presented were considered significant when $P < 0.05$.

DP-5-CT (3 nmol kg^{-1};n=6) caused a rise in MAP (10 ± 1mmHg) and HR (124 ± 16 beats min^{-1}) associated with a decrease in mesenteric (-10 ± 2%) and renal conductances (-14 ± 4%). Sumatriptan (10 nmol kg^{-1};n=5) caused a rise in MAP (14 ± 1mmHg) and HR (56 ± 24 beats min^{-1}) but decreases in conductance (C) in all beds (MC -10 ± 1%; RC -10 ± 2%; HC -9 ± 2%). In the presence of the 5-HT_{1A} antagonist WAY-100635 (10 µg kg^{-1};i.c.v.;n=6) DP-5-CT caused a delayed decrease in MAP (9 ± 2mmHg), associated with increases in renal (11 ± 5%) and hindquarters conductance (31 ± 9%). While 100 µg kg^{-1} of WAY-100635 (n=6) blocked the effect of DP-5-CT and the pressor effects of sumatriptan. However sumatriptan now caused a fall in MAP (7 ± 3mmHg) which was associated with increases in renal (7 ± 3%) and hindquarters conductance (15 ± 5%). These changes induced by sumatriptan could be blocked by additional pretreatment with the 5-$HT_{1D/1B}$ antagonist GR127935 (300 µg kg^{-1} i.v.,n=5; Skingle et al., 1993).

The present study indicates that sumatriptan can activate 5-HT_{1A} receptors which causes a rise in MAP associated with vasoconstriction in the mesenteric, renal and hindquarters vascular beds. Further if forebrain and hindbrain 5-HT_{1A} receptors are blocked a 5-$HT_{1D\alpha/1B}$ receptor mediated depressor effect of i.c.v sumatriptan is unmasked. The site of these receptors, peripheral or central, remains to be determined.

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152P 5-HT_{1D} RECEPTORS MEDIATE INHIBITION OF CYCLIC AMP ACCUMULATION IN MDCK CELLS

P. Schoeffter & I. Bobirnac, Preclinical Research 386/744, Sandoz Pharma Ltd, CH-4002 Basel, Switzerland

5-Hydroxytryptamine 5-HT_{1B}/5-HT_{1D} receptors are members of the same receptor subfamily, but display a different pharmacology (Hartig et al., 1992). Whereas several cell lines have been reported to contain 5-HT_{1B} receptors, none has been described that endogenously expresses 5-HT_{1D} receptors. The present study deals with the characterization of 5-HT_{1D} receptors inhibiting cyclic AMP accumulation in Madin-Darby canine kidney (MDCK) cells.

MDCK cells were obtained from American Tissue Type Collection (Rockville, MD, USA) and propagated in Dulbecco's modified Eagle Medium (with sodium pyruvate and 1 g/l glucose; Gibco BRL) supplemented with 5 % foetal calf serum. The cells were passaged once a week using trypsin/EDTA and grown to confluency in 24-well plates. Cyclic AMP accumulation was measured as [^{3}H]adenine conversion to [^{3}H]cyclic AMP as previously described (Murphy & Bylund, 1989). Mean values ± s.e.mean were calculated from at least 3 individual experiments.

5-HT (1 nM - 10 µM) induced a concentration-dependent inhibition of cyclic AMP accumulation stimulated by prostaglandin E_1 (1 µM) in MDCK cells. The maximal effect of 5-HT averaged 50 % inhibition. The effect of 5-HT was abolished after a pre-treatment of the cells with pertussis toxin (100 ng/ml for 20 h). Other agonists mimicked the effects of 5-HT, with the following rank order of potency (pEC_{50}): 5-carboxamidotryptamine (8.36 ± 0.48) > PAPP (p-aminophenylethyl-m-trifluoromethylphenyl piperazine, 7.44 ± 0.36) = 5-HT (7.35 ± 0.05) > sumatriptan (6.59 ± 0.33) > 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin, ≈6). Among them, PAPP and 8-OH-DPAT behaved as partial agonists. Methiothepin, GR127935 (Skingles et al., 1993), (-)propranolol, rauwolscine and ketanserin were all devoid of intrinsic activity (up to 10 µM or 0.1 mM). Methiothepin (10 nM, 0.1 µM and 1 µM) antagonized 5-HT effects (pA_2 8.54, Schild slope 1.17). GR127935 (1 and 10 nM) shifted the curve of 5-HT to the right, yielding a pK_B value of 9.77 ± 0.17. From the shifts obtained with rauwolscine (1 µM) and (-)propranolol (10 µM), respective pK_B values were estimated 6.68 ± 0.30 and ≈5.5. PAPP, when tested as an antagonist at 1 µM, also shifted the curve of 5-HT to the right, with a pK_B of 8.27 ± 0.16. Finally, ketanserin (10 µM) also antagonized the effects of 5-HT, the pK_B being 6.54 ± 0.16.

The rank orders of agonist and antagonist potencies strongly suggest 5-HT receptors mediating inhibition of cyclic AMP accumulation in MDCK cells to be 5-HT_{1D} receptors. This is the first cell line reported to express these receptors endogenously. The partial agonist activity of PAPP indicates a lesser degree in coupling than in e.g. calf substantia nigra (Schoeffter & Hoyer, 1989). With regard to the 5-HT_{1D} receptor subtype involved, the relatively high potency of ketanserin would be in favour of 5-$HT_{1D\alpha}$ or a mixture of 5-$HT_{1D\alpha}$/5-$HT_{1D\beta}$ subtypes (Kaumann et al., 1993).

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153P CHARACTERISATION OF [^{35}S]GTPγS BINDING TO CHO CELL MEMBRANES EXPRESSING HUMAN 5-HT$_{1DA}$ RECEPTORS: EVIDENCE FOR NEGATIVE EFFICACY OF 5-HT RECEPTOR ANTAGONISTS

D.R. Thomas, S.A. Faruq and A.M. Brown, Dept. of Psychiatry Research, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW.

Two human 5-HT$_{1D}$ receptor subtypes, 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$, have been cloned, which show similar receptor binding profiles (Weinshank et al., 1991). The function of the 5-HT$_{1D\beta}$ subtype has been extensively investigated (Levy et al., 1992) but that of the 5-HT$_{1D\alpha}$ receptor is less well characterised. In the present study we have used a [^{35}S]-GTPγS binding assay to study the function of the human 5-HT$_{1D\alpha}$ receptor stably expressed in chinese hamster ovary (CHO) cells and to investigate further apparent negative efficacy at this receptor (Thomas et al., 1994).

Table 1. Drug effects on basal [^{35}S]-GTPγS binding to 5-HT$_{1D\alpha}$/CHO membranes (mean ± s.e.mean, n≥3).

Compound	pEC$_{50}$ or *pIC$_{50}$	%Emax or * % inhibition of basal	pKi[a]
5-CT	8.7 ± 0.2	115 ± 4.9	9.2
Metergoline	8.7 ± 0.1	88 ± 6.0	8.2
Sumatriptan	8.5 ± 0.1	92 ± 9.2	8.5
5-HT	8.1 ± 0.1	100	8.4
Mianserin	7.6 ± 0.2	66 ± 12	-
Yohimbine	7.5 ± 0.2	49 ± 5.1	7.7
8-OHDPAT	7.0 ± 0.1	69 ± 2.8	6.9
Methiothepin	*8.1 ± 0.2**	*70 ± 6.4**	8.0
Ketanserin	*6.9 ± 0.1**	*73 ± 3.0**	7.1
Ritanserin	*7.8 ± 0.1**	*64 ± 4.5**	7.3

[a] pKi data from Weinshank et al., (1991) for comparison.

The method used was as described previously (Thomas et al., 1994) using washed CHO membranes showing a high 5-HT$_{1D\alpha}$ receptor density (5.0 pmoles/mg protein). 5-HT stimulated basal [^{35}S]-GTPγS binding (170±8.8 fmoles/mg protein) by 39±4.4%. 5-CT, metergoline and sumatriptan were full agonists compared with 5-HT, and yohimbine, 8-OHDPAT and mianserin were partial agonists, whereas methiothepin, ketanserin and ritanserin produced marked inhibition of basal [^{35}S]-GTPγS binding (table 1). Drug potencies were similar to their affinities for the receptor (Weinshank et al., 1991). The inhibition by methiothepin was not significantly altered by three additional membrane washes, suggesting that the effect was not due to antagonism of endogenous 5-HT. In addition, the GDP and Na^+ ion dependence of the methiothepin effect was opposite to that expected for an agonist. GDP produced an increase in the inhibitory potency of methiothepin (pIC$_{50}$'s were 7.2±0.04, 7.7±0.1 and 8.2±0.14 in the presence of 0.1, 1 and 10μM GDP respectively). Furthermore, the degree of inhibition by methiothepin was maximal at low (<50mM) Na^+ concentrations, whereas stimulation by 5-HT was maximal at high (>100mM) Na^+ concentrations.

The reduction of basal binding by the above mentioned 5-HT antagonists is consistent with an inhibition of spontaneous receptor-G-protein coupling to promote an uncoupled receptor state. These 5-HT antagonists therefore appear to show negative efficacy at the human 5-HT$_{1D\alpha}$ receptor.

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154P BLOCKADE OF PHENCYCLIDINE-INDUCED HYPERLOCOMOTION IN RATS BY CLOZAPINE, MDL 100,907 AND OTHER ANTIPSYCHOTICS CORRELATES WITH AFFINITY AT 5-HT$_{2A}$ RECEPTORS

S. Maurel-Remy, V. Audinot, F. Lejeune, K. Bervoets and M.J. Millan (Introduced by M. Spedding), Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy-sur-Seine (Paris, France).

Although the primary mechanism of action of the psychotomimetic agent, phencyclidine (PCP), is probably a non-competitive blockade of the ion channel coupled to N-methyl-D-aspartate receptors, several other mechanisms, notably dopaminergic and serotoninergic, may intervene in its effects. The suggestion (Nabeshima *et al.*, 1988) that serotonin (5-HT)$_2$ receptors may be involved is of interest in that a blockade of these sites may contribute to the 'atypical' antipsychotic profile of clozapine (Meltzer, 1989) Here, we examined the potential role of 5-HT$_{2A}$ and dopamine D$_2$ receptors in the modulation of PCP- as compared to amphetamine-induced locomotion in rats. Male Wistar rats of 200-220 g were used. Drug affinities were determined as previously (Millan *et al.*, 1994). Conditions were: 5-HT$_{2A}$, cortex [^{3}H]-ketanserin (1.0 nM) and D$_2$ striatum, [^{3}H]-spiperone (0.2 nM). Ambulation was monitored (over 60 min) automatically with rats placed in chambers of 45 cm x 55 cm x 20 cm. The interruption (within 3 sec) of 2 infrared beams placed 24 cm apart 4 cm above the chamber floor was equivalent to one "count". Rats received PCP (20.0 mg/kg, s.c.) or amphetamine (2.5 mg/kg, i.p.) immediately before placement in chambers and were pretreated with other ligands 30 min prior thereto. Drugs were dissolved in sterile water and given s.c.. Clozapine and several other, potentially 'atypical' antipsychotics more potently blocked PCP- than amphetamine-evoked locomotion (Table 1). This was not the case for haloperidol and raclopride. The Pearson Product-Moment correlation coefficient between potency (log μmoles/kg) for inhibition of PCP-induced locomotion and affinity (pK$_i$) was high for 5-HT$_{2A}$ ($r = + 0.97$, $p < 0.001$) but not D$_2$ (+ 0.14, $p > 0.05$) sites. Conversely the coefficient for inhibition of amphetamine-induced locomotion was high for D$_2$ (+ 0.94, $p < 0.001$) but not 5-HT$_{2A}$ (- 0.09, $p > 0.05$) sites. An antagonist at 5-HT$_{2C}$ receptors (SB 200,646, 20.0 mg/kg, p.o.) as well as antagonists at α_1- and α_2-adrenergic receptors (prazosin, 0.63 mg/kg, s.c. and RX 821,002, 0.63 mg/kg, respectively) did not significantly ($p > 0.05$) modify the actions of PCP or amphetamine. In conclusion, although a possible role of other receptors should *not* be excluded, the data suggest that amphetamine- and PCP-induced locomotion are modulated by D$_2$ and 5-HT$_{2A}$ receptors, respectively. The precise mechanism of action of PCP remains to be further explored. These data support the concept of a functional relationship between 5-HT$_{2A}$ receptors and the etiology and treatment of psychotic disorders.

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Table 1: Inhibition of PCP- as compared to amphetamine-induced locomotion as compared to affinity at 5-HT$_{2A}$ and D$_2$ sites.

	MDL 100,907	*Risperidone*	*Olanzapine*	*Clozapine*	*Haloperidol*	*Amperozide*	*Seroquel*	*Raclopride*
5-HT$_{2A}$	9.23	9.16	8.43	7.63	7.08	7.02	6.43	4.39
D$_2$	5.37	8.34	7.82	6.65	8.73	6.13	6.54	8.10
PCP-Loc	0.0009	0.002	0.003	0.04	0.13	0.15	0.33	0.87
	(0.0003-0.003)	(0.0008-0.007)	(0.0008-0.01)	(0.02-0.07)	(0.09-0.2)	(0.04-0.50)	(0.08-1.4)	(0.7-1.1)
Amph-Loc	> 10.0	0.20 (0.1 - 0.4)	1.2 (0.6-2.3)	8.8 (6.4-12.1)	0.04 (0.03-0.1)	5.3 (2.7-10.3)	21.7 (10.8-43.3)	0.18 (0.10-0.24)

In vitro affinities are pK$_i$s. *In vivo* values are Inhibitory Dose$_{50}$s (95 % Confidence Limits) in mg/kg base, s.c..

155P THE 5-HT2C/2B RECEPTOR ANTAGONIST SB 200646A IS A POTENT AND SELECTIVE ANTAGONIST OF THE HUMAN 5-HT2C RECEPTOR

M.D. Wood, T.L.Gager, D.R. Thomas, R.A. Newton*, S.L. Phipps* and J.M. Elliott*. SmithKline Beecham, New Frontiers Science Park, Harlow, CM19 5AW and *Oxford University SmithKline Beecham Centre for Applied Neuropsychobiology, Oxford, OX2 6HE

SB 200646A is the first antagonist which shows significant selectivity for the 5-HT_{2C} receptor compared to the 5-HT_{2A} receptor (Forbes et al, 1993). These studies were carried out in tissues from laboratory animals. We have now extended these studies to determine whether SB 200646A retains its profile against the cloned and native human receptors.

Binding studies to 5-HT_{2A} and 5-HT_{2C} receptors used ^{3}H-ketanserin and ^{3}H-mesulergine respectively in cloned (HEK 293 cells, Wood et al, In Press) and human (frontal cortex and choroid plexus, Elliott et al, 1993) receptor preparations. Phosphoinositide (PI) hydrolysis was measured in HEK 293 cells expressing the human 5-HT_{2C} receptor pre incubated with ^{3}H-myo-inositol. Cells were exposed to antagonist 20 min prior to agonist in the presence of 10 mM lithium. The reaction was terminated after 20 min by methanol (-40°C) and the ^{3}H-inositol phosphates isolated using Dowex AG1-X8 columns. Results are means±SEM from (n) separate experiments.

Compared to mianserin, SB 200646A showed selectivity for the human 5-HT_{2C} receptor over the human 5-HT_{2A}receptor (Table 1). In PI studies on cloned human 5-HT_{2C} receptors, SB 200646A (10^{-6}M) lacked agonist activity but antagonised the effects of 5-HT in a competitive manner with an apparent pA_2 of 7.1 ± 0.2(3). Mianserin (10^{-7}M) also attenuated the effects of 5-HT without altering the maximum response with an apparent pA_2 of 8.2 ± 0.3 (3).

Table 1: Ligand affinities at human 5-HT_{2A} and 5-HT_{2C} receptors

Human receptor	pK_I	
	SB 200646A	Mianserin
Cloned 5-HT_{2C}	6.96 ± 0.10 (3)	8.95 ± 0.07(5)
Native 5-HT_{2C}	6.28 ± 0.05(4)	8.78 ± 0.18(3)
Cloned 5-HT_{2A}	< 5.2	8.24 ± 0.12(9)
Native 5-HT_{2A}	4.72 ± 0.08(4)	7.88 ± 0.04(3)

In conclusion, SB 200646A remains a potent and selective (over 5-HT_{2A}) 5-HT_{2C} receptor antagonist at the human 5-HT_{2C} receptor, the final therapeutic target. Its activity at the human 5-HT_{2B} receptor awaits to be determined.

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156P S 16924: A PYRROLIDINE-SUBSTITUTED BENZODIOXANE WITH HIGHER AFFINITY AT 5-HT1A/2A/2C THAN D2 RECEPTORS, AND AN 'ATYPICAL' ANTIPSYCHOTIC PROFILE IN THE RAT

M.J. Millan, V. Audinot, M. Brocco, S. Maurel-Remy, F. Joly, A. Newman-Tancredi, H. Canton, P. Hautefaye, M. Spedding, O. Muller & G. Lavielle, Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy-sur-Seine (Paris, France).

In addition to dopaminergic receptors, adrenergic and serotoninergic receptors are of importance in the etiology of schizophrenia. An interaction of clozapine with 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors, as well as with D_4 receptors (Van Tol *et al.*, 1991), may contribute to its distinctive profile as compared to that of haloperidol. Here, we examined the potential antipsychotic profile of the novel ligand, S 16924 ((-)-(1-(benzodioxane-5y-3-[3-(4-fluorophenacyl)pyrrolidine] -1-oxapropane)) in rodents. All studies employed male Wistar rats of 200 - 220 g. Drug affinities in tissue homogenates were determined as previously (Millan *et al.*, 1994). Conditions were: 5-HT_{1A}, rat hippocampus, [^{3}H]-8-OH-DPAT (0.4 nM); 5-HT_{2A}, rat cortex, [^{3}H]-ketanserin (1.0 nM); 5-HT_{2C}, [^{3}H]-mesurlergine (1.0 nM), pig choroid plexus and D_2, rat striatum [^{3}H]-spiperone (0.2 nM). Affinities at cloned human hD_2 and hD_4 receptors transfected into CHO cells were determined using [^{125}I]-iodosulpride (0.1 nM) and [^{3}H]-spiperone (0.2 nM), respectively (Sokoloff *et al.*, 1992; Van Tol *et al.*, 1991). For *in vivo* studies, S 16924, haloperidol and clozapine were dissolved in sterile water and injected s.c. 60 min prior to testing. Their influence upon the locomotor ambulation elicited by amphetamine (2.5 mg/kg, i.p.) was determined (automated counting of beam-brakes) over the 60 min following administration of amphetamine. For catalepsy, the time over which rats kept a posture with the right hind-paw crossed over the ipsilateral front-paw was measured (Waldmeier & Delini-Stula, 1979): the cut-off was 30 sec and the mean of 3 measures was taken. Further, the influence of S 16924 and clozapine upon the induction of catalepsy by haloperidol (2.5 mg/kg, s.c., 30 min pre-testing) was also determined. The conditioned avoidance procedure was as described by Janssen and Niemegeers (1961). Like clozapine, S 16924 showed higher affinity for 5-HT_{2A} and 5-HT_{2C} than D_2 receptors, and for hD_4 than hD_2 receptors. In addition, S 16924 showed higher affinity for 5-HT_{1A} sites than clozapine. S 16924 mimicked haloperidol and clozapine in inhibiting amphetamine-induced locomotion and in reducing conditioned avoidance responses. Like clozapine, S 16924 failed to evoke catalepsy and blocked its induction by haloperidol. The distinctive receptorial profile of S 16924 may underlie its 'atypical' pattern in tests of potential antipsychotic activity in the rat.

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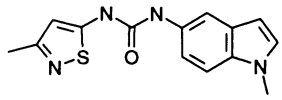
Table 1: *In vitro* and *in vivo* actions of S 16924 as compared to haloperidol and clozapine

	5-HT1A	*5-HT2A*	*5-HT2C*	*D2*	*hD2*	*hD4*	*Amph. locom.*	*Conditioned avoidance*	*Cat.: induction*	*Cat.: inhibition*
Haloperidol	5.6	7.1	5.2	8.7	9.2	8.7	0.1 (0.03 - 0.2)	0.1 (0.05 - 0.3)	0.2 (0.1 - 0.4)	-
Clozapine	6.5	7.6	8.1	6.7	7.1	7.4	8.8 (6.4 - 12.1)	4.4 (2.5 - 7.1)	> 40.0 (-)	6.4 (1.2 - 33.8)
S 16924	8.4	8.6	8.1	7.3	7.5	8.2	2.3 (1.5 - 3.6)	2.9 (1.5 - 6.1)	> 40.0 (-)	3.2 (1.5 - 6.7)

In vitro affinities are pK_is. *In vivo* values are Inhibitory $Dose_{50}$s (95 % Confidence Limits) in mg/kg base, s.c., except for induction of catalepsy, where they are Active $Dose_{50}$s for induction of a half-maximal effect. Amph. Loc. = amphetamine locomotion. Cat. = catalepsy.

G. S. Baxter E.S. Ellis, I.T. Forbes, G.E. Jones, G.A. Kennett, O.E. Murphy & N. Tilford SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, 3rd Avenue, Harlow, Essex CM19 5AD

5-HT2B and 5-HT2C receptors display a high level of pharmacological similarity and can be readily distinguished from other 5-HT receptors using ligands such as SB 200646A which shows high affinity and selectivity for the 5-HT2B and 5-HT2C receptor subtypes (*for references see* Baxter *et al.*, 1994). We now report the identification of, SB 204741, an antagonist with high affinity and selectivity for 5-HT2B receptors.



SB 204741

Rat stomach fundus, caudal artery and jugular vein were set up as described previously (Baxter *et al.*, 1994; Ellis *et al.*, 1994). SB 204741 did not show agonism in any preparation but caused a concentration-dependent dextral shift of concentration-effect curves to 5-HT at 5-HT2B receptors in rat stomach fundus (pA_2 = 7.96, slope = 0.96). SB 204741 (1 μM) also caused a dextral displacement of concentration-effect curves to 5-HT at putative 5-HT2B receptors in rat jugular vein (mean concentration-ratio ± s.e.m. = 20.7 ± 2.9) suggesting a pA_2 in the order of 7.3. In contrast, SB 204741 (1-10 μM) caused little or no antagonsim of 5-HT induced contractions mediated via 5-HT2A receptors in rat caudal artery and possessed low affinity for radiolabelled human cloned 5-HT2C receptors (pKi < 6.0) and for the following binding sites; 5-HT1A, 5-HT1Dα, 5-HT1Dβ, 5-HT1E, 5-HT3, 5-HT4 D1, D2, D3, H1 and $GABA_A$ (pKi < 5.0). SB 204741 also demonstrated low affinity for α_1- and α_2-adrenoceptors (pKi < 6.0).

SB 204741 thus appears to act as a selective antagonist at 5-HT2B receptors. The use of SB 204741 should allow discrimination of 5-HT2B receptors in preparations containing mixed 5-HT receptor populations and may facilitate a greater understanding of the role of this receptor subtype in physiological and pathophysiological processes.

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158P CHARACTERIZATION OF THE FUNCTIONAL RESPONSES MEDIATED BY TWO SPLICE VARIANTS OF THE CLONED RAT 5-HT4 RECEPTOR IN HETEROLOGOUS EXPRESSION SYSTEMS

N. Adham, C. Gerald, P. J.-J. Vaysse, R. L. Weinshank, and T. A. Branchek, Synaptic Pharmaceutical Corp. Paramus, New Jersey 07652 USA

We have isolated two splice variants of the pharmacologically-defined 5-HT_4 receptor, 5-HT_{4L} and 5-HT_{4S}, differing in the length and sequence of their carboxy termini (Gerald et al., 1994). To date, however, we have found no major differences in receptor pharmacology of the long and short forms of the 5-HT_4 receptor in binding assays using [^{3}H]5-HT or [^{3}H]GR113808 as the radioligand. This is not surprising since the amino acid sequences of these two clones are identical, apart from the cytoplasmic carboxy tail, a region that is important for G protein-coupling, where the 5-HT_{4L} receptor carries an extra phosphorylation site. Therefore, we investigated the possibility of differences in functional characteristics of the two clones.

When transiently expressed in COS-7 cells, each receptor stimulated adenylate cyclase activity by approximately 20-fold in the absence of forskolin; no inhibition of either the basal or forskolin-stimulated cAMP release was observed. Addition of 10 μM forskolin together with 1 μM 5-HT stimulated cAMP accumulation about 10-fold more than either agent alone. Neither of these receptors was found to modulate either intracellular Ca^{++} levels or the accumulation of total inositol phosphates.

In functional assays measuring cAMP release, 5-HT_{4S} and 5-HT_{4L} clones exhibited very similar pharmacological profiles. Some differences were noted: 1) In general, the potency of agonists for stimulating cAMP release was greater for 5-HT_{4S} clone. For example, the mean EC_{50} values for 5-HT were significantly different (5-HT_{4L}, 51 ± 7 nM; 5-HT_{4S}, 26 ± 3 nM (n=3), $p < 0.05$). This difference could be due to higher expression levels (approximately 2-fold) of 5-HT_{4S} clone as compared to 5-HT_{4L} clone (B_{max}s of 5-HT_{4S} using [^{3}H]GR113808, 2.9 ± 0.5 pmol/mg protein; 5-HT_{4L}, 1.8 ± 0.37 pmol/mg protein (n=3) $p < 0.05$). An inverse relationship between receptor number and EC_{50} value for β-adrenergic receptor-mediated stimulation of cAMP levels have been previously shown in several heterologous expression systems (Bouvier et al., 1988; Samama et al., 1993; Whaley et al., 1994). 2) The maximal stimulation elicited by 5-HT_{4L} receptor was significantly greater than that produced by 5-HT_{4S} (E_{max}s of 5-HT_{4L}, 2,598 ± 154%; 5-HT_{4S}, 2,107 ± 52% basal cAMP release (n=3) $p < 0.05$) despite the lower expression levels. 3) BRL24924 and zacopride displayed significantly lower potency (approximately 3-fold and 10-fold, respectively) for 5-HT_{4S}-mediated cAMP responses than those elicited by 5-HT_{4L} clone. Whether this disparity is due to differences in the degree of receptor reserve of these compounds for the two receptors needs to be investigated further.

These data indicate that the splice variants, 5-HT_{4S} and 5-HT_{4L} receptors, which possess very similar pharmacological profiles and differ only at their C-terminal tails, may possess different coupling efficiencies to elicit functional responses. Whether 5-HT_{4S} and 5-HT_{4L} couple to different isoforms of G_s and/or adenylyl cyclase remains to be investigated.

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159P RS-17017, RS-67333 AND RS-67506: POTENT AND SELECTIVE 5-HT$_4$ RECEPTOR PARTIAL AGONISTS *IN VITRO* AND *IN VIVO*

R.M. Eglen, D.W. Bonhaus, R.D. Clark[1], L.G. Johnson & E. Leung. Institutes of Pharmacology and Organic Chemistry[1], Syntex Discovery Research, Palo Alto, CA 94304, USA.

5-HT$_4$ receptors (see Ford and Clarke, 1993, for review) are stimulated by indoles, (5-HT; 5-methoxytryptamine), benzamides (SC-53116, renzapride, zacopride) or benzimidazolones, (BIMU-1, BIMU-8). In the present study, the pharmacology of three novel, compounds, RS-17017 (1-(4-amino-5-chloro-2-methoxyphenyl)-5-(piperidin-1-yl)-1-pentanone HCl), RS-67506 (1-(4-amino-5-chloro-2-methoxyphenyl)-3-[1-(2-methylsulfonylamino)ethyl-4-piperidinyl]-1-propanone HCl) and RS-67333 (1-(4-amino-5-chloro-2-methoxy phenyl)-3-[1-(n-butyl)-4-piperidinyl]-1-propanone HCl) has been assessed. The data were compared to RS-70678 (ML10302; 2-(1-piperidinyl) ethyl 4-amino-5-chloro-2-methoxybenzoate HCl, Elz & Keller, 1993), reported by Langlois et al. (1994) to act as a potent 5-HT$_4$ receptor agonist (-log EC_{50}=8.4; α=0.8) in guinea-pig isolated ileum. RS-17017 and RS-70678 are analogues of the 5-HT$_4$ antagonist, RS-23597 (Eglen et al. 1993).

The methods used to evaluate 5-HT$_4$ agonism have been previously described (Eglen et al. 1993; 1994). The selectivity of these agonists at several neurotransmitter receptors was assessed using methods described in Eglen et al. (1994) and Grossman et al. (1993).

RS-17017, RS-67333 and RS-67506 were partial 5-HT$_4$ agonists *in vitro* and *in vivo* (Table 1). In rat OMM all compounds were antagonized by 10 nM GR113808 (-log K_B values 9.1-9.3). RS-70678, was also a potent, but partial, 5-HT$_4$ receptor agonist in rat OMM (-log EC_{50}=8.0 ± 0.1; α=0.5). This value was similar to that reported by Langlois et al. (1994) in rat OMM (-log EC_{50}=8.6). All compounds exhibited low apparent affinities at several neurotransmitter receptors including 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2A}$, 5-HT$_{2C}$, 5-HT$_3$, dopaminergic D_1, D_2, or muscarinic M_1-M_2 receptors (-log K_i < 6.0).

In conclusion, replacement of the ester in the antagonist RS-23597 (Eglen et al. 1993) by a ketone group, resulted in the partial 5-HT$_4$ receptor agonist, RS-17017. Modification, in turn, of RS-17017 resulted in higher potency partial 5-HT$_4$ agonists, RS-67506 and RS-67333. These novel agonists may prove useful to study 5-HT$_4$ receptor function *in vitro*. The lack of the ester moiety in these novel agonists, in contrast to RS-70678, may enhance their utility *in vivo*.

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Table 1. Pharmacology of novel agonists at 5-HT$_4$ receptors

	Guinea-Pig Striatum[a]		Rat OMM[b]		Micropig Tachycardia	
Agonist	-log K_i	nH	-log EC_{50}	α	ED_{50}; $\mu g \cdot kg^{-1}$	max response; beats min^{-1}
5-HT	7.5 ± 0.08	0.7	8.2 ± 0.1	1.0	3.1 (2.3-4.2)	66 (61-72)
RS-17017	7.7 ± 0.1	1.2	7.4 ± 0.1	0.7	27 (15-51)	35 (26-44)
RS-67333	8.7 ± 0.1	1.0	8.4 ± 0.1	0.5	4.9 (2.3-10)	35 (27-43)
RS-67506	8.8 ± 0.03	1.0	8.6 ± 0.1	0.6	5.4 (4.0-7.2)	47 (43-52)

Values are mean ± s.e. mean or mean (95% confidence intervals), n=4-8. [a]displacement of specific [^{3}H]GR 113808 binding, [b]relaxation of rat, isolated oesophageal muscularis mucosae (OMM). α-maximum response relative to 5-HT.

160P EXCITATORY ACTIONS OF 5-HT IONTOPHORETICALLY APPLIED TO DORSAL VAGAL PREGANGLIONIC NEURONES IN ANAESTHETISED RATS ARE ATTENUATED BY 5-HT$_{1A}$ RECEPTOR ANTAGONISTS

Yun Wang, James F.X. Jones, *Andrew G. Ramage & David Jordan, Departments of Physiology & *Pharmacology, Royal Free Hospital School of Medicine, Hampstead, London NW3 2PF

In the rat, *in vitro* studies have provided evidence that 5-HT increases the excitability of neurones in dorsal vagal nuclei (DVN); the slow depolarisations are due to 5-HT directly acting on postsynaptic 5-HT$_2$ receptors (Albert and Brooks,1994). However, *in vivo* investigations have shown that microinjection of 5-HT$_{1A}$, but not 5-HT$_2$ agonists into the DVN could evoke a vagally mediated bradycardia (Sporton et al., 1991). In addition, ionophoretically administered 8-OH-DPAT inhibits the majority of dorsal vagal preganglionic neurones and this effect is not antagonised by the 5-HT$_{1A}$ antagonist WAY-100635 (Wang et al., 1994). The present study investigates these incongruities more and directly tests the hypothesis that 5-HT$_{1A}$ and 5-HT$_2$ receptors increase the excitability of DVN neurones.

In pentobarbitone sodium (60 mg kg^{-1}, i.p.) anaesthetised Sprague-Dawley rats, single unit activity was recorded from identified preganglionic neurones in the DVN using 5-barrel microelectrodes. Of the 43 DVN neurones tested, 37 exhibited a bi-phasic response to ionophoretic administration of 5-HT (5-HT maleate, 50 mM, pH = 4): excitation at "low" ejection currents (2-10nA) and inhibition at "high" currents (10-40nA). The initial excitatory effect could be attenuated by ionophoretic application of either (±)pindolol (20 mM), pH = 4, 5-20nA), WAY-100635 (10mM, pH = 4, 5-20nA), ketanserin (10mM, pH = 4, 5-20nA) or cinanserin (50 mM, pH = 4, 5-20nA). However, ionophoretic application of 8-OH-DPAT (20 nM, pH = 4, 1-40nA) onto DVN neurones caused either inhibition alone (67/104) or much less often excitation alone (19/104). The excitatory effect of 8-OH-DPAT, like that of 5-HT, could be attenuated by (±)pindolol.

In addition, 4 out of 4 DVN neurones excited by "low" currents of 5-HT were inhibited both by 8-OH-DPAT and by a "high" current of 5-HT. The inhibitory effects of both 5-HT and 8-OH-DPAT were mimicked by (±)pindolol and WAY-100635.

In conclusion, these results are in agreement with the *in vitro* study that 5-HT increases the excitability of DVN neurones. However, *in vivo* the excitatory response of DVN neurones is mediated by both 5-HT$_2$ and 5-HT$_{1A}$ receptors. What remains unexplained is the underlying mechanism of the powerful inhibitory effects of 5-HT and 8-OH-DPAT on DVN neurones observed *in vivo*, and which is resistant to and indeed mimicked by 5-HT$_{1A}$ antagonists.

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161P 5-HT RECEPTORS MEDIATING INHIBITION OF PRESSOR RESPONSES TO ELECTRICAL SYMPATHETIC STIMULATION IN PITHED RATS

C.M. Villalón*, J. Contreras, E. Ramírez-San Juan, & J.A. Terrón. Department of Pharmacology, CINVESTAV, I.P.N., Apdo. Postal 22026, 14000 Mexico D.F., Mexico.

5-Hydroxytryptamine (5-HT) inhibits contractile responses to adrenergic nerve stimulation in several blood vessels (Saxena & Villalón, 1990). We have now investigated the effects of 5-HT and the receptor type involved in the inhibition of pressor responses caused by preganglionic sympathetic stimulation (T7-T9) in pithed rats, using the method described by Gillespie *et al.* (1970). Baseline values of heart rate and diastolic blood pressure (DBP) were 244±5 beats min^{-1} and 56±4 mmHg, respectively (n=55). Sympathetic stimulation (0.03, 0.1, 0.3, 1 and 3 Hz) resulted in frequency-dependent increases in DBP (11±2, 14±2, 25±3, 41±4 and 55±3 mmHg, respectively), with small and inconsistent changes in heart rate. During i.v. infusion of 5-HT (3-100 μg/kg.min), but not of saline, the increases in DBP were inhibited in a dose-dependent manner, both in control and desipramine (50 μg/kg) treated rats. Table 1 shows that the inhibitory effect of 5-HT remained unaffected after i.v. metergoline, ritanserin, MDL 72222 or tropisetron (n=5 each) at doses that are high enough to block 5-HT_{1D}, 5-HT_2, 5-HT_3 and/or 5-HT_4 receptors (Saxena & Villalón, 1990; Villalón et al., 1993), but was antagonized by methysergide (n=5) and mimicked by 5-carboxamidotryptamine and sumatriptan (data not shown). Taken together, the above findings suggest an operative 5-HT_1-like receptor-mediated prejunctional inhibition of sympathetic nerve activity on the systemic vasculature *in vivo*.

Table 1. Δ DBP (mmHg) by sympathetic stimulation during infusion of 5-HT (5.6 μg/kg.min) in desipramine-treated rats.

Compound	*Dose*	*0.03 Hz*		*0.1 Hz*		*0.3 Hz*		*1.0 Hz*		*3.0 Hz*	
	mg/kg	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
Saline[a]	-	5±1	5±1	5±1	5±1	10±1	10±1	23±4	22±4	48±6	44±7
Metergoline	1.0	8±1	12±2	8±2	13±4	16±2	18±3	43±3	46±2	84±2	78±4
Ritanserin	0.1	5±1	3±1	5±1	3±1	10±2	7±1	26±5	20±4	59±7	50±5
MDL 72222	0.15	5±1	3±1	5±1	3±1	10±2	7±1	26±5	25±6	59±7	58±6
Tropisetron	3.0	5±1	1±1	6±2	1±1	11±2	8±1	28±7	21±4	61±10	47±8
Methysergide	0.3	6±1	12±2*	8±1	13±3*	11±3	24±6*	32±8	46±6*	61±8	78±6

*[a], 1 ml/kg. *, P<0.05 vs corresponding value before antagonists, which did not affect responses to sympathetic stimulation.*

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162P A COMPARISON OF THE CONTRACTILE EFFECTS OF 5-HT, SUMATRIPTAN AND MK-462 ON HUMAN CORONARY ARTERY *IN VITRO*

A. Ferro**, J. Longmore*, R.G. Hill* & M.J. Brown. Department of Clinical Pharmacology, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge, * Merck Sharp & Dohme, Eastwick Road, Harlow, Essex, **Present address Department of Cardiology, Bristol Royal Infirmary, Bristol.

MK-462 (N,N-dimethyl-2-{5-(1,2,4-triazol-1-ylmethyl)-1H-indol-3-yl}ethylamine) is a new $5HT_{1D}$-receptor agonist (Beer *et al.*, 1994) which, like sumatriptan, is effective in the treatment of migraine (Ferrari *et al.*, 1994). As angiographic studies have shown that sumatriptan can cause coronary artery constriction in patients, we compared the effects of MK-462 on human isolated coronary artery with those of sumatriptan.

Coronary arteries were removed from ex-planted hearts from patients undergoing cardiac transplantation (2 females, 20 males, aged 21-60 years). Ring segments (endothelium-denuded) were mounted in organ baths for isometric tension recording. For each segment a cumulative concentration-effect curve to either 5-HT, sumatriptan or MK-462 was performed and on obtaining the maximal response to each agonist, ketanserin (0.6μM) was added to the tissue bath followed by methiothepin (0.6μM).

5-HT evoked a contractile response in 10 of the 22 arteries studied (all arteries responded to 45mM KCl). From these ten vessels, four arteries responded to all three agonists (ie individual segments from the same artery responsed to 5-HT, sumatriptan or MK-462) with EC_{50} values (μM; geometric mean with [95% confidence limits]) of 0.2 [0.06-1.0], 0.6 [0.2-1.7] and 0.9 [0.3-3.1] and E_{max} values (%, relative to 45mM KCl) of 90.7 ± 9.1, 61.8 ± 9.9 and 28.9 ± 7.2 for 5-HT, sumatriptan and MK-462 respectively (n=4). Of the ten "5-HT responding" vessels ten were exposed to MK-462 and seven to sumatriptan. One artery contracted to MK-462 but not to sumatriptan and two arteries did not contract to MK-462 but contracted in response to sumatriptan. The mean data obtained from all 10 "5-HT responding" arteries reflected the data obtained from arteries which responded to all three agonists (see above) ie EC_{50} values (μM) were 0.4 [0.2-0.7], 0.6 [0.3-1.1] and 1.0 [0.6-1.7] and E_{max} values were 78.0 ± 7.2, 57.6 ± 9.5 and 32.5 ± 6.7 for 5-HT (n=10), sumatriptan (n=6) and MK-462 (n=8) respectively. E_{max} values for MK-462 and sumatriptan were significantly less than the E_{max} value for 5-HT ($P < 0.03$) and the E_{max} value for MK-462 was significantly less than that for sumatriptan ($P < 0.04$, Students t-test). Ketanserin (0.6μM) partially reduced the response to each agonist and the remaining contraction was abolished by methiothepin (0.6μM).

In conclusion, not all human coronary arteries contracted in response to 5-HT; those which responded to 5-HT tended to respond also to sumatriptan and MK-462. The maximum contractile response to MK-462 was significantly less than that to sumatriptan.

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163P A POSSIBLE ROLE FOR ARACHIDONIC ACID IN 5-HYDROXYTRYPTAMINE-INDUCED CONTRACTION OF RABBIT MESENTERIC ARTERY

S. J. W. Parsons, M. Sumner[1] & C. J. Garland, Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol. BS8 4PT and [1]Glaxo Group Research, Ware. Herts. SG12 0DP.

A component of the contraction to 5-hydroxytryptamine (5-HT) in the mesenteric artery reflects sensitization of the cellular myofilaments to cytoplasmic calcium (Ca^{2+}; Seager *et al.*, 1994). It has been suggested that Ca^{2+} sensitization in smooth muscle is a consequence of increased myofilament phosphorylation which may be due to an inhibition by arachidonic acid (AA) of protein phosphatase activity (Gong *et al.*, 1992). This suggestion was based mainly on the ability of exogenous AA to evoke Ca^{2+} sensitization in permeabilized smooth muscle cells and to dissociate purified myosin light chain phosphatase, inhibiting the enzyme's activity. In the present study, we have investigated the role of AA in sensitization induced by 5-HT in the mesenteric artery, using the phospholipase A_2 (PLA_2) inhibitor quinacrine.

Helical strips 2mm long and 100-200μM in diameter were cut from the third branch of the rabbit mesenteric artery, and attached to a sensitive force transducer. The strips were bathed in mock intracellular solution (0 Ca^{2+}, 10mM EGTA) and permeabilized by incubation with Staphylococcus aureus α-toxin for 15-20 mins at room temperature. The extent of permeabilization was assessed by the ability of Ca^{2+} to stimulate contraction in concentrations (0.01-100μM) which had no effect before exposure to α- toxin (Seager *et al.*, 1994).

1μM 5-HT with 10μM GTP increased Ca^{2+}-activated force (0.3μM) by 58.2 ± 6.9 % mean ± s.e. mean (n=8). The PLA_2 inhibitor, quinacrine (10-50μM) inhibited the component of sensitization to 5-HT by 98.1 ± 5.2 %, but did not alter the sensitization to GTP (n=4 $p< 0.05$). The α-agonist phenylephrine (PE) also induced a similar Ca^{2+} sensitization (n=6) in permeabilized strips of the mesenteric artery, but in contrast to 5-HT this effect was unaltered in the presence of quinacrine. Exogenous AA (300μM), applied in the presence of BW755C, indomethacin and miconazole (lipoxygenase, cycloxygenase and cytochrome P_{450} inhibitors), also caused sensitization, increasing the contraction at constant Ca^{2+} concentration by 32.5 ± 3.1 % (n =5).

These observations show that in the rabbit mesenteric artery both 5-HT and PE can cause myofilament Ca^{2+} sensitization. However, the effect of these contractile agonists appears to be mediated by separate mechanisms. While the activation of PLA_2 and the generation of AA appears to contribute to contraction with 5-HT, this mechanism is apparently not of importance in α_1- adrenoceptor mediated responses.

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164P THE EFFECT OF NOVEL NONPEPTIDE RECEPTOR ANTAGONISTS ON ARGININE VASOPRESSIN-INDUCED CONTRACTIONS OF THE HUMAN ISOLATED CORONARY ARTERY

Willem A. Bax*, Dino Nisato[2], Egbert Bos[1] & Pramod R. Saxena, Dept. of Pharmacology, Laboratory for Human Pharmacology and [1]Thorax Centre, Erasmus University Rotterdam, Post Box 1738, 3000 DR Rotterdam, The Netherlands; [2]Sanofi Recherche, Montpelier Cedex, France.

Arginine vasopressin (AVP) may contribute to cardiovascular homeostasis by causing vasoconstriction and stimulating renal water absorption. In the present study we investigated the effect of the non-peptide receptor antagonists OPC-21268 and SR49059 (both selective for vasopressin V_1 receptors: Yamamura et al., 1991; Serradeil-Le Gal et al., 1994), and OPC-31260 (selective for V_2 receptors: Yamamura et al., 1992), on AVP-induced contractile responses of the human coronary artery (HCA). We obtained right coronary artery segments from 12 organ donors (4F, 8M, 10-54 yrs), whose heart valves were used for homograft transplantation. Contraction was measured isometrically in 15 ml oxygenated organ baths (37 °C), and expressed as a percentage of K^+ (100 mM)-induced contraction (all data: mean ± s.e.m.). AVP caused concentration-dependent contraction of the HCA (pD_2: 9.19 ± 0.17; E_{MAX}:10.2 ± 1.6%; n=12). Removal of the endothelium (as verified by the relaxant response to 1 nM substance P after precontraction with 1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)) did not result in significantly altered responses to AVP (endothelium intact: pD_2: 9.06 ± 0.24; E_{MAX}: 10.7 ± 2.0%; endothelium removed: pD_2: 9.30 ± 0.32; E_{MAX}: 14.8 ± 4.3%; n=8 paired segments). Furthermore, we did not observe relaxation after adding 0.1-1 nM AVP to coronary artery segments (n=3) precontracted (15.4 ± 1.1% of K^+, 100 mM) with 0.1 μM $PGF_{2\alpha}$. By contrast, addition of 1 nM AVP induced an additional contractile response (6.7 ± 2.9% of K^+, 100 mM). These segments subsequently completely relaxed to 1 nM substance P. In endothelium-intact coronary artery segments, SR49059 (3-30 nM) induced a concentration dependent parallel rightward shift of the concentration response curve (CRC), resulting in a pA_2=9.76 ± 0.16 (n=7; slope in Schild analysis: 1.08 ± 0.10). However, only a concentration of 3 μM OPC-21268 caused a small rightward shift of the AVP CRC (approx. pK_B=5.6 ± 0.3; n=6). OPC-31260 (0.3-3 μM) induced a parallel rightward shift of the AVP CRC with a pA_2=7.31 ± 0.18 (n=7; slope in Schild analysis: 1.07 ± 0.07). We conclude that AVP contracts the HCA with high potency but low E_{MAX}, and that the response to AVP is mediated via smooth muscle V_1 receptors with higher affinity for SR49059 than for OPC-21268, suggesting that the reported high V_1 receptor affinity of OPC-21268 may be confined to rat V_1 receptors (Serradeil-Le Gal et al., 1994). The pA_2 value of OPC-31260 is possibly explained by previously observed additional affinity for V_1 receptors (Yamamura et al., 1992).

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165P EFFECTS OF TREATMENT WITH AN ANGIOTENSIN CONVERTING ENZYME INHIBITOR ON AGONIST- AND FLOW-INDUCED DILATIONS IN PERFUSED CORONARY ARTERIES OF SHRs

J.L. Freslon & F. Pourageaud, Department of Pharmacology, Faculty of Pharmacy, University of Bordeaux II, 146 Rue Léo Saignat, 33076 Bordeaux cedex, France.

The aim of the study was to assess the effects of a one-month treatment period with the angiotensin converting enzyme (ACE) inhibitor trandolapril (0.3 mg/kg/day) on the endothelial reactivity of epicardial right coronary arteries taken from 26-30 week-old SHRs.
For this purpose, segments of coronary arteries were mounted in an arteriograph where wall thickness and internal diameter were continuously monitored while intraluminal pressure and flow were controlled. In the absence of flow and under an intraluminal pressure of 30 mm Hg, internal diameters were not significantly different in control SHRs arteries compared to those of treated SHRs (μm, 250 ± 8 vs 240 ± 7). In preconstricted preparations (5HT, 10 μM extraluminally) C/E curves were constructed by adding acetylcholine (ACh, 0.01-10 μM) or bradykinin (BK, 0.01-10 μM) in the bath. On the other hand, the effect of a stepwise increase in intraluminal flow (5-45 μl/min, intraluminal pressure=30 mm Hg) of perfusion solution was observed. The effects of subsequent additions of sodium nitroprusside (SNP) were assessed. All experiments were performed in the presence of indomethacin (10 μM). Maximal dilations were expressed as percent of maximal constrictions and results are collected in table 1.

Table 1. Maximal dilations induced by agonists, flow and sodium nitroprusside (SNP) in treated compared to control SHRs.

	ACh (10 μM)	BK (10 μM)	Flow (45 μl/min)	SNP (10 μM)
Control SHRs	67 ± 6	22 ± 5	42 ± 8	97 ± 3
Treated SHRs	88 ± 5 *	44 ± 5 **	47 ± 9	98 ± 2

Values significantly different from those of control SHRs: *: $p<0.05$; **: $p<0.01$; Student's t test

These results show that the endothelium-dependent dilations induced by ACh and BK were significantly increased in coronary arteries of treated compared to control SHRs whereas the flow-induced dilation seemed to remain unaffected. From these data, it can be concluded that a short period of ACE inhibition in SHRs is able to improve the endothelium-dependent vasodilation induced by agonists in the coronary arterial bed.

166P CALCIUM DEPENDENCY OF ANGIOTENSIN II-INDUCED CONTRACTION IN RABBIT RENAL ARTERY PREPARATIONS

J. Zhang, M. Pfaffendorf & P.A. van Zwieten, Department of Pharmacotherapy, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Angiotensin II (AII)-induced contractions have found to be calcium dependent in a variety of target tissues (van Heiningen & van Zwieten, 1993; Zhang *et al.*, 1994). Recently, we have shown that AII causes vasoconstrictor effects which are mediated by AT_1-receptors in the rabbit isolated renal artery (Zhang *et al.*, 1994). However, the role of calcium movements triggered by the stimulation of AT_1-receptors in rabbit renal artery has so far not been studied.

Isolated renal artery ring preparations (endothelium-denuded) of the rabbit were set up as described previously (Zhang *et al.*,1994). The following protocols were performed: 1) Cumulative concentration-response curves for AII were constructed 1h before and after addition of different drugs. 2) contractions were induced by a single addition of AII ($10^{-7}M$), drugs to be studied were present for 1 h and the AII-induced contraction was re-examined after the Krebs solution had been replaced by nominally Ca^{+2}-free, EGTA-containing saline (obtained by omitting Ca^{+2} and adding 0.5 m*M* of Na_2EGTA) for 7 min.

AII ($3X10^{-10}$-$3X10^{-7}M$) caused concentration-dependent contractions. The maximal contraction (E_{max}) and EC_{50} values amounted to 4.3±0.5 g and $3.5X10^{-9}M$, respectively. Four different calcium antagonists depressed the concentration-response curves for AII by maximally 63±4% (diltiazem $10^{-6}M$), 61±3% (verapamil $3X10^{-7}M$), 47±3% (nifedipine $3X10^{-7}M$) and 42±3% (manidipine$10^{-7}M$), respectively. The residual responses to angiotensin II could be completely suppressed by the intracellular calcium release inhibitors W-7 (10^{-4}M) (Asano, *et al.*, 1985) and TMB-8 ($10^{-5}M$) (Malagodi & Chiou, 1974). In Ca^{2+}-free, EGTA-containing medium, AII-induced initial transient contractions (ITC) could be blocked by TMB-8 ($10^{-5}M$), W-7 ($10^{-4}M$) and losartan ($10^{-7}M$) but not by ($10^{-5}M$) diltiazem, verapamil, nifedipine or PD123177 ($10^{-6}M$).

We conclude that the AII-induced vasoconstrictor effects in rabbit renal artery are the consequence of both extracellular Ca^{2+} influx and intracellular Ca^{2+} release. Manidipine proved more effective than the other calcium antagonists used. Contractions evoked by intracellular Ca^{2+} release after AII stimulation are mediated by AT_1-receptors.

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167P GENERATION OF ANGIOTENSIN I AND II IN THE ISOLATED RAT HEART

L.M. de Lannoy*, A.H.J. Danser, [1]A.M.B. Bouhuizen, R.G. Schoemaker, P.R. Saxena and [1]M.A.D.H. Schalekamp.
Depts. of Pharmacology and [1]Internal Medicine I, Erasmus University, P.O. Box 1738, 3000 DR, Rotterdam, The Netherlands.

We previously studied the uptake of renin (R), angiotensinogen (Ao) angiotensin I (AngI) and II (AngII) from plasma in isolated rat hearts (De Lannoy *et al.* 1994) by using a modified Langendorff model (De Deckere & Ten Hoor, 1977), which allows the separate collection of coronary (CE) and interstitial (ISF) effluent. It was the aim of the present study to investigate angiotensin production during R and AngI infusion in this heart model. R (porcine or rat, concentrations corresponding with a V_{max} of 12 and 17 nmol AngI/min per ml, respectively, n = 8) was infused for 40 min, and 10-min fractions of CE and ISF were collected for the determination of AngI and AngII. AngI (arterial concentration of 6.1 ± 2.4 pmol/ml) was infused during 15 min in the absence (n=5) or presence (n=5) of the ACE inhibitor captopril (0.1 mM), and 1-min fractions of CE and ISF were collected for the determination of AngI and AngII. AngI and AngII were measured by RIA after SepPak extraction and HPLC separation.

Prior to the infusions R, AngI and AngII were below the detection limit in both CE and ISF; Ao in CE was undetectable and in ISF it was 0.5-5 pmol/ml (n=5). *R infusion:* AngI appeared in ISF in concentrations of 3.0 ± 1.6 and 6.9 ± 5.3 fmol/ml (mean and SD of 40 min period) for porcine and rat renin, respectively (difference not significant). Towards the end of the infusion period the AngI levels in ISF tended to fall, suggesting Ao exhaustion. AngI in CE was close to or below the detection limit of the assay. AngII remained below the detection limit of the assay in both CE and ISF. *AngI infusion:* Without captopril, the AngI levels in CE and ISF were 59.9 ± 10.3 and 16.8 ± 4.6 % of arterial AngI, respectively. AngII appeared in both CE and ISF in concentrations that were 7.5 ± 6.5 and 9.5 ± 3.4 % of arterial AngI. With captopril, AngI in CE was not significantly different from the concentrations found in the absence of captopril (53.3 ± 12.5 % of arterial AngI), whereas AngI in ISF rose to 33.6 ± 8.8 % of arterial AngI ($p < 0.05$ vs. without captopril). AngII was still present in CE and ISF, although in much lower concentrations (3.8 ± 3.8 and 1.9 ± 1.7 % of arterial AngI, respectively; $p < 0.05$ vs. without captopril).

Conclusion: AngI is generated in the heart outside the vascular compartment from Ao that is either synthesized locally or taken up from the circulation. This local cardiac generation of AngI appears to depend on renin taken up from the vascular compartment. Our results with captopril do not support the contention that cardiac AngII generation depends on enzymes other than ACE. The finding that, during AngI infusion, AngII levels in CE and ISF are equal raises the possibility that cardiac ACE is not limited to the coronary endothelium and the endocardium.

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168P COMPONENTS OF THE RENIN-ANGIOTENSIN SYSTEM IN NORMAL AND FAILING HUMAN HEARTS: UPTAKE OR CARDIAC SYNTHESIS OF RENIN?

A.H. Jan Danser, Willem A. Bax, [1]Monique Tavenier, Maarten A.D.H. Schalekamp[2] & Pramod R. Saxena. Depts. of Pharmacology, [1]Cardiology and [2]Internal Medicine I, Erasmus University, Rotterdam, The Netherlands.

The beneficial effects of ACE inhibitors in heart failure appear to be independent, at least partly, from their blood pressure lowering effect (Linz *et al.*, 1992). This suggests that these drugs do not only inhibit the circulating renin-angiotensin system (RAS), but the cardiac RAS as well. Evidence however for the existence of a cardiac RAS operating independently from the circulating RAS is lacking. Under normal circumstances uptake from plasma largely accounts for the presence of RAS components in the heart (Danser *et al.*, 1994), whereas under pathological conditions local synthesis may also occur. To investigate this possibility we measured renin (R), prorenin (PR), angiotensinogen (Ao) and ACE in normal and failing human hearts. In addition, we studied R binding by cardiac membranes. Left ventricular (LV) tissue was obtained from 10 healthy subjects who died of non-cardiac disorders (age 3-54 yr) and 10 subjects with end-stage dilated cardiomyopathy (DCM) who underwent cardiac transplantation (age 30-64 yr). PR (after conversion to R) and R were measured by enzyme-kinetic assay in LV tissue homogenates in the presence of excess sheep Ao and in the presence and absence of the specific renin inhibitor remikiren in order to distinguish between R and pseudorenins. R and PR were also measured by an immunoreactive sandwich assay using 2 monoclonal antibodies. Ao was measured as the maximum amount of angiotensin I (AI) generated in the presence of excess R. ACE was measured using a synthetic substrate. R binding was studied by incubating cardiac membranes prepared from freshly obtained porcine cardiac LV tissue (n=8) with increasing concentrations of semi-purified porcine kidney R (640-6400 pM AI/sec or 250-2500 mU/L). Non-specific binding was determined by incubating boiled membranes with R.

Results (mean and SD, *$p < 0.05$):

	R (fmol AI/g.min)	PR (fmol AI/g.min)	Ao (pmol/g)	ACE (mU/g)
Normal	109 ± 53	15 ± 28	40.4 ± 10.3	7.5 ± 5.0
DCM	741 ± 535*	285 ± 312*	17.6 ± 5.7*	9.5 ± 4.6

Remikiren inhibited cardiac renin by > 90 %, with an IC_{50} (0.3 nM) similar to that for human plasma and kidney renin. Immunoreactive R and PR measurements corresponded with enzyme-kinetic R and PR measurements. Cardiac R levels correlated with plasma R levels, but were too high to be explained by contamination with blood. Ao in cardiac tissue was more than 10 times lower than in plasma and correlated inversely with cardiac R ($r=0.72$, $p < 0.05$). Cardiac membranes bound R with a B_{max} of 202 ± 115 μU per mg protein and a K_d of 81 ± 41 U/L (≈ 1 nM R). We conclude that cardiac R, but not ACE or Ao, is increased in subjects with DCM. Binding to cardiac membranes is a possible mechanism by which circulating R might be taken up by the heart. Ao appears to be a rate-limiting determinant of angiotensin generation in the heart.

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169P CONTRACTILE PROPERTIES OF PORCINE ISOLATED AORTIC VALVE LEAFLETS BEFORE AND AFTER CRYOPRESERVATION AS USED FOR VALVE HOMOGRAFT TRANSPLANTATION

Claes Wassenaar, Willem A. Bax*[1], Robert-Jan van Suylen[2], Egbert Bos & Pramod R. Saxena[1], Depts. of Thoracic Surgery, [1]Pharmacology, and [2]Pathology, Erasmus University Rotterdam, Post Box 1738, 3000 DR Rotterdam, The Netherlands.

Human homograft transplantation has become an established choice of treatment for valvular replacement surgery. Aortic and pulmonary valves are obtained from organ donors by standardized preparation procedures, and stored in nitrogen vapour until a suitable recipient is identified. Heart valves consist of fibroblasts; contractile responses of individual myofibroblasts have only recently been described (Messier et al., 1994). In the present study we investigated the response to 5-HT (1 nM-100 μM), noradrenaline (NA: 1 nM-100 μM), endothelin-1 (ET-1: 0.01 nM-0.3 μM) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ 0.1 nM-10 μM) on porcine intact aortic heart valve leaflets (VL) before and after standard preparation and cryopreservation (CP) procedures (Thijssen et al., 1992). For comparison we included 2x20 mm strips of adjacent porcine aortic wall (AW). Tension was measured isometrically in 15 ml organ baths. Responses were expressed as a percentage of the response to 100 mM K^+. NA, ET-1, and $PGF_{2\alpha}$ all caused contraction of both VL and AW, whereas 5-HT contracted VL, but induced concentration dependent relaxation of the AW. Furthermore, ET-1 was more potent in fresh VL (EC_{50}: 2.3±0.5 nM) than in the AW (EC_{50}: >44±23 nM). After cryopreservation, contraction to 100 mM K^+ was decreased from 1.61±0.14 mN to 0.38± 0.06 mN (n=25) in VL, and from 26.6±2.6 mN to 3.63±0.55 mN (n=26) in AW. When expressed as a percentage of 100 mM K^+-induced contractions, responses remained unchanged in both VL and AW.

Table 1. E_{MAX} (% 100 mM K^+) and EC_{50} of VL and AW to 5-HT, NA, ET-1 and $PGF_{2\alpha}$ (mean ± s.e.m.). N.C., not calculated. [1],exact EC_{50} or E_{MAX} could not be calculated because maximum was not reached. *, significantly different ($p<0.05$) from AW. n=6-7.

	VL before cryopreservation		VL after cryopreservation		AW before cryopreservation		AW after cryopreservation	
	EC_{50}	E_{MAX}	EC_{50}	E_{MAX}	EC_{50}	E_{MAX}	EC_{50}	E_{MAX}
5-HT	137±27 nM	84±10*	81±40 nM	63±12	N.C.[1]	<-30±10[1]	N.C.[1]	-5±9[1]
NA	3.7±0.9 μM	44±10	2.2±1.3 μM	52±15	>5.1±1.4 μM[1]	>93±18[1]	>2.1±0.6 μM[1]	>73±18[1]
ET-1	2.3±0.5 nM*	134±27	2.2±0.9 nM	182±26	>19±4 nM[1]	>85±9[1]	>44±23 nM[1]	>82±24[1]
$PGF_{2\alpha}$[1]	N.C.	>41±15	N.C.	>70±17	N.C.	>78±9	N.C.	>43±8

The present data provide evidence for contractile and relaxant responses of intact isolated heart valve leaflets. Responses of valve leaflets appear to be different from those of the adjacent aortic wall. Although the physiological relevance of valve leaflet contraction and relaxation requires further investigation, their functioning may provide an additional model to verify the consequences of alternative methods of (cryo-) preservation of human heart valves. W.A.B. is supported by the Netherlands Heart Foundation, grant 89.252.

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170P ANGIOTENSIN II-STIMULATED MITOGENESIS OF VASCULAR SMOOTH MUSCLE DERIVED CELLS MAY INVOLVE TYROSINE KINASE-REGULATED PHOSPHOLIPASE D

C. Morton, L.L. Ng[1], N. Wilkie and M.R. Boarder, Dept of Cell Physiology & Pharmacology, and [1]Dept of Medicine, University Road, Leicester, LE1 9HN.

It has previously been reported that vascular smooth muscle cells from the spontaneously hypertensive rat (SHR) gave a larger mitogenic response to angiotensin II (AII) than their normotensive (WKY) controls Bunkenberg et al, 1992). A variety of studies have suggested that in some cases phospholipase D (PLD) may be involved in agonist stimulated mitogenesis (Boarder, 1994; Kondo et al, 1994). Here we investigate a possible role for PLD in AII stimulated mitogenesis of cultured vascular smooth muscle derived cells of SHR origin.

Primary smooth muscle cell cultures were established from aortae of 12 week old SHR and WKY rats. For determination of [^{3}H]thymidine incorporation into nucleic acids cells were maintained serum free for 24h, stimulated with 100 nM AII for 1h, then incubated without agonist for 23h, the last 4h of which included [^{3}H]thymidine, followed by extraction and counting of [^{3}H]nucleic acids. In some experiments butanol at 10-50 mM was included with the agonist to divert the product of PLD away from phosphatidic acid. For the measurement of PLD cells were labelled for 16h with 5 μCi ml^{-1} [^{33}P]orthophosphate and stimulated in the presence of butanol; production of [^{33}P] phosphatidylbutanol was taken as an index of PLD activity. Tyrosine phosphorylation was estimated by Western blotting with a anti-phosphotyrosine antibody.

Exposure to 100 nM AII for 1h stimulated [^{3}H]thymidine incorporation into SHR cells (control, 1.13 ± 0.023; AII, 5.32 ± 0.474) but not into WKY cells (control, 1.07 ± 0.035; AII, 0.9 ± 0.077). Butanol inhibited this response in a dose dependent manner, with a maximal effect at 30 mM (e.g control, 1.01 ±0.05; AII, 6.8 ± 0.21; AII + 30 mM butanol, 3.79 ± 0.30). (Thymidine incorporation data are dpm x 10^{-3} mg^{-1} protein, n = 3, throughout). AII stimulation of PLD activity was greater in SHR cells than WKY cells (significant at $p < 0.001$ by analysis of variance of dose response curve between 3 and 1000 nM). The stimulation of PLD by AII was attenuated by the tyrosine kinase inhibitor genistein (control, 0.15 ± 0.01; AII 1μM, 0.51 ± 0.08; AII + 100 nM genistein, 0.19 ± 0.03; dpm x 10^{-3} mg^{-1} protein) with an EC_{50} of 1.45 ± 0.07 nM. Tyrosine phosphorylation in SHR cells was stimulated by AII.

These results are consistent with the hypothesis that AII stimulation of mitogenesis of vascular smooth muscle involves activation of tyrosine kinases and subsequent stimulation of PLD.

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171P ROLE OF cGMP IN ANGIOTENSIN II-STIMULATED PHOSPHOLIPASE C RESPONSES OF VASCULAR SMOOTH MUSCLE-DERIVED CELLS FROM NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS

R.J. Baines, L.L. Ng[1]& M.R. Boarder, Dept. of Cell Physiology & Pharmacology and [1]Dept. of Medicine & Therapeutics, University of Leicester.

The control of mitogenesis by agonists acting through 7 transmembrane receptors is complex. It may be related to such factors as changes in free intracellular Ca^{2+} ($[Ca^{2+}]_i$) and/or phospholipase D activity. We have previously shown that a spontaneously hypertensive rat (SHR) vascular smooth muscle derived cell line (VSMC) exhibits an enhanced mitogenic response to angiotensin II (AII) compared to a normotensive (WKY) control. This SHR cell line shows an increased accumulation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), larger increases in $[Ca^{2+}]_i$ and increased phospholipase D activity in response to AII compared to the WKY control (Morton et al., 1994). Several reports suggest that one role of cGMP is as a modulator of phospholipase C (PLC) activity (Rapoport & Murad, 1983). Here, we describe studies in permeabilised and intact cells designed to further our understanding of the enhanced PLC activity and consequent larger increases in $[Ca^{2+}]_i$ observed in the SHR derived cells.

Primary smooth muscle cell cultures were established from the thoracic aortae of 12 week old rats. Mass $Ins(1,4,5)P_3$ and cGMP were determined using a competitive binding assay and $[Ca^{2+}]_i$ was monitored fluorometrically with fura2. Permeabilisation was achieved using α-toxin.

The basal accumulation of cGMP was significantly lower in SHR VSMC compared to WKY VSMC (SHR, 2.63 ± 0.48; WKY, 14.80 ± 3.89 fmol/μg protein, n = 3, $P<0.05$). Approximate equilibration of cGMP content in the two cell types using the NO donor S-nitroso-N-acetylpenicillamine (SNAP) resulted in a significant reduction of the accumulation of $Ins(1,4,5)P_3$ in response to AII in the SHR VSMC (AII, 220 ± 12 vs AII+SNAP, 78 ± 9 pmol/mg protein, n = 3, $P<0.05$) but no significant effect in the WKY VSMC. L-NMA and L-NAME had no significant effect on the AII stimulated $Ins(1,4,5)P_3$ response or basal accumulation of cGMP respectively in either cell type. The $Ins(1,4,5)P_3$ response to AII was still present in permeabilised SHR cells (basal 4.8 ± 0.7 vs AII, 25.3 ± 3.8 pmol/mg, n = 6, $P<0.04$) but not in WKY cells. However, the $Ins(1,4,5)P_3$ response to GTPγS was approximately equal in the two cell types. The $Ins(1,4,5)P_3$ response to increased $[Ca^{2+}]_i$ was greater in the SHR derived cells (SHR 27.7 ± 1.4 vs WKY 7.5 ± 6.0 pmol/mg, n = 6, $P<0.05$).

These results confirm that cGMP can exert a modulatory effect on PLC activity. A possibility could be that the reduced basal cGMP levels in SHR cells may contribute to the enhanced PLC activity. However, the studies in permeabilised cells indicate that other factors also contribute to the differences in PLC activity between the two cell types.

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172P INHIBITION OF THE ANTIHYPERTENSIVE EFFECT OF ENALAPRIL AND THE ANGIOTENSIN AT_1 RECEPTOR ANTAGONIST, GR138950, BY Nω-NITRO-L-ARGININE METHYL ESTER IN CONSCIOUS RENAL HYPERTENSIVE RATS

I.K. Anderson, J.S. Polley, A. Hilditch & G.M. Drew, Pharmacology 2, Glaxo Research & Development Ltd., Ware, Hertfordshire, SG12 0DP, U.K.

The effect of Nω-nitro-L-arginine methyl ester (L-NAME) on the anti-hypertensive response to enalapril, hydralazine and the angiotensin AT_1 receptor antagonist, GR138950 (Hilditch *et al.*, 1993) has been examined in conscious renal artery ligated hypertensive (RALH) rats. In addition, the effect of GR138950 on urine and plasma cGMP levels has been determined in conscious RALH rats. Hypertension was induced in male AH/A rats (300-400g) by ligation of the left renal artery and animals were used 6 days after ligation (Travers & Hilditch, 1992). Cannulae were placed in the jugular vein and tail artery for administration of drugs and measurement of blood pressure, respectively. In animals in which cGMP was assayed, blood samples were collected, and blood pressure was measured from a carotid artery cannula. These rats were placed in metabolism cages for urine collection.

Administration of GR138950 (1mg kg^{-1}, i.v.), enalapril (1mg kg^{-1}, i.v.) or hydralazine (1mg kg^{-1}, i.v.) caused significant reductions in mean arterial blood pressure (MAP). The anti-hypertensive effects of GR138950 and enalapril were significantly attenuated in animals pretreated (30 min) with L-NAME (10mg kg^{-1}, then 1mg $kg^{-1}h^{-1}$, i.v.). The fall in MAP caused by hydralazine was not affected by pretreatment with L-NAME. D-NAME (10mg kg^{-1}, then 1mg $kg^{-1}h^{-1}$, i.v.) did not significantly affect the anti-hypertensive effect of GR138950 (Table 1). L-NAME alone caused a transient increase in MAP (25 ± 5mmHg; 5-15min; n=5) after which MAP stabilised at pre-L-NAME levels. GR138950 (1mg kg^{-1}, i.v., n=8) did not significantly change plasma (basal 13.3 ± 1.8, 6h 10.7 ± 1.6pmol ml^{-1}) or urine (basal 491 ± 121, 6-24h 519 ± 85pmol ml^{-1}) cGMP levels up to 24h after administration despite causing a marked fall in MAP (basal 166 ± 7, 6h Δ of -90 ± 6mmHg).

The ability of L-NAME to inhibit the anti-hypertensive action of GR138950 or enalapril suggests that these agents release nitric oxide (NO) and/or enhance the cardiovascular effects of NO as part of their mechanism of action. However, the inability of GR138950 to increase urine and plasma cGMP levels is not consistent with this view. Attenuation of the response to GR138950 or enalapril, but not hydralazine, suggests a selective interaction between L-NAME and inhibitors of the renin-angiotensin system, although the nature of this interaction is unknown.

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MAP (mmHg)	GR138950 1 mg kg^{-1}			Enalapril 1 mg kg^{-1}		Hydralazine 1 mg kg^{-1}	
	Control	L-NAME	D-NAME	Control	L-NAME	Control	L-NAME
Basal (n)	173 ± 4 (9)	180 ± 4 (7)	165 ± 8 (5)	180 ± 4 (7)	165 ± 5 (7)	168 ± 6 (4)	168 ± 6 (4)
Maximum Δ	-96 ± 4	-41 ± 5**	-76 ± 7	-85 ± 3	-22 ± 5**	-63 ± 10	-59 ± 13

Table 1: The effect of L-NAME on the maximum change (Δ) in MAP (mean ± s.e.mean) following intravenous administration of test compound in conscious RALH rats. ** $P<0.001$ compared (Student's unpaired *t* test) with the relevant control.

173P CHARACTERISATION OF THE FUNCTIONAL PROPERTIES AND DISTRIBUTION OF THE MOUSE TYPE 1 *MYO*-INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR OVER-EXPRESSED IN MOUSE L-FIBROBLASTS

R.A. Wilcox; J.J. Mackrill; S. R. Nahorski and R.A.J. Challiss. Department of Cell Physiology and Pharmacology, University of Leicester, Leicester.

We have characterized the functional consequences of over-expression of the mouse type I *myo*-inositol 1,4,5-trisphosphate receptor [IP_3R] in a stably transfected mouse L fibroblast line ("L15 cells") (Miyawaki *et al.*, 1990).

Using a polyclonal antibody (CT-1) raised against a synthetic peptide identical to a unique region of the C-terminal of the type 1 IP_3R (Wojcikiewicz *et al.*, 1994), Western blot analysis revealed ~10-fold higher expression in L15 cells compared to control vector transfected L-fibroblasts ("Lvec cells"). The overexpression of IP_3R was found to be maintained over many passages, when cells were grown in Hepes-buffered Dulbecco's Modified Eagles media supplemented with 10% (v/v) foetal calf serum and fed daily.

These observations were confirmed by [^{3}H]-IP_3 binding studies using L15 and Lvec membrane preparations as described (Wilcox *et al.*, 1993). L15 cells (10.2 ± 1.9 nM; B_{max} 3.56 ± 0.28 pmol/mg of membrane protein; n = 4) exhibited an identical affinity but >8-fold more binding sites than the Lvec cells (10.7 ± 1.4 nM; B_{max} 0.44 ± 0.05 pmol/mg of membrane protein; n = 4).

In order to characterize the sub-cellular location of the over-expressed IP_3R, L15 and Lvec membranes were sub-fractionated on a sucrose gradient (Nigam & Blobel, 1989). These fractions were then subjected to Western blotting using both CT-1 (anti-type 1-IP_3R) and a polyclonal antibody raised against the sarcoplasmic / endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump. The SERCA pump is known be structurally and functionally associated with the intracellular Ca^{2+} stores. Using this approach we were able to demonstrate that the overexpressed IP_3R in L15 cells was predominantly co-localized to the same sub-fraction as the SERCA pump, indicating that the over-expressed IP_3R was appropriately located to access intracellular calcium stores.

The functional consequences of increased IP_3R expression in L15 (compared to Lvec) cells was examined in saponin-permeabilized cells exposed to increasing concentrations of IP_3 using a $^{45}Ca^{2+}$ uptake and release assay (Wilcox *et al.*, 1993). When permeabilized in suspension and centrifuge-washed, L15 cells (EC_{50} = 45 nM) were ~ 4-fold more sensitive to IP_3 than Lvec cells (EC_{50} = 171 nM; $p < 0.05$) and they also exhibited a small but significant increase in the apparent size of their IP_3-sensitive Ca^{2+} pool. Ionomycin (5 μM) was used to define the total mobilizable Ca^{2+} pool (100%) which was identical in both cell types, but the IP_3-sensitive Ca^{2+} pool for L15 and Lvec cells was 74.1 ± 0.2 % and 68.1 ± 0.4 %, respectively. In contrast when adherent saponin-permeabilized L15 (EC_{50} = 165 nM) and Lvec (EC_{50} = 311 nM) cells were used the increased sensitivity of the L15 cells was retained, but the extent of the IP_3-sensitive-Ca^{2+} pool was identical (L15 67.0 ± 3.8 % compared to Lvec 67.5 ± 1.1% IP_3 sensitive). This suggests a degree of store fragmentation was produced in the suspension-permeabilized cells. Thus, in L15 cells the over-expressed IP_3Rs are appropriately localized to the IP_3-sensitive Ca^{2+} pool(s) and are able to functionally increase the sensitivity of the cell to IP_3-induced Ca^{2+} mobilization.

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174P DISTRIBUTION OF DOPAMINE D_{1A} RECEPTOR IN THE KIDNEY

C.J. Vaughan, M.B. Murphy & D.P.O'Connell. Department of Pharmacology, University College Cork, Ireland.

DA receptors have been classically divided into D_1 and D_2 in the central nervous system (Kebabian *et al.* 1979). Cardiovascular and renal DA receptors have been classified more recently into DA_1 and DA_2 subtypes on the basis of their synaptic localisation (Goldberg *et al.* 1986). Recently, molecular biological techniques have allowed further differentiation of DA receptor subtypes (Zhou *et al.* 1990). Intra-renal dopamine mediates a variety of functions including vasodilation, natriuresis and diuresis. Abnormalities of the renal dopaminergic system are implicated in the pathophysiology of some forms of hypertension.

A combination of immunohistochemical and non-radioactive *in situ* hybridisation studies were conducted on rat kidney sections. The antisera were raised against a synthetic peptide sequence derived from the predicted D_{1A} rat dopamine receptor amino acid sequence, corresponding to an epitope located on the third extracellular loop of the receptor [GSEETQPFC]. The specificity of the antisera was verified by attenuation of the ELISA response following preincubation of the antisera with its immunisation peptide and by the ability of the anti-peptide antisera to recognise the native receptor which had been expressed in a murine fibroblast (LTK$^-$) cell-line stably transfected with a full-length rat D_{1A} cDNA. Electron microscopic immunocytochemistry was performed using a standard protein A gold technique.

In situ hybridisation was conducted using 24-mer HPLC purified antisense and sense oligonucleotides complementary to D_{1A} mRNA sequence coding for the N-terminal amino acid residues (14-21). The oligonucleotides were labelled by tailing the 3' end with digoxigenin-11-dUTP using terminal transferase. Immuno-reactive hybridised digoxigenin-labelled probe was detected with an alkaline phosphatase-NBT/BCIP technique. In addition to sense control, other slides had no oligonucleotide added or no anti-digoxigenin secondary antibody added, both of which serve to control for non-specific hybridisation and background. Receptor protein was localised to the renal vasculature and parenchyma. Immunostaining was detected in the juxtaglomerular apparatus in renin containing granules in the smooth muscle layer. In the proximal tubules, staining was present both on brush border and basolateral membranes. Staining was also noted in collecting ducts and throughout the renal vasculature. D_{1A} mRNA colocalised to the renal vasculature, proximal tubular cells, juxtaglomerular apparatus, and collecting ducts.

Table 1. Renal D_{1A} receptor

	Protein	mRNA
Glomerulus	-	-
Proximal tubule	++	++
Distal tubule	+	+
JGA	++	++
Arterial	++	++

The previously described DA_1 receptor is homologous to the recently described D_{1A} receptor and is localised in the rat kidney atp sites known to be fundamental in sodium and water homeostasis and blood pressure control. Such knowledge may, in the future, lead to a greater understanding of pathophysiological mechanisms underlying hypertension and the generation of more specific receptor agonists and antagonists.

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175P INHIBITION OF L-DOPA UPTAKE INTO OK CELLS IN CULTURE BY CYANINE-RELATED COMPOUNDS

P. Soares-da-Silva & M.A. Vieira-Coelho, Institute of Pharmacology & Therapeutics, Faculty of Medicine, 4200 Porto, Portugal.

The rate of formation of dopamine, an endogenous natriuretic agent, in epithelial cells of renal tubules largely depends on the rate of uptake of circulating or filtered L-3,4-dihydroxyphenylalanine (L-DOPA). In isolated rat renal tubules, L-DOPA uptake has been shown to occur through non-saturable and saturable mechanisms (Soares-da-Silva et al., 1994), the latter one being sensitive to inhibition by the organic cation inhibitor cyanine 863 (1-ethyl-2-[(1,4-dimethyl-2-phenyl-6-pyrimidinylidene)methyl]-quinolinium) and two other cyanine derivatives, 1,1'-diethyl-2,4'-cyanine and 1,1'-diethyl-2,2'-cyanine (Soares-da-Silva & Pinto-do-Ó, 1994). Opossum kidney (OK) cells in culture also take up L-DOPA through a saturable transporter and the utilization of these cells in culture offers the possibility to limit the access of L-DOPA to the cell either from the basal or the apical cell border (Vieira-Coelho & Soares-da-Silva, this meeting). The present work reports on the effect of a series of cyanine derivatives on the uptake of L-DOPA in OK cells in culture when applied from the apical cell border. OK cells (ATCC 1840-CRL) were grown as a monolayer for 6-8 days at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524). In uptake studies, OK cells were preincubated (30 min) with Hanks medium with added pargyline (100 μM), tolcapone (1 μM) and benserazide (50 μM) in order to inhibit the enzymes monoamine oxidase, catechol-O-methyltransferase and aromatic L-amino acid decarboxylase, respectively, and increasing concentrations (0.1 to 500 nM) of compounds to be tested. Determination of initial rate of uptake was performed in OK cells incubated with L-DOPA (250 nM) during 1, 3, 6, 12, 30, 60 and 120 min. The initial rate of transfer of L-DOPA amounted to 78±8 pmol mg protein^{-1} during a 6 min incubation period; the effect of cyanine derivatives was tested on these experimental conditions. The maximal inhibitory effect of L-DOPA uptake was obtained with 100 nM cyanine 863 (74% decrease in L-DOPA accumulation). Listed below are the IC_{25}'s or IC_{50}'s (n=5) with the corresponding 95% confidence intervals for all compounds tested.

Cyanine 863	IC_{50}=6 (3, 9) nM
1,1'-diethyl-2,4'-cyanine iodide	IC_{50}=7 (2, 11) nM
1,1'-diethyl-2,2'-cyanine iodide	IC_{50}=8 (3, 12) nM
1,1'-diethyl-4,4'-cyanine iodide	IC_{25}=20 (8, 32) nM
1,1'-diethyl-2,2'-carbocyanine iodide	IC_{25}=35 (23, 70) nM
1,1'-diethyl-4,4'-carbocyanine iodide	IC_{25}=38 (22, 86) nM
3,3'-diethyloxacarbocyanine iodide	IC_{25}=1 (0.3, 3) nM
3,3'-diethylthiacarbocyanine iodide	IC_{50}=22 (14, 33) nM
3,3'-dipropyloxacarbocyanine iodide	IC_{50}=21 (13, 39) nM
3,3'-dipropylthiacarbocyanine iodide	IC_{50}=53 (38, 67) nM
Gallocyanine	IC_{50}=28 (15, 34) nM
Astrazon orange G	IC_{50}=10 (5, 16) nM
Astrazal blue	IC_{50}=14 (7, 18) nM
Basic blue 41	IC_{50}=18 (9, 21) nM
(compounds were obtained from Sigma and Aldrich Chemical Co.)	

It is concluded that the cyanine related compounds studied, particularly cyanine 863, 1,1'-diethyl-2,4'-cyanine and 1,1'-diethyl-2,2'-cyanine, are potent inhibitors of L-DOPA uptake in OK cells. These compounds constitute a new class of drugs which might prove useful in uptake studies of neutral amino acids and related substances.

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176P OVALBUMEN-INDUCED AIRWAY HYPERREACTIVITY IN ANAESTHETISED GUINEA-PIGS: DEPENDENCE ON THE IV SPASMOGEN

Alexia Johnson & K. J. Broadley, Division of Pharmacology, Welsh School of Pharmacy, University of Wales, Cardiff CF1 3XF.

Airway hyperreactivity has been demonstrated in anaesthetized ovalbumin sensitized guinea-pigs approximately 60 min after aerosol challenge with ovalbumin (OA). Fewer studies have examined the effects in anaesthetized guinea-pigs 24h after the OA inhalation, hyperreactivity having been demonstrated as increases in the magnitude of bronchoconstriction to iv histamine and acetylcholine,(Coyle *et al.*, 1988; Havill *et al.*,1990). We have examined a wider range of spasmogens in anaesthetized guinea-pigs in an attempt to demonstrate hyperreactivity as a leftward shift of the dose-response curve (DRC).

Guinea-pigs received OA (10μg) and $Al(OH)_3$ (100mg) in 1ml normal saline, ip, approx. 14 days before challenge with OA aerosol (10mgml^{-1} for 2min). Mepyramine (10mgkg^{-1} ip), protected from fatal anaphylaxis. 24h later, a time we have shown conscious animals to be hyperreactive to bronchoconstriction to the thromboxane-mimetic U46619 (Lewis *et al.*,1993), they were anaesthetized with pentobarbitone (90mgkg^{-1} ip). The trachea, a jugular vein and carotid artery were cannulated. Under artificial respiration (58 strokes min^{-1},10mlkg^{-1}), pulmonary inflation pressure (PIP) was recorded. Spasmogens were given iv, PIP being measured as a percentage of maximum, determined by complete occlusion of the tracheal cannula. Mean ED_{50} values, dose for 50% of maximum, (N=4-7) were determined. Controls received a saline aerosol challenge. 5-Hydroxytryptamine (5-HT),methacholine (Mch), U46619, histamine (Hist) and substance P (SP), produced dose-related bronchoconstriction. OA challenged guinea-pigs displayed hyperreactivity to 5-HT. The DRC was significantly ($P<0.05$) shifted to the left (ED_{50},0.78(0.43-1.43)μgkg^{-1}) compared with controls (1.9(0.03-3.2)μgkg^{-1}). Similarly, the ED_{50} for U46619 after OA challenge (24(19-30)ngkg^{-1}) was significantly ($P<0.05$) less than for controls (56(37-137)ngkg^{-1}).

In contrast, DRCs after OA and saline challenge were virtually superimposable when MCh,Hist and SP were spasmogens. ED_{50} after OA challenge (1.9(0.77-4.83), 2.2(1.7-3.1) and 2.6(1.3-5.0)μgkg^{-1}, respectively) were not significantly different from their corresponding controls (3.6(2.3-5.6), 2.6(1.7-3.8) and 1.8(0.25-12.2)μgkg^{-1}).

Thus, we have demonstrated airway hyperreactivity 24h after OA challenge as parallel leftwards shifts of 5-HT and U46619 DRCs. This does not appear to be a non-specific effect since no change was seen with Mch, Hist or SP. The reason for this selectivity remains to be determined.

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177P EFFECT OF BUDESONIDE ON ANTIGEN-INDUCED AIRWAYS HYPERRESPONSIVENESS IN NEONATALLY IMMUNIZED RABBITS

Ahmed Z. El-Hashim, Caroline M. Herd & Clive P. Page, Dept. of Pharmacology, King's College, Manresa Road, London SW3 6LX.

The introduction of inhaled corticosteroids is regarded as a major advance in the management of asthma. This is due to their potent anti-inflammatory actions (Weiss *et al.*, 1984). We assessed the effect of budesonide, one of the most widely used corticosteroids, on antigen -induced airways hyperresponsiveness (AHR) in neonatally immunized rabbits.

Litter-matched New Zealand White rabbits were immunized from birth until 3 months of age with *Alternaria tenuis* in $Al(OH)_3$ gel (*i.p.*). On day 1, rabbits were premedicated with diazepam (5 mg kg^{-1}, *i.p.*) followed 15 minutes later by hypnorm (0.4 mg kg^{-1} *i.m.*). Airways responsiveness to aerosolised histamine was determined as a measure of lung function. Cumulative dose-response curves were performed to establish the provocative concentration of histamine that produces a 50% (PC_{50}) increase in airways resistance (R_L) and 35% (PC_{35}) decrease in dynamic compliance (C_{dyn}). Bronchoalveolar lavage (BAL) was then performed by administration of 5 ml sterile saline directly into the airways. Rabbits were administered either 50µg of budesonide in 50% ethanol (n=10) or vehicle (n=10) by aerosol. On day 2, rabbits were challenged with *Alternaria tenuis* aerosol (20,000 PNU ml^{-1} in saline for 20 min). On day 3, airways responsiveness to histamine and BAL were performed as on day 1 (Herd *et al.*, 1994).

The early onset response was evidenced by an increase in % R_L at 15 and 30 min following antigen challenge (22.2±6.6 and 33.6±20.0 respectively) and a decrease in % C_{dyn} (-32.0±5.6 and -20.9±9.1, respectively) in the vehicle-treated group. Budesonide inhibited the fall in C_{dyn} at both 15 and 30 mins (P=0.0004 and 0.01, respectively). Antigen-induced changes in R_L were unaffected by budesonide pre-treatment.

Antigen challenge of animals pre-treated with vehicle resulted in AHR to inhaled histamine (table 1). In animals pre-treated with budesonide, antigen-induced AHR was inhibited. Total cell numbers were elevated in BAL fluid 24 h following antigen challenge in the vehicle-treated group (P=0.02). This cell recruitment was not observed in rabbits pretreated with budesonide. Eosinophil and neutrophil numbers were significantly inhibited in the budesonide treated groups (P=0.001 and 0.0003 respectively, table 2).

Table 1.

TREATMENT	PC 50 (mg ml^{-1})		PC35 (mg ml^{-1})	
	Pre	Post	Pre	Post
VEHICLE	18.5±1.2	4.1±1.2***	10.1±4.2	2.6±1.3**
BUDESONIDE	17.3±1.3	17.7±1.2	7.3±1.1	9.5±1.2

**P<0.01 Vs Pre (paired t-test) *** P<0.001 Vs Pre (paired t-test)

Table 2.

		x 10^4 cell ml^{-1}			
		TOTAL CELLS	MONONUCLEAR	NEUTROPHILS	EOSINOPHILS
VEHICLE n=10	PRE ALLERGEN	17.7 (7.3-32.0)	17.3 (16.2-17.2)	0.38 (0-1.4)	0.008 (0-0.08)
	POST ALLERGEN	33.2 ¥ (11.5-61.0)	27.0 (22.2-30.5)	4.3 ¥ (1.7-7.6)	1.2 ¥ (0.5-3.6)
BUDESONIDE n=10	PRE ALLERGEN	22.8 (10.5-41.5)	21.9 (18.5-22.8)	0.87 (0.1-4.3)	0.01 (0-0.1)
	POST ALLERGEN	17.1 * (3.2-25.0)	15.8 (11.9-16.9)	1.2 * (0.17-7.6)	0.08 * ¥ (0-0.3)

* indicates p<0.05 Vs Post Veh (unpaired t-test)
¥ indicates p <0.05 Vs pre antigen (paired t-test)

In rabbits immunized from birth, budesonide inhibited the C_{dyn} component of the acute bronchoconstriction, AHR and cellular infiltration induced by antigen.

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178P MECHANISMS INVOLVED IN THE MULTIPHASIC RESPONSE TO HYPOXIA IN RAT INTRAPULMONARY ARTERY RINGS

V.S.N.M.R. Karamsetty, K.A. Kane & R.M. Wadsworth, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, Scotland.

Evidence for the role of NO, prostacyclin and K channels in hypoxic pulmonary vasoconstriction is contentious. The purpose of the present study was to assess the importance of EDRF/NO, prostaglandins and potassium channels in the contractile and relaxant responses to hypoxia in rat IPA rings. Isometric tension was recorded from IPA rings (Diameter ~1.2mm, Length 2.5mm) suspended on L-shaped hooks in organ baths filled with Krebs solution maintained at 37°C and bubbled with 20% O_2+5% CO_2 in N_2. Phenylephrine (PE $6x10^{-7}$M) was used to induce tone and hypoxia was introduced by gassing with 95%N_2+5%CO_2 for 30 min which yielded a PO_2 value of 7±1 mm Hg in the baths. This hypoxic challenge was carried out in control and endothelium denuded preparations and in the presence of LNOARG(10µM, $3x10^{-4}$M), DNOARG($3x10^{-4}$M), Flurbiprofen(1µM) or Glibenclamide(10µM) added to the baths 30 min prior to the phenylephrine.

Acute hypoxia generated a triphasic response which includes a rapidly developing phasic contraction followed by a relaxation and a slowly developing tonic contraction. LNOARG ($3x10^{-4}$M) reduced carbachol induced relaxation from 85±6% to 47±4%(P<0.05). Table 1 summarises the contractile and relaxant response to hypoxia in control and pretreated IPA rings measured relative to the PE induced tone. Denudation of the endothelium abolished both phases of the contractile response to hypoxia and potentiated the relaxation phase. Since LNOARG ($3x10^{-4}$M) potentiated the response to PE, in these experiments the concentration of PE was reduced so that the initial tone was matched with the controls. Under these conditions, LNOARG markedly reduced the phasic contraction, abolished the tonic contraction and potentiated the relaxation phase. DNOARG ($3x10^{-4}$M) had no effect nor did the lower dose of LNOARG (10µM). Neither flurbiprofen (1µM), the cyclooxygenase inhibitor nor glibenclamide (10µM), a K_{ATP} channel blocker modified the contractile or relaxant responses to hypoxia. It can be concluded from this data that the response to acute hypoxia in isolated rat pulmonary arteries is not dependent upon prostaglandins nor K_{ATP} channel opening. Both phases of the contraction are endothelium dependent and phasic contraction is partially mediated by the hypoxic inhibition of EDRF/NO synthase. However, the relaxation phase is not dependent on either endothelium or EDRF/NO.

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Table 1. The hypoxic response was measured relative to the PE induced tone and expressed as g/cm^2. Mean values ± s.e.mean are shown. *P<0.05 compared with the respective control value.

Treatment	*n*	*Hypoxic Contraction*		*Hypoxic Relaxation*
		Phasic	Tonic	
Control	23	36±4	42±4	5±3
Endothelium (-)	6	5±1*	1±2*	-12±3*
LNOARG$3x10^{-4}$M	8	20±3*	-8±6*	-23±4*
DNOARG$3x10^{-4}$M	4	59±18	67±21	3±10
LNOARG10 µM	4	55±13	60±17	-3±13
Flurbiprofen 1 µM	6	37±14	52±17	3±9
Control	6	32±7	39±7	8±3
Glibenclamide10µM	8	45±7	56±10	14±5

179P BACTERIAL LIPOPOLYSACCHARIDES AND TUMOUR NECROSIS FACTOR$_\alpha$ INDUCE MARKED MORPHOLOGICAL CHANGES ON RAT EPITHELIAL TRACHEAL CELLS IN CULTURE

A. Reimann, A. Freitag, I. Wessler* & K. Racké, *Department of Pharmacology, University Mainz; Department of Pharmacology, University-Hospital, J.W. Goethe-University, Theodor-Stern-Kai 7, D-60590 Frankfurt, F.R.G.

Marked alterations of the airway epithelium are frequently observed in inflammatory airway diseases and may play an important role in the pathogenesis of the broncho-obstructive symptoms (see Webber & Corfiled, 1993). There is evidence that bacterial toxins and cytokine may alter the airway epithelial function. Thus, we recently reported that bacterial lipopolysaccharides (LPS) and tumor necrosis factor$_\alpha$ (TNF$_\alpha$) can induce nitric oxide (NO) synthase in rat tracheal epithelial cells (Hey *et al.*, 1994). The aim of the present experiments was to study the effects of LPS and TNF$_\alpha$ on the morphology of isolated rat tracheal epithelial cells in culture.

Tracheae of newborn rats, cut into small pieces, were explanted onto 60 mm culture dishes (Lechner et al. 1985) and cultured in low-calcium (60-80 μmol/l) RPMI-1640 medium, containing 16 % fetal calf serum, supplemented with epidermal growth factor and few other growth hormones, conditions allowing the selective proliferation of epithelial cells (Emura *et al.*, 1990). In the present study confluent monolayers of 1st or 2nd passage were used.

When LPS (10 μg/ml) and TNF$_\alpha$ (500 U/ml) were added to the culture medium marked morphological changes developed. Within 24-48 hrs the polygonal, honeycomb-like structure of the culture was loosened, the cell density, determined by counting the cells in 4 defined areas in each well (initial sum 274 $\pm$ 5 cells, n=8), was reduced by about 20 %, and an increasing number of cells showed a longish orientation. After 3-4 days many cells with dendrite-like outgrowths were observed. All changes were reversible after 3 days culture in LPS- and TNF$_\alpha$-free medium.

Dexamethasone (10 μmol/l, present with LPS and TNF$_\alpha$) largely attenuated these changes. Since treatment with LPS and TNF$_\alpha$ has been shown to induce NO synthase in these cells in a dexamethasone sensitive manner (Hey *et al.*, 1994), the effect of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) was also tested. L-NMMA (100 μmol/l, present with LPS and TNF$_\alpha$) tended to augment rather than to inhibit the reduction in cell densitiy and the appearance of "dendritic" outgrowths.

In conclusion, exposure of airway epithelial cells to LPS and TNF$_\alpha$ caused marked morphological changes which may reflect fundamental shifts in the cell function, alterations sensitive to glucocorticoids. Although these changes are paralleled by an induction of NO synthase, an enhanced formation of NO appears not to be responsible for the observed changes. It is hypothesized that the appearance of the dendritic morphology could reflect the induction of antigen presenting and/or immunostimulatory properties of the airway epithelial cells.

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180P HYPORESPONSIVENESS TO NORADRENALINE INDUCED BY GROUP B *STREPTOCOCCUS* IS MEDIATED BY NITRIC OXIDE IN ISOLATED PULMONARY ARTERIES OF NEONATAL PIGLETS

F. Pérez-Vizcaíno, E. Villamor[1], T. Ruiz[1], J.C. Leza, M. Moro[1] & J. Tamargo. Depts of Pharmacology and [1]Pediatrics. School of Medicine. Universidad Complutense. 28040 Madrid. Spain.

E. coli, a Gram-negative bacterium, and group B *Streptococcus* (GBS), a Gram-positive bacterium, are the most common causal agents of neonatal sepsis. Enhanced production of nitric oxide (NO) following the NO synthase (NOS) induction is responsible for the loss of systemic and pulmonary vascular responsiveness that occurs in Gram-negative sepsis in the adult (Thiemermann, 1994).

Isolated pulmonary artery rings (3 mm in length, 1 mm internal diameter) or lung parenchymal segments of neonatal piglets were incubated in oxygenated Krebs solution (37° C) containing endotoxin (*E. coli* lipopolysaccharide, 1 μg ml^{-1}, LPS) or heat inactivated GBS for 1, 5 or 20 h (3 x 10^7 colonies forming units ml^{-1}). The contractile responses to noradrenaline (NA) were recorded under isometric conditions (resting tension 0.5 g). NOS activity of the constitutive (cNOS) and inducible (iNOS) isoforms was determined by the conversion of radiolabelled L-arginine to citrulline as described by Salter et al. (1991).

Short-term ($\leq$ 5 h) incubation with LPS or GBS did not modify the vascular responsiveness to NA (10^{-8}M - 10^{-4}M) in isolated intrapulmonary arteries. However, long-term incubation (20 h) with LPS or GBS produced a significant reduction in the maximal contractile responses to NA (E_{max} = 923 $\pm$ 80 mg, 492 $\pm$ 47 mg and 613 $\pm$ 52 mg in the control, LPS and GBS groups, respectively, $P < 0.01$). Endothelium removal or the cyclooxigenase inhibitor meclofenamate (10^{-5}M) did not affect the GBS- and LPS-induced hyporesponsiveness to NA. The presence of the NO precursor L-arginine (10^{-5}M), 30 min prior to the contractility challenge increased the LPS- and GBS-induced pulmonary vascular hyporesponsiveness to NA. In contrast, the addition, prior to the challenge with NA, of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4}M) or the coincubation with dexamethasone (3 x 10^{-6}M), an inhibitor of NOS induction, or with the protein synthesis inhibitor cycloheximide (10^{-5}M) completely restored the reactivity to NA in LPS- and GBS-treated pulmonary arteries. Incubation for 20 h of pulmonary parenchymal segments with LPS or GBS produced a significant increase in iNOS (but not cNOS) activity which was abolished by coincubation with dexamethasone (3 x 10^{-6}M).

These results demonstrated that prolonged incubation with GBS and LPS of pulmonary arteries of neonatal piglets causes an induction of iNOS activity which results in vascular hyporesponsiveness to NA.

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181P PROLONGED PROSTACYCLIN PRODUCTION BY BOVINE AORTIC ENDOTHELIAL CELLS IS INCREASED EQUALLY BY BRIEF AND PROLONGED PERIODS OF STRETCH

Carolyn L. Hynes, Timothy D. Warner, Elizabeth G. Wood and John R. Vane. *The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.*

Mechanical stretching for periods of up to 360 min increases the release of prostacyclin (PGI_2) from bovine aortic endothelial cells (BAEC) in culture (Macarthur *et al.*, 1994). Here we have extended these observations to investigate the effects of various degrees and frequencies of stretch on the release of PGI_2 from BAEC at 360 minutes.

BAEC (from primary cultures to passage 5) were seeded onto 6-well culture plates with flexible bottoms (Flex I, Flexcell USA,) and grown to confluence. The medium was removed from the cells and replaced with warmed (37 °C) HEPES-buffered Locke's solution (pH 7.4) containing 5% BSA. The plates were then placed on a Flexercell strain unit, which consists of a base plate of vacuum ports over which are centred the Flex I plates, contained within an incubator (37 °C) . The plates were stretched (1 Hz, 5 or 10% elongation) for 1, 5, 10, 20 or 360 min or left unstretched as control (basal). After 360 min samples were collected from each well and the content of 6-keto-prostaglandin F1α (6-keto-PGF1α, the stable hydrolysis product of PGI_2) determined by specific radioimmunoassay. In some experiments $8x10^{-7}$ M actinomycin D (an inhibitor of mRNA transcription) was added to some of the culture wells.

The amount of 6-keto-PGF1α accumulated over 360 min under basal conditions was 42±9.3 ng/ml (n=44). Stretch for 20 min at 5% or 10% elongation increased the accumulation of 6-keto-PGF1α at 360 min by more than 50% (Table 1). Similarly, all durations of stretch (1Hz, 10% elongation) significantly increased the accumulation of 6-keto-PGF1α. In the presence of actinomycin D both the basal and stretched-enhanced accumulations of 6-keto-PGF1α were greatly depressed.

Table 1. *Accumulation of 6-keto-PGF1α from BAEC over 360 min. *, p 0.05 from basal by one sample t-test.*

Duration of stretch (min)	% elongation	% of basal release (n)	
		control	+actinomycin D
basal	0	100 (44)	34±7* (24)
1	10	144±16* (18)	8±2* (6)
5	10	128±18* (14)	6±1* (6)
10	10	130±17* (8)	8±1* (6)
20	5	174±27* (9)	n.d.
20	10	151±20* (30)	8±2* (6)
360	10	176±33* (9)	49±11* (15)

Thus, stretching of endothelial cells elevates the release of PGI_2 at 360 min. This increase in accumulation is dependent upon mRNA transcription and is due to early effects for it is produced by stretching for periods as short as 1 min.

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182P THE EFFECTS OF TOXIC SHOCK SYNDROME TOXIN ADMINISTRATION TO CONSCIOUS RABBITS USING TELEMETRY EQUIPMENT

P.S.Lidbury, C.E.Bryant & J.R.Vane, The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.

Toxic Shock Syndrome Toxin (TSST), an endotoxin produced by *Staphylococcus aureus*, causes multi-organ dysfunction *in vivo* (Arko, 1984; Parsonnet, 1987) and induction of nitric oxide synthase (NOS) *in vitro* (Zembowicz, 1992). Here, we investigated the effect of subcutaneous administration of TSST (30 μg/kg per day, for 4 days) to conscious male rabbits (2 kg) implanted with telemetry equipment continuously to monitor mean arterial blood pressure (MAP), heart rate (HR), temperature and activity. In addition to recording body weight, blood samples were withdrawn daily for haematological and serum biochemical analysis. NOS activity was measured in lung and spleen homogenates by the conversion of [3H]-arginine to [3H]-citrulline (Mitchell *et al.*, 1991).

Basal telemetry measurements recorded a MAP of 74 ± 5 mmHg, a HR of 250 ± 16 beats/min and a body temperature of 38.84 ± 0.10 °C. Although the MAP remained unaffected by TSST (70 ± 4 mmHg after 4 days), the toxin caused a marked rise in HR (296 ± 13 beats/min at 24 h) and temperature (40.34 ± 0.13 °C at 24 h) that was sustained for 36 h. Further injections of TSST produced only transient rises in these parameters. The blood samples revealed a gradual decrease in packed cell volume from 0.41 ± 0.01 to 0.27 ± 0.01 after just 4 days. In addition, TSST caused hypoalbuminaemia and rises in aspartate aminotransferase and glutamate dehydrogenase, indicative of muscle and liver damage. The activity and dietary intake were all dramatically reduced. Consequently, the body weight of the rabbits decreased by 5.6 % over the 4 day experimental period, while vehicle-treated animals gained 1.7 %.

Upon termination, in rabbits that had been given TSST, the weight of the spleen and lung had increased by 246 % and 30 % in comparison to control animals, while the heart, liver and kidney remained unchanged. However, TSST did not induce the calcium-independent isoform of nitric oxide synthase (iNOS) in either spleen or lung tissue.

In this study, TSST administration to conscious rabbits caused tachycardia, fever and anaemia without significant hypotension or induction of NOS. It is possible that the hypotension seen in other species in acute models of shock occurs as only the final event in the development of Toxin Shock Syndrome.

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183P INTERDEPENDENCE OF NORADRENALINE (NE)-INDUCED CONTRACTILE RESPONSES OF ISOLATED PERFUSED RAT KIDNEY (IPRK) ON NITRIC OXIDE (NO) AND CYCLO-OXYGENASE PRODUCTS OF ARACHIDONIC ACID

*H.Luz-Rodrigues, **B.Silva-Lima, *J.M.Gião T-Rico. *Institute of Pharmacology, Faculty of Medicine, and **Faculty of Pharmacy. University of Lisbon. Portugal.

NE is known to release both endothelium-dependent relaxing and contracting factors from rat arteries (Nigro et al., 1990). The contracting factors described are cyclo-oxygenase-dependent while the relaxing factors are the NO and cyclo-oxygenase-dependent metabolites. The balance between these mediators is pivotal in the control of renal vasoactivity. We have examined the relative roles and the interrelationship of cyclo-oxygenase products of arachidonic acid (AA) and NO in the contractility of IPRK.

Right kidney from male Wistar rats was isolated and perfused with a Tyrode's solution. The influence on the NE dose-effect curve of blocking the NO formation with N^{ω}-nitro-L-arginine (L-NOARG) 10 μM or endothelium removal with CHAPS, and of blocking cyclo-oxygenase products of AA production with indomethacin (IND) 5,6 μM was studied. The dose-response curve parameters were calculated by nonlinear regression analysis.

IND attenuated NE responses (E_{max}: 239±5 vs 217±7 mmHg, P<0.002; n=10) and the inhibitory effect of IND persisted following endothelial removal (E_{max}: 252±9 vs 234±7 mmHg, P<0.02; n=6), suggesting the presence of a cyclo-oxygenase-derived vasoconstrictor endothelium-independent released by NE. L-NOARG potentiated NE-induced contraction (E_{max}: 217±18 vs 239±18 mmHg, P<0,05; n=6) suggesting that NO was released by contraction with NE. The inclusion of L-NOARG in the perfusions with IND attenuated the effect of IND on the NE-induced contraction (E_{max}: 215±11 vs 224±12 mmHg, P<0,01; n=6). The inclusion of IND in the perfusion containing L-NOARG suppressed the potentiating effects of L-NOARG on the NE vasoconstriction (E_{max}: 239±18 vs 180±15 mmHg, P<0,005; n=6).

The existence of a relationship between NO and a cyclo-oxygenase-derived endothelium independent vasoconstrictor released by NE was shown. L-NOARG potentiated the constriction induced by NE more markedly in the absence than in presence of IND. The depressive effect of IND on the NE response was more marked in the presence of L-NOARG. The results indicate that in IPRK the reduction of NO facilitates the formation of cyclo-oxygenase-derived vasoconstrictor (Kanner *et al.*, 1992); the inhibition of cyclo-oxygenase, probably, enhances NO production through inhibition of superoxide production (Semb & Vaage, 1991).

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Nigro, D., Fortes, Z.B., Scivoletto, R. *et al.*(1990) *Gen. Pharmacol.* 21, 443-446.
Semb, A.G. & Vaage, J.(1991) *Scand. J. Clin. Lab. Invest.* 51, 377-384.

184P ARTERIAL BLOOD PRESSURE, ALPHA-PRESSOR RESPONSES AND NITRIC OXIDE IN RATS FED ON A CALCIUM-FREE DIET

M. Puerro, V. López-Miranda, J.C. Leza, I. Lizasoain, & M.A. Aleixandre (Introduced by J. Tamargo) Dpto. Farmacología, Fac. Medicina, U. Complutense, 28040 Madrid, España.

Various mechanisms seem to be implicated in the calcium hypotensive effect and it has been suggested that calcium administration may cause, among other changes, variations in the pressor responses mediated by the stimulation of α vascular adrenoceptors (Hatton et al., 1993) and in endothelial nitric oxide (NO) production (Porsti et al., 1990). Some researchers have tried to develop hypertension in rats by decreasing dietary calcium to study the mechanisms implicated in the calcium hypotensive effect (Togari et al., 1989). We evaluated the arterial blood pressure (ABP) in 9-week-old Sprague-Dawley rats (SDR) fed on a calcium-free (0.01%) or a normal calcium (1%) diet from weaning (3 weeks). We also evaluated the methoxamine and B-HT 920 pressor responses in pithed rats, and the plasma nitrite (NO_2^-) and nitrate (NO_3^-) levels by the Griess reaction (Green et al., 1982) in both groups of animals. On the other hand, we recorded the development of hypertension in spontaneously hypertensive rats (SHR) fed from weaning to 20 weeks on the different diets, and we measured plasma NO_2^- and NO_3^- levels of these rats at 20 weeks.

The calcium-free diet caused a decrease in the ABP of the SDR and the SHR, but it did not increase the plasma NO_x^- levels of the rats (see table 1). On the other hand, the removal of dietary calcium provoked a marked decrease in methoxamine and B-HT 920 pressor responses in pithed rats, with a significant difference ($p < 0.001$ for methoxamine and $p < 0.01$ for B-HT 920) between the dose-response curves of the increases in systolic and diastolic blood pressure of animals fed on the different diets. Therefore, the calcium-free diet used in this study caused a reduction in the ABP and sensitivity of α vascular adrenoceptors in normotensive SDR, and lessened the development of hypertension in SHR. These effects seem not to be linked to endothelial NO production.

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Table 1. ABP (in conscious and pentobarbital sodium 50 mg/kg i.p. anesthetized animals) and plasma NO_x^- concentration in 9-week-old SDR and 20-week-old SHR fed on calcium-free and normal calcium diets.

	Calcium-free SDR	Normal calcium SDR	Calcium-free SHR	Normal calcium SHR
ABP conscious (mm Hg)	148.70 ± 4.05	143.45 ± 2.34	154.00 ± 2.17	237.16 ± 12.06
ABP anesthetized (mm Hg)	81.10 ± 14.93	158.13 ± 10.09	136.57 ± 15.13	170.30 ± 9.56
NO_x^- (μmol/l)	16.46 ± 2.08	16.36 ± 2.19	4.16 ± 0.66	7.08 ± 1.29

185P CONTRIBUTION OF ENDOTHELIUM AND SMOOTH MUSCLE CELLS TO VASCULAR CONTRACTION IN A CHOLESTEROL-ENRICHED ENVIRONMENT

P. Tavares, C. A. Fontes Ribeiro, A. Barbero[1], H. Silva, P. Wulfroth[2], J. M. Lopez Novoa[1], F. Teixeira.
Institute of Pharmacol. and Exp. Ther.- Faculty of Medicine, Coimbra, Portugal; 1Department of Physiol. and Pharmacol.- Fac. of Medicine, Salamanca, Spain; 2Merz & Co. Germany.

Hypercholesterolaemia is a main risk factor of atherosclerosis, acting through several mechanisms. In spite of the existence of many animal models, no one has yet reproduced the polymorphism of atherosclerosis. On the other hand, the influence of high cholesterol diets on endothelium and/or smooth muscle cells is controversial. Therefore, this study was undertaken to verify the influence of cholesterol diets on vascular reactivity, as well as on the contractility of smooth muscle cells in culture.

NZW rabbits were fed a normal diet (control) or low-level cholesterol-enriched diet (0.1%) for 12 months (mo). This diet significantly increased the serum cholesterol and thus induced atherosclerosis (Teixeira et al., 1991). After 6,9 or 12 mo the rabbits were killed, the thoracic aorta removed cleaned, cut in rings and connected for isometric force recording. Finally, segments of thoracic aortae were obtained from adult rabbits and after several procedures smooth muscle cells were grown in culture (Alipui et al., 1991). Thereafter, the isolated cells were fused with cholesterol enriched liposomes and contractility studies performed.

5-HT-induced contractile responses increased (-log EC_{50}-values of 6.53±0.13, 6.25±0.10 and 5.90±0.12 at 6, 9 and 12 months for rabbits on diet, and 5.60±0.20 for controls at 6 months) and the acetylcholine-induced relaxing response (endothelium-dependent) decreased (the maximal relaxation was 20.1±3.0, 27.1±4.3 and 13.4±3.4 % at 6, 9 and 12 mo for rabbits on diet; 35.2±4.4 for controls at 6 mo); the noradrenaline-induced contractions and the relaxations elicited by nitroprusside or papaverine (endothelium-independent) were not significantly modified. Since intimal early lesions were detected at 6 mo, and some early and advanced lesions appeared at 9 and 12 mo, we conclude that endothelial functional alterations are enough to induce the changes we found. With regard to the contractility of smooth muscle cells in culture, angiotensin II was less potent in the presence of cholesterol-enriched membranes (-log EC_{50}=6.90±0.13) than in control ones (-log EC_{50}=7.30±0.15).

Our results suggest that a rich cholesterol environment decreases the smooth muscle cell contractility and EDRF production and/or transport. Since this last effect seems to predominate, the final cholesterol-influenced response is an increased vascular contractility.

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Alipui C, Tenner TE Jr, Ramos K, (1991) J. Pharmacol. Meth. 26: 211-222

186P CHRONIC ADMINISTRATION OF NO-SYNTHASE INHIBITOR CAUSES CARDIOVASCULAR STRUCTURAL CHANGES

A.Albino-Teixeira & P. Soares-da-Silva. Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200-Porto, Portugal

The acute administration of inhibitors of NO-synthase results in vasoconstriction and systemic hypertension (Rees et al., 1989). The present study has evaluated the blood pressure profile as well as the cardiovascular structural changes induced by the chronic administration of NO-synthase inhibitor N^G -Nitro-L-arginine methyl esther (L-NAME) in drinking water.

After a seven day period of stabilization, normotensive male Wistar rats were selected and given L-NAME in drinking water (mean intake of about 10 mg kg^{-1} day^{-1}). Blood pressure (systolic, SBP; diastolic, DBP) were measured using a photoelectric tail cuff pulse detector; SBP and DBP were in normotensive rats, respectively, 108±3 and 81±2 mmHg (n=6). During the four weeks of treatment both SBP and DBP increased progressively reaching respectively, 186±2 and 125±3 mmHg.

On day 28, under pentobarbitone anaesthesia, blood from the renal vein was colled for plasma renin activity determination and fragments of left ventricle, mesenteric and caudal arteries were processed for morphological studies.

The heart to body weight ratio was significantly increased in L-NAME treated rats (control: 3.28±0.05; L-NAME: 3.48±0.08) ($P<0.01$). A decrease in renin plasma activity (control: 16.4±3.4 ng h^{-1} ml^{-1}; L-NAME 4.3±5.2 ng h^{-1} ml^{-1}) ($P<0.05$) was verified. Structural changes caused by NO-synthase inhibition included increase in smooth muscle cell dimension; increase in the thickness of mesenteric artery (control 45.0±10.0 μm; L-NAME 54.9±7.2 μm) ($P<0.05$) and of tail artery (control 28.1± 10.7 μm; L-NAME 41.9±7.8 μm) ($P<0.05$).

Thus the hypertension determined by long-term nitric oxide synthesis inhibition caused cardiovascular hypertrophic changes which were not associated with activation of the renin-angiotensin system.

Rees D.D., Palmer R.M.J. and Moncada S. (1989). Proc. Nat. Acad. Sci. U.S.A. 86, 3375-3378.

187P ENDOTHELIUM-DEPENDENT, NITRIC OXIDE-INDEPENDENT INHIBITION OF NORADRENALINE RELEASE FROM THE CANINE PULMONARY ARTERY

M.J. Vaz-da-Silva, S. Guimarães & D. Moura, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200-Porto, Portugal.

This study aims at characterising the prejunctional influence of endothelium on noradrenaline (NA) release from the pulmonary artery removed from dogs anaesthetized with 30 mg/kg i.v. pentobarbital. Tritium overflow from intact or endothelium-free pulmonary arteries preloaded with [^{3}H]NA (0.2 μM) was evoked by electrical stimulation (1Hz, 300 pulses) or KCl (25-100 mM for 5 min). The fractional release of [^{3}H]NA induced by electrical stimulation was increased by removing the endothelium (from 1.89 ± 0.29 to 2.78 ± 0.19 x 10^{-5}/pulse, $n=10$, $P<0.05$). This effect was not mimicked by either N^G-nitro-L-arginine methyl ester (3-300 μM) or indomethacin (3-30 μM). However, the facilitatory effect of tetraethylammonium (TEA) on NA release was much higher in endothelium-free than in intact arteries (Figure 1).

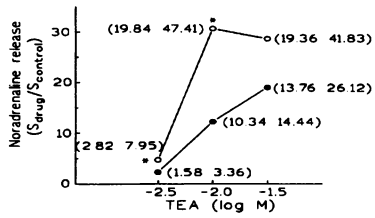


Figure 1. Effect of TEA on [^{3}H]NA release induced by electrical stimulation of endothelium-free (open circles) or intact (closed circles) arteries. Shown are geometric means and 95% confidence limits (n = 5 or 6) of the ratios of [^{3}H]NA overflow in the presence of TEA (S_{drug}) to that in its absence ($S_{control}$). *P < 0.05

In contrast to stimulation-induced overflow, the KCl-induced overflow was not changed by rubbing (Figure 2).

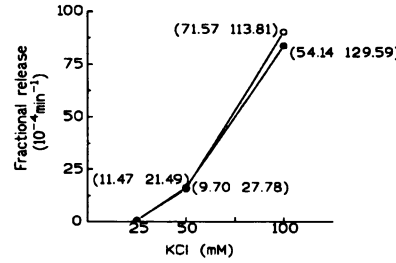


Figure 2. [^{3}H]NA release induced by KCl in endothelium-free (open circles) and intact (closed circles) arteries. Shown are geometric means and 95% confidence limits (n = 7 or 8).

In conclusion, neither NO nor a prostanoid appears to be a mediator of the endothelium-dependent inhibition of NA release from the pulmonary artery. The data also suggest that an endothelium-derived K-channel opener identical to EDHF (endothelium-derived hyperpolarizing factor: Weston *et al.*, 1988) may be involved in reducing NA release.

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188P OXIDISED LOW DENSITY LIPOPROTEIN AFFECTS PROSTAGLANDIN PRODUCTION AND NITRIC OXIDE SYNTHASE ACTIVITY IN MACROPHAGES

K.E. Matthys, C.E. Van Hove, P.G. Jorens, B. Marescau[1], M. Rosseneu[2], A.G. Herman & H. Bult, Div. Pharmacol. and [1]Neurochem., Univ. of Antwerp (UIA), Wilrijk, and [2]Dept. of Clin. Chem., A.Z. St. Jan, Bruges, Belgium.

Oxidized low density lipoprotein (oxLDL), found extracellularly and intracellularly in macrophages in atherosclerotic lesions, is currently regarded as a tentative key player in atherogenesis. We investigated the effect of oxLDL pretreatment on the secretion of two inflammatory mediators, prostacyclin (PGI_2) and nitric oxide (NO), by immune-stimulated macrophages.

OxLDL was prepared by oxidation with $CuCl_2$ (Matthys *et al.*, 1994). J774 mouse macrophage-like cells (10^6) were cultured in 1 ml Dulbecco's modified Eagle medium and exposed to oxLDL 20 μg ml^{-1} for 18 h or 200 μg ml^{-1} for 6 h. Cells were then washed and stimulated with interferon-γ (IFNγ, 100 U ml^{-1}) in the absence of oxLDL. After 24 h the NO metabolite nitrite (Jorens *et al.*, 1992) and PGI_2 (radioimmunoassay of 6-oxo-$PGF_{1\alpha}$) were determined in the supernatant and expressed per mg cell protein.

PGI_2 and nitrite were elevated upon stimulation with IFNγ (Table 1). Pretreatment with 20 μg oxLDL ml^{-1} for 18 h resulted in a decreased (31 ± 2 %, n = 4) nitrite release, whereas PGI_2 was not influenced. The shorter pre-exposure to a high dose of oxLDL had the opposite effect: it significantly potentiated both PGI_2 and nitrite production. Indomethacin 10 μM abolished PGI_2 production and largely prevented the enhanced nitrite release due to oxLDL, while only slightly affecting nitrite formation in control cells (Table 1). The unchanged cellular protein content excluded direct cytotoxic effects of ox-LDL. Measurements of PGE_2 and the NO synthase metabolite citrulline yielded similar results.

The data show that the immune-induced release of NO is stimulated or suppressed, depending on the conditions of the oxLDL exposure. The inhibition of NO synthase induction by the prolonged oxLDL exposure confirms our original report (Jorens *et al.*, 1992). The down-regulation was presumably not due to PGI_2, as PGI_2 release was hardly affected by oxLDL in this situation. Shorter exposure to oxLDL potentiates the production of both inflammatory macrophage products, PGI_2 and NO. The inhibitory effect of indomethacin on both processes suggests the involvement of prostaglandins in the upregulation of inducible NO synthase activity.

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Matthys, K.E., Jorens, P.G., Marescau, B. et al. (1994) *Mediators of Inflammation* 3, 323-327.

Table 1. Effect of oxLDL (200 μg ml^{-1}, 6 h) and indomethacin (indo, 10 μM) on the release of PGI_2 and nitrite.

	PGI_2 pmol mg^{-1}		nitrite nmol mg^{-1}	
treatment	control	oxLDL	control	oxLDL
no stimulus	1.1±0.0	4.4±0.4*	< 10	< 10
IFNγ	2.0±0.2	10.7±1.6*	98±5	148±10*
IFNγ + indo	< 0.02	< 0.02	81±4	103±12$^+$

mean ± s.e.mean, n = 4; * different from control; $^+$ different from oxLDL alone; P < 0.05, Student's t-test

189P HYPERREACTIVITY OF AORTAE FROM THE OBESE ZUCKER RAT: IMPAIRMENT OF SMOOTH MUSCLE FUNCTION?

J. W. Growcott, B. Cox, V. Torr, R. Hatton[1], & M. Hollingsworth[2], VIM, [1] Cardiovascular & Metabolism Departments, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG and [2], School of Biological Sciences, University of Manchester, Manchester, M13 9PT

Obese Zucker rats have been shown to be hypertensive and insulin-resistant (Zemel *et al.*, 1992). The elevated blood pressure in these animals could be due to enhanced vascular reactivity to spasmogens observed *in vitro* and *in vivo* (Cox and Kikta, 1992 and Johnson *et al.*, 1994). The properties of isolated aortae from obese and lean Zucker rats were further investigated to gain an insight into the mechanisms involved in any changes in vascular smooth muscle function.

Aortic rings from male obese and lean Zucker rats (5 months old) were set up in gassed Krebs' solution at 37°C, under 2g tension, to record isometric tension changes to cumulative additions (10nM-100μM) of 5-hydroxytryptamine (5-HT), in the absence and presence of N^G-nitro-arginine methyl ester (L-NAME). Relaxations to cumulative additions of acetylcholine (ACh) and glyceryltrinitrate (GTN) were also recorded in 5-HT pre-tensioned aortae. Mean pD_2 values (±s.e.m.) and maximal tensions (±s.e.m.) were calculated. $P<0.05$ represented statistical significance.

The aortae from obese rats were significantly more sensitive ($P<0.05$) and responsive ($P<0.01$) to 5-HT than those from their lean counterparts (pD_2's: 6.14±0.15 and 5.63±0.09; Max. tension: 0.3±0.02 and 0.2±0.03 g/mg, respectively, n = 5). These differences were maintained in the presence of L-NAME (100μM) which itself, increased maximal tension by 23±3% and 35±8% in aortae from obese and lean rats respectively. Sensitivity to ACh of aortae was not significantly different between obese and lean rats (pD_2's: 6.59±0.15 and 6.97±0.04, n=4), whereas sensitivity to GTN was significantly reduced ($P<0.05$) in aortae from obese rats (pD_2's: 6.32±0.50 and 8.04 ±0.23, in aortae from obese and lean rats respectively, n=4). Maximal responses to both agents were unchanged.

These results suggest that the increased sensitivity and responsiveness to 5-HT of aortae from obese Zucker rats was not due to altered endothelial function *per se*. The reduced sensitivity of the aortae to the relaxant effects of GTN indicates that smooth-muscle relaxation-coupling may be impaired in the obese rats. This reduction in function could contribute to the increased reactivity of the vessels when challenged by spasmogens and consequently, the hypertension observed *in vivo*.

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190P INFLUENCE OF OXYSTEROLS ON ENDOTHELIUM-DEPENDENT RELAXATIONS IN RABBIT ISOLATED AORTA

A.M. Gleeson, A.C. Hubbard, K. R. Bruckdorfer[1] & M. Jacobs. Departments of Pharmacology and Biochemistry[1], Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF.

Accumulation of oxidatively-modified low-density lipoproteins (LDL) in the arterial wall is thought to contribute to the pathogenesis of atherosclerosis by impairment of endothelium-dependent vasodilation, mediated by nitric oxide (Jacobs *et al.*, 1990). The oxidation of LDL leads to the formation of a complex array of products, several of which could play a role in endothelial dysfunction. These potential cytotoxins include oxidised fatty acids, lysophosphatidylcholine (LPC) and oxysterols. Previous studies have shown inhibition of acetylcholine (ACh)-evoked relaxations in isolated rabbit aorta by LPC (Plane, 1992) and oxidised fatty acid derivatives (Buckley, 1994). The aim of this study was to examine the vascular effects of three oxysterols present in oxidised LDL; 7β-hydroxycholesterol, 25-hydroxycholesterol and 7-ketocholesterol to see whether they might contribute to the inhibition of relaxation.
Endothelium-dependent relaxation was assessed in intact aortic rings from New Zealand White rabbits as previously described (Jacobs *et al.*, 1990). Tissues were precontracted with phenylephrine (0.1-0.3μM) and relaxed with cumulative concentrations of ACh (1nM-10μM) After washout, tissues were incubated for 30 min with 7-hydroxycholesterol, 25-hydroxycholesterol and 7-ketocholesterol and the contraction/relaxation cycle was repeated. A third cycle of contraction/relaxation was carried out after a further 30 min washout to determine the reversibility of any effects. Statistical analysis was performed by use of a Student's t-test for unpaired observations, where $p<0.05$ was considered significant. In the presence of 7-ketocholesterol (100nM-10μM), ACh evoked relaxations were attenuated. This was demonstrated by a significant reduction in the maximal relaxation to ACh from 81±3.7% (n=7) in control tissues, compared to 59±8%(n=12) in the rings treated with 7-ketocholesterol ($p<0.0001$). After washout, responses were not restored to control values (60±9%; n=12). Incubation with 25-hydroxycholesterol (100nM-10μM) also resulted in a significant reduction in the maximal relaxation evoked by ACh from 93±5.7% (n=12) in control tissues to 83 ±4.5% (n=12) in rings treated with 25-hydroxycholesterol ($p<0.001$). After washout, responses were not restored when compared to control values (88±8.6%; n=12). Incubation with 7-hydroxycholesterol (100nM-10μM) had no effect on relaxations with maximum responses in control and treated tissues of 81±7.0% and 81±9.2% respectively.
In conclusion, this study demonstrates that 7-ketocholesterol and 25-hydroxycholesterol inhibits endothelium-dependent relaxations mediated by nitric oxide. 7-β-hydroxycholesterol had no significant effect on ACh evoked relaxations. This study suggests that both 7-ketocholesterol and 25-hydroxycholesterol in oxidised LDL may contribute to the impairment of nitric oxide mediated endothelium-dependent vasodilation.

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Jacobs, M., Plane, F. & Bruckdorfer, K.R. (1990). *Br. J. Pharmacol. 100, 21-26.*
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191P THE HUMAN MEGAKARYOBLASTIC CELL LINE MEG-01 PREDOMINANTLY CONTAINS THE ET_B ENDOTHELIN RECEPTOR SUBTYPE

R.D. Hamroun, M-N. Mathieu, M-F. Laliberté & C. Chevillard, INSERM U.300, Faculté de Pharmacie, Avenue Charles Flahault, Montpellier, France

In a previous paper (Diochot et al., 1992), we have shown that endothelins (ET) increased the intracellular ionized calcium concentration ($[Ca^{2+}]i$) and inositol phosphate levels in the MEG-01 megakaryoblastic cell line. The present study was designed to determine the subtype of ET receptor present on these cells and to investigate its expression, using radioligand binding techniques, measurements of $[Ca^{2+}]i$ and reverse transcriptase-polymerase chain reaction (RT-PCR) procedure.
Binding was assayed following incubation of 1 x 10^6 cells in PBS buffer containing 5.55 mM glucose, 1 h at 30°C with ^{125}I-ET-1 (1 nM) in absence or in presence of increasing concentrations of either ET-1, ET-3, the selective ET_B agonist SRTX6c or the selective ET_A antagonist BQ123.
For $[Ca^{2+}]i$ measurement, cells were loaded with INDO-1-AM and Ca^{2+} transients induced by ET-1 and SRTX6c were determined as previously described (Diochot et al., 1992). The effect of ET-1 on $[Ca^{2+}]i$ was also measured in presence of 10 μM BQ 123 and that of SRTX6c in presence of 1 and 10 μM of the non-selective antagonist PD 142893 (Haynes et al., 1993), administered 5 min before the agonists.
For ET_B receptor expression, total RNA was extracted from MEG-01 cells using a guanidinium isothiocyanate method. RT-PCR analysis was performed with two 29 and 31 oligonucleotide primers, specific for the human ET_B receptor sequence (Ogawa et al., 1991). First strand cDNA was synthesized using 1.5 μg total RNA and amplification was carried out for 30 cycles (2 min at 74°C, 1 min at 92°C and 1 min at 65°C) and a final extension of 10 min at 74°C.
The products were analysed by agarose gel electrophoresis.

The specific binding of ^{125}I ET-1, which constituted about 90% of total radioligand binding was inhibited with the relative potency profile of ET-1 ≥ ET-3 ≥SRTX6c but not affected by BQ 123.
Both ET-1 and SRTX6c increased $[Ca^{2+}]i$ in a concentration dependent fashion with a threshold at 1 nM and a maximal response at 100 nM. BQ 123 did not affect the ET-1 induced responses whereas PD 142893 caused a rightward shift of the SRTX6c concentration-response curve.
RT-PCR analysis showed that the amplified products of the cDNA from MEG-01 cells were at the expected size of 238 bp. Conversely, when using RNA from the ET unresponsive megakaryoblastic DAMI cell (Vittet et al., 1992), no such an amplified product could be detected.

These results demonstrate that the MEG-01 cell line predominantly contains the ET_B receptor subtype.

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192P CHARACTERIZATION OF ENDOTHELIN RECEPTORS MEDIATING VASOCONSTRICTION IN HUMAN PLACENTAL COTYLEDONS

S.R. Howarth & C.A. Wilson, Department of Obstetrics & Gynaecology, St. George's Hospital Medical School, London SW17 0RE.

The endothelin (ET) family consists of three structurally related peptides, ET-1, ET-2 and ET-3 with ET-1 being the most powerful vasoconstrictor yet described. Currently the effects of these peptides are considered to be mediated by two receptor subtypes, ET_A and ET_B (Battistini & Botting, 1993). Experiments *in vitro* suggest that vasoconstriction in humans is predominantly mediated via the ET_A receptor (Davenport & Maguire, 1994). The present study was designed to clarify which receptor subtype is responsible for producing ET mediated vasoconstriction in human placentae.

Isolated placental cotyledons obtained from normotensive women after elective caesarean section or spontaneous vaginal delivery were perfused at a constant rate (5ml min^{-1}) with oxygenated (95% O_2, 5% CO_2) Krebs' Ringer solution (pH 7.4; 37°C). Bolus injections of ET-1, ET-3 and S6c (selctive ET_B agonist; Kloog et al, 1988) were applied to the perfusion system in cumulative graded doses (20fmols - 1nmol) in the presence of vehicle, selective ET_A and ET_B receptor antagonists, BQ123 (Ihara et al, 1992) and RES 701-1 (Tanaka et al, 1994), respectively, and a non-selective $ET_{A/B}$ antagonist, PD145065 (Cody et al, 1993), applied for 30min at 1μM. Responses were expressed as an increase in perfusion pressure (mmHg) and PP_{20} values (the concentration of agonist required to produce a 20mmHg increase in pressure) were calculated. Data were compared by ANOVA and $p<0.05$ was considered to be significant.

All three agonists caused a dose-dependent increase in perfusion pressure with ET-1 being the most potent (Table 1). BQ123 failed to shift the curves to ET-1 or ET-3 suggesting that their actions are ET_B mediated. PD145065 and RES 701-1 significantly shifted the curves rightward to ET-3 (44 fold, $p<0.001$; 8 fold, $p<0.05$) and S6c (188 fold, $p<0.0001$; 9 fold, $p<0.0001$) respectively. However neither of these agents had any effect upon the ET-1 induced dose response curve.

Table 1 Mean PP_{20} values (pmols) and maximum responses (mmHg) obtained. 95% Confidence intervals are shown in parentheses.

Agonist	(n)	PP_{20}	Maximum Response
ET-1	8	33.4 (16.4 - 50.4)	122 (96 - 149)
ET-3	7	736.0 (348.0 - 1124.0)	52 (28 - 75)
S6c	7	157.2 (68.0 - 382.3)	52 (25 - 78)

These data indicate that ET-induced vasoconstriction in the human placenta is predominantly ET_B mediated. However, it also suggests that ET-1 may act through a non ET_A/ET_B receptor.

We are very grateful to Dr. K. Clark of Glaxo Research and Development U.K. for his assistance, to Parke-Davis, Ann Arbor and Kyowa Habbo Kogyo Co Ltd, Tokyo for their gifts of PD 145065 and RES 701-1, respectively.

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193P THE GUINEA-PIG TAENIA COLI: A PREPARATION VERY SENSITIVE TO ENDOTHELIN ET_A RECEPTOR ANTAGONISTS

C.F.C. Smith, C. Baker, T. Hayden and C. Wilson; ZENECA Pharmaceuticals, Cardiovascular and Metabolism Department, Alderley Park, Macclesfield SK10 4TG.

Wiklund *et al.* (1989) showed that in the guinea-pig taenia coli, endothelin-1 produced only relaxation, contrasting markedly with the predominantly contractile effects reported for the peptide in most other smooth muscle preparations. Sadaharu *et al.* (1991) later reported that the relaxation was mediated via an effect on a Ca^{2+}-activated K^+-channel. We have investigated the preparation to determine the receptor sub-types responsible for the effects of endothelin.

Strips of taenia coli were set up for isotonic recording in 20ml organ baths containing Krebs' solution (32°C) gassed with 95% O_2, 5%CO_2. After a 90 min rest period, partial cumulative response curves were constructed to the test agonist. After a 2 h washout period, a second response curve was constructed in the presence of antagonist and pA_2 values determined from the concentration ratios obtained.

Endothelin-1 (ET-1) and endothelin-3 (ET-3) produced concentration-related relaxations, ET-1 being approximately 100 times more potent than ET-3. Responses were inhibited by the selective ET_A antagonists, BQ123, FR139317 and BMS182874, and the non-selective ET antagonists, bosentan and Ro46-2005 (Table 1). Antagonist pA_2 values against ET-1 were invariably higher than those reported in other isolated tissue preparations: e.g. pA_2 values for BQ123 in a range of blood vessel preparations were found to be between 6.4 and 7.4 (Smith *et al.*, 1994). Antagonist pA_2 values were 3 to 9 times higher against ET-3 than against ET-1 as found previously in vascular tissues (Smith *et al.*, 1994). In some preparations, the ET_A selective antagonists unmasked a contractile effect of the endothelins. Furthermore, the selective ET_B agonist, BQ3020 (Ihara *et al.*, 1992b), was either inactive or produced contractile effects.

Table 1

Antagonist	pA_2 (mean ± s.e.mean; n ≥ 4) vs. ET-1	vs. ET-3
BMS182874 (Stein *et al.*, 1994)	7.0 +0.12	7.3 + 0.30
BQ123 (Ihara *et al.*,1992a)	8.4 +0.10	9.0 + 0.13
FR139317 (Sogabe *et al.*,1992)	7.9 +0.08	8.8 + 0.11
Bosentan (Clozel *et al.*, 1994)	7.4 +0.11	8.0 + 0.10
Ro46-2005 (Clozel *et al.*, 1993)	6.9 +0.12	7.6 + 0.19

Thus, this preparation possesses ET_A receptors that mediate only relaxation and a variable ET_B receptor population that mediates contraction. It is the most sensitive isolated tissue preparation yet reported for assaying ET_A antagonists.

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194P CHARACTERISATION OF CLONED HUMAN ENDOTHELIN ET_A AND ET_B RECEPTORS EXPRESSED IN MOUSE ERYTHROLEUKAEMIC CELLS

E. Whiting, C. Bath, E. Tang and C. Wilson, ZENECA Pharmaceuticals, Cardiovascular and Metabolism Department, Alderley Park, Macclesfield SK10 4TG

Characterisation of sub-type selectivity of ligands for endothelin (ET) receptors was initially performed using cells expressing endogenous ET receptors (e.g. rat A10 cells). More recently, molecular biology has enabled expression of human ET receptors in e.g. CHO cells (Buchan *et al.*, 1994) allowing evaluation of each receptor sub-type in isolation.

In the present study, we have cloned the human endothelin ET_A and ET_B receptors and stably expressed them in mouse erythroleukaemic (MEL) cells as described by Needham *et al.* (1992). This report describes the binding characteristics of these receptors using membrane preparations derived from these cells.

Saturation binding studies with these membranes and [^{125}I]-ET-1 (Amersham International) yielded values for equilibrium constant (K_d) of 28.7 ± 4.1 pM and 20.7 ± 3.3 pM for ET_A and ET_B receptors respectively. The saturation binding constants B_{max} for these membrane preparations were 2927 ± 103 fmol mg^{-1} and 1717 ± 49 fmol mg^{-1} respectively. The binding affinities for a range of ET receptor ligands of differing selectivity are shown in Table 1. The data are in good agreement with affinities reported using human ET receptors transfected into CHO cells (Buchan *et al.*, 1994) except that BMS182874 showed a 1.4 log unit higher affinity for the ET_A receptor in the present study. The reason for this difference remains unclear.

The results of the present study indicate that the MEL cell expression system represents a versatile tool which will facilitate the identification of novel antagonists of human endothelin receptors.

Table 1. Binding affinity (pIC_{50}) for human receptors

Competing Agent	ET_A	ET_B
ET-1	9.9 ± 0.1	9.8 ± 0.1
ET-2	9.6 ± 0.5	9.9 ± 0.2
ET-3	8.3 ± 0.2	10.5 ± 0.2
Sarafotoxin S6c	< 4.0	9.0 ± 0.2
BQ123 (Ihara *et al.*, 1992a)	7.9 ± 0.2	<4.0
BQ3020 (Ihara *et al.*, 1992b)	6.7 ± 0.2	9.3 ± 0.3
IRL 1620 (Takai *et al.*, 1992)	5.8 ± 0.2	8.9 ± 0.2
FR139317 (Sogabe *et al.*, 1992)	8.7 ± 0.4	4.4 ± 0.2
BMS182874 (Stein *et al.*, 1994)	7.2 ± 0.3	<4.0
Ro46-2005 (Clozel *et al.*, 1993)	6.9 ± 0.2	6.9 ± 0.2
Bosentan (Clozel *et al.*, 1994)	8.0 ± 0.1	6.9 ± 0.1

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195P CHARACTERISATION OF ENDOTHELIN RECEPTORS IN CULTURED HUMAN CEREBRAL VASCULAR SMOOTH MUSCLE CELLS

J.C.M. Yu, J.D. Pickard* & A.P. Davenport. Clinical Pharmacology Unit and *Department of Neurosurgery, University of Cambridge, Box 110, F & G Block, Level 2, Addenbrooke's Hospital, Cambridge, CB2 2QQ, U.K.

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide. Oxyhaemoglobin stimulates secretion of ET-1 from vascular endothelial and smooth muscle cells (Kasuya et al., 1993), and is thought to be one of the principal pathogenic agents for cerebral vasospasm following subarachnoid haemorrhage, possibly via activation of ET-1. ET antagonists have also been shown to reverse vasospasm in animal models of subarachnoid haemorrhage. Thus, it has been hypothesised that ET-1 plays a role in cerebral vasospasm. The aim of our study was to characterise ET receptors on cerebral vascular smooth muscle cells.

Cerebral vessels (< 300 μm in diameter) were dissected from tissue removed from patients undergoing neurosurgical procedures. Following explantation, human cerebral vascular smooth muscle cells (CVSMCs) were grown to confluence in the absence of heparin to prevent growth of endothelial cells, which was confirmed by negative staining for von Willebrand factor (Dako) and positive staining for smooth muscle α-actin (Sigma). ET and big ET-1 were measured directly by radioimmunoassay over 12 hours with standard curves constructed using ET-1 and big ET-1 (Plumpton et al., 1992). Reverse transcriptase - polymerase chain reaction (RT - PCR) assays were carried out as previously described (Davenport et al., 1993). For saturation binding experiments, CVSMCs were incubated with increasing concentrations of [^{125}I]ET-1 and [^{125}I]-PD151242 (concentration range: 10^{-12}M - 10^{-8}M). For competition experiments CVSMCs were incubated with 100 pM [^{125}I]ET-1 and increasing concentrations of unlabelled PD151242 (concentration range: 2 x 10^{-11}M - 10^{-4}M). Non-specific binding was determined by the inclusion of 1 μM unlabeled ET-1 as previously described (Davenport et al., 1993; Davenport et al., 1994). Receptor autoradiography was performed using [^{125}I]ET-1, [^{125}I]-PD151242 and [^{125}I]-BQ3020 (Amersham International, plc) as previously described (Davenport et al., 1993, 1994). CVSMCs secreted comparable levels of immunoreactive (IR) big ET-1 and IR ET (12.7 ± 10.3 and 8.3 ± 5.6 pM/10^6 cells respectively; mean ± s.e.m., n=3), while RT - PCR assays demonstrated the presence of messenger RNA for ET_A and ET_B receptors. Saturation binding assays revealed K_D and B_{max} values of 0.8 ± 0.1 nM and 690 ± 108 fmol mg^{-1} using [^{125}I]ET-1 whilst ET_A selective [^{125}I]-PD151242 yielded similar values of 0.4 nM ± 0.1 nM and 388 ± 68 fmol mg^{-1} (mean ± s.e.m., n=3). Autoradiography illustrated the presence of specific binding sites for [^{125}I]ET-1 and [^{125}I]-PD151242, but no binding was detected using ET_B subtype-selective [^{125}I]-BQ3020. In agreement with results obtained from saturation binding and autoradiography, ET_A subtype-selective antagonist PD151242 competed for the binding of [^{125}I]ET-1 monophasically and analysis of the competition curves indicated that a one-site fit was preferred over a two-site model.

Thus, CVSMCs secrete both IR big ET-1 and IR ET. Messenger RNA encoding both ET_A and ET_B receptors were detected in these cultured cells. However, ligand binding demonstrated that CVSMCs mainly express ET_A receptor protein and thus antagonism of this sub-type may be necessary to block the actions of ET-1 in the human cerebral vasculature.

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196P PHARMACOLOGICAL CHARACTERISATION OF ENDOTHELIN RECEPTORS IN A HUMAN NEUROBLASTOMA (SK-N-MC) CELL LINE

S.L. Wood, K.L. Clark & D.G. White, Pharmacology 2, Glaxo Research & Development Ltd., Ware, Hertfordshire, SG12 0DP, U.K.

Studies using endothelin (ET) isopeptides suggest that SK-N-MC cells, derived from a human neuroblastoma cell line, contain ET_A receptors (Wilkes & Boarder, 1991). We have further characterised these receptors using a range of both peptide and non-peptide ET receptor ligands and have compared the data with that obtained at cloned human ET_A receptors expressed in Chinese hamster ovary (CHO) cells (Buchan *et al.*, 1994).

SK-N-MC cells (passages 36-56) were grown to confluence in Dulbecco's minimum essential medium F12 mixture (with 10% v/v foetal calf serum and 1mM glutamine). Membranes from SK-N-MC and CHO cells were prepared as described by Buchan *et al.* (1994). For competition experiments, SK-N-MC or CHO membranes (20μg.ml^{-1} and 1.5μg.ml^{-1}, respectively) were incubated (25°C, 3h) in buffer (Triss 50mM, BSA 0.1% w/v, $CaCl_2$ 1mM, bacitracin 100μg.ml^{-1}) with varying concentrations of unlabelled ET receptor ligands and [^{125}I]-ET-1 (20pM). Specific binding was defined as the difference between binding in the absence and presence of unlabelled ET-1 (1μM). Saturation analysis was performed using [^{125}I]-ET-1 (1-200pM).

Scatchard plots from saturation isotherms revealed a single population of [^{125}I]-ET-1 binding sites in SK-N-MC membranes (mean K_d ±s.e.mean =29±3 pM, B_{max} = 232±64 fmol.mg protein^{-1}, n=3).

All ligands tested produced concentration-dependent and complete inhibition of [^{125}I]-ET-1 binding with pIC_{50} values shown in Table 1.

Table 1: Mean pIC_{50} values ±s.e. mean (n=4-6) for ET receptor ligands at ET_A receptors in SK-N-MC and CHO membranes.

	CHO	SK-N-MC
ET-1	10.1±0.1	10.1±0.1
ET-3	7.0±0.1	7.2±0.2
S6c	<6.0	<6.0
BQ123	7.8±0.2	7.9±0.1
*SB209670	8.8±0.1	9.0±0.1
*Ro 462005	7.1±0.1	7.1±0.1
*BMS182874	6.3±0.1	6.3±0.1

* See Buchan *et al.* (1994) for structure and selectivity.

In conclusion, these results indicate the presence of a homogenous population of ET_A receptors in SK-N-MC cells. These receptors show an identical ligand binding profile to human cloned ET_A receptors expressed in CHO cells.

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197P RAPID DEGRADATION OF ENDOTHELIN-1 BY AN ENZYME RELEASED BY THE RAT ISOLATED PERFUSED MESENTERY

Andrea C. Cooper, Francisco Pérez-Vizcaíno, Roger Corder, Alain Fournier and Timothy D. Warner. *The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.*

In vivo the effects of endothelin-1 (ET-1) are limited by its rapid removal from the circulation and possibly by its metabolism by enzymes such as neutral endopeptidase 24.11, deamidase or carboxypeptidase A. Here we have examined the involvements of these enzymatic activities in regulating the responses to ET-1 of the rat isolated perfused mesentery.

Rat isolated aortic rings and isolated perfused mesenteric beds were prepared as described before (Warner et al., 1993). The isolated mesentery was perfused at a constant flow of 2 ml min^{-1} with warmed (37 °C) and gassed (95% O_2:5% CO_2) Krebs' solution. After a 60 min equilibration period the effluent from the mesentery was collected and reperfused, producing a closed perfusion system (total volume, 10 ml). Responses of the aortic rings were normalised to the contraction caused by 80 mM KCl.

Samples of Krebs' buffer which had been recirculated through the mesenteric arterial bed for 30 min (recirc-K) rapidly destroyed the activity of ET-1 over 15 min as assessed either by bioassay on rings of rat thoracic aorta (control contraction, 72±12% KCl; recirc-K, 7±3 %, n=10-12) or by HPLC. Contractions induced by sarafotoxin (SX6b, $3x10^{-9}$ M, 70±21% KCl, n=3) were similarly suppressed by pre-incubation with recirc-K (4±2 %, n=3) whereas those to arg-vasopressin ($3x10^{-9}$ M, n=4) were unaffected (n=4). The degradation of ET-1 by recirc-K was prevented by 1,10-phenanthroline (10^{-3} M, n=6), abolished by heating the recirc-K solution to 90 °C for 15 min (n=3), and reduced by EGTA ($5x10^{-3}$ M) (71% of control, n=4) or ET-1(16-21) (10^{-5} M). For instance, in the presence of ET-1(16-21) the contraction induced by ET-1 was only reduced by 40% after 15 min incubation with recirc-K buffer (n=6). Leupeptin ($3x10^{-4}$ M), dichloroisocoumarin ($5x10^{-5}$ M), phenymethyl-sulfonyl fluoride (10^{-3} M), a combination of bacitracin (300 mg ml^{-1}), bestatin (10^{-5} M), captopril (10^{-5} M), phosphoramidon (10^{-4} M) and thiorphan (10^{-4} M) or Polypep® (a proprietary protein digest) did not inhibit the degradation of ET-1 by recirc-K (n=3-5). In experiments examining directly the vascular responses of the isolated perfused mesentery of the rat the addition of cumulative concentrations of ET-1 to the recirculating Krebs' solution caused small concentration-dependent increases in perfusion pressure. The inclusion of ET-1(16-21), ET-1(17-21), or ET-1(18-21) (10^{-5} M) greatly potentiated these responses, but not those to arg-vasopressin or methoxamine. For instance, 10^{-8} M ET-1 increased the perfusion pressure by 8.9±2.3 mm Hg (n=14) in control conditions and by 42.5±10.8, 45.8±5.1 or 34.0±4.7 mm Hg in the presence of ET-1(16-21), ET-1(17-21), or ET-1(18-21), respectively (n=4-6).

Thus, the rat mesentery releases an enzyme that very rapidly destroys ET-1 and the very closely related peptide SX6b but not arg-vasopressin. This enzyme is most probably a metallo-peptidase because of its sensitivity to inhibition by 1,10-phenanthroline or EGTA. It is particularly interesting that a simple vascular bed such as the mesentery produces such a powerful endothelin metabolising enzyme and it is tempting, therefore, to speculate that the endothelin degrading enzyme active at neutral pH that we have found is important in the metabolism of ET-1 throughout the vasculature.

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198P ABILITY OF NON-PEPTIDE ENDOTHELIN ANTAGONISTS TO REVERSE AN ESTABLISHED ENDOTHELIN-1 PRESSOR RESPONSE IN THE PITHED RAT

C.A. Sargent, R. Brazdil, T.J. Brown & A.G. Roach. Rhône-Poulenc Rorer, Dagenham Research Centre, Rainham Road South, Dagenham, Essex.

Endothelin-1 (ET-1) has been shown to bind almost irreversibly to its receptor in assay systems utilising cell membranes (Waggoner et al, 1992). Therefore, endothelin (ET) antagonists may be less effective when an established ET-1 response is present. However, Warner and co-workers recently showed that infusion of the peptide endothelin antagonists, BQ 123 and PD 145065 reversed an established ET-1 pressor response in ganglionic blocked rats (Warner et al, 1994). In their study the mixed antagonist PD 145065 was less effective than the ET_A selective antagonist, BQ 123. The present study was designed to investigate if other mixed ET antagonists (bosentan and SB 209670 (Clozel et al, 1994 ; Elliott et al, 1994)) were less effective than the ET_A selective antagonist BMS 182,874 (Stein et al, 1994) in reversing an established ET-1 mediated pressor response in the pithed rat. Male Sprague Dawley rats (250-350g) were anaesthetised with isoflurane and the trachea was cannulated. The rats were pithed, immediately connected to a respirator and both jugular veins were cannulated, one for administration of vehicle or antagonist and the other for continuous infusion of ET-1. The carotid artery was cannulated and connected to a pressure transducer for measurement of blood pressure and heart rate. A bolus i.v. injection of ET-1 (0.3 nmol//kg) followed by a continuous ET-1 infusion (10 pmol/kg/min) resulted in a pressor response of 44 ± 4 mm Hg (n=10) which was sustained for 90 min. In order to generate dose-dependent inhibitory responses for ET antagonists, vehicle or drug was administered as an i.v. bolus dose and pressure monitored until a plateau was reached. If no effect was observed after 15 min, the next cumulative dose of antagonist was administered. This protocol was repeated until the ET-1 mediated pressor response could be reversed no further. The reversal for each antagonist was calculated as percent change from the established ET-1 pressor response. ID_{50} values were calculated and are expressed as geometric mean plus 95 % confidence limits (Table 1). In these experiments, vehicle had no effect on the sustained pressor response. BMS 182,874 (n=5), bosentan (n=4) and SB 209670 (n=5) all demonstrated a complete dose-dependent reversal of the ET-1 pressor response. The mixed antagonists appeared to be more effective than the ET_A selective antagonist BMS 182,874. Interestingly, the ID_{50} in the pithed rat has a similar rank order of potency to the IC_{50} for ET_A receptor binding (Table 1). In conclusion, these results demonstrate that selective or non-selective ET antagonists can reverse an established ET-1 pressor response and that potency appears to be related to their binding affinity for ET_A receptors.

Table 1. The dose ranges for endothelin antagonists tested, their ID50 values in the pithed rat and their published IC50 values in ETA binding experiements

Compound	Dose range (μmol/kg i.v.)	Pithed rat ID50 (95 % confidence limits) (μmol/kg)	ETA Binding assay IC50 (nM)	
BMS 182874	2.5 - 75	10.9 (4.81,25.03)	150	(Stein et al, 1994)
Bosentan	0.25 - 2.5	1.09 (0.47,2.54)	4.7	(Clozel et al, 1994)
SB 209670	0.025-0.25	0.13 (0.06,0.25)	0.43	(Elliott et al, 1994)

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199P SELECTIVE ET_A AND MIXED ET_A/ET_B RECEPTOR ANTAGONISTS INHIBIT BALLOON CATHETER-INDUCED SMOOTH MUSCLE CELL PROLIFERATION IN THE RAT THORACIC AORTA

D.J. Hele, M. Birrell, R.C. Bush, D.A. Flynn, T.J. Brown & A.G. Roach.
Rhône-Poulenc Rorer, Dagenham Research Centre, Rainham Road South, Dagenham, Essex.

The proliferation of arterial smooth muscle cells is recognised as an important factor in the genesis and development of atherosclerotic lesions and has been implicated in the restenosis of coronary arteries following angioplasty (Liu et al., 1989). Endothelin-1 (ET-1) is a potent vasoactive agent whose effects are mediated by two receptor subtypes, the ET_A and ET_B receptor located on vascular smooth muscle. ET-1 has also been shown to be a potent mitogen for smooth muscle cells *in vitro* (Hirata et al., 1989). Douglas et al (1994) using the mixed receptor antagonist SB 209670 have shown a reduction in lesion size in the rat carotid artery 14d after a balloon injury. In this study we have examined the effects of an ET_A receptor selective antagonist, BMS 182874 (Stein et al., 1994), and a mixed ET_A and ET_B antagonist, Bosentan (Ro 47-0203) (Clozel et al., 1994) on balloon catheter induced smooth muscle cell proliferation in the rat thoracic aorta.

Male Sprague-Dawley rats (380-420g) were anaesthetised with a combination of fentanyl-droperidol (0.5 ml/kg s.c.) and alphaxalone / alphadolone (0.6 ml/kg of a 1/3 dilution i.v.), a 2-F balloon catheter was introduced into a femoral artery and passed to the aortic arch, it was then inflated with 0.25 ml of air and withdrawn to the aortic-iliac bifurcation. This procedure was repeated three times, the balloon was then deflated, removed and the wound closed. Groups of 9-10 rats received vehicle or compound orally b.i.d. for 3d starting 18h prior to catheterisation. Forty seven hours after catheterisation rats were given [3H]-thymidine (0.5 mCi/kg s.c.) and 1h later they were killed and the thoracic aorta removed. The intima/media was stripped from the adventitia and incorporation of [3H]-thymidine into DNA of the intima/media was measured. DNA was assayed (Burton, 1956), and tritium incorporation was measured by liquid scintillation counting. Results are expressed as dpm/µg of DNA (mean ± sem). A separate study showed that the incorporation of [3H]-thymidine into the intima/media of sham operated rats was minimal (2.55 ± 0.45 dpm/µg DNA, n=12). This study shows that both the selective ET_A receptor antagonist, BMS182874, and the mixed ET_A/ET_B receptor antagonist, Bosentan, inhibit [3H]-thymidine incorporation in the balloon injured rat thoracic aorta (Table 1). There was no effect on other fast proliferating tissues, bone marrow, testis and duodenum (testis results shown in Table 1). This data suggests a role for ET-1 in the proliferative response to injury, and further suggests that ET receptor antagonists may be of use in the treatment of restenosis following angioplasty.

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Table 1. Antagonist induced inhibition of [3H]-thymidine incorporation in the balloon injured rat thoracic aorta and non injured testis.

			Thoracic aorta		Testis	
Compound	Dose (mg/kg p.o.)	n	Mean ± sem (dpm/µg DNA)	% change from control	Mean ± sem (dpm/µg DNA)	% change from control
Control	-	9	50.7 ± 5.7	-	21.1 ± 0.4	-
BMS 182874	30	9	22.8 ± 1.4**	-55 ± 6%	20.9 ± 0.9	-0.6 ± 4%
Bosentan	30	10	32.7 ± 3.2*	-35 ± 10%	24.5 ± 1.2	+16 ± 5%

* $P < 0.05$, ** $P < 0.001$ (One way analysis of variance with a correction for multiple comparisons using Dunnett's critical values).

200P EFFECTS OF ARGATROBAN, A SPECIFIC THROMBIN INHIBITOR, IN A CANINE MODEL OF CORONARY ARTERY THROMBOSIS

N. Duval, C. Lunven, A. Grosset, D.P. O'Brien, C.N. Berry & S.E. O'Connor, Department of Preclinical Research, Synthelabo Recherche, 31 Avenue P.V. Couturier, 92225 Bagneux, France

Thrombin plays a key role in blood coagulation and is an important mediator of platelet aggregation. Hence thrombin inhibitors have potential in the treatment of a variety of thrombotic disorders. We have studied the effects of argatroban, a specific, direct, active-site thrombin inhibitor in a dog model of unstable angina (Folts, 1991) in which cyclic blood flow variations (CFV) are established following intimal damage and critical stenosis of the circumflex coronary artery. A comparison was made with heparin which inhibits thrombin indirectly following association with antithrombin III.

Male Foxhound dogs (22 - 35 kg) were anaesthetised with pentobarbitone and prepared for measurement of blood pressure, heart rate and ECG. The left circumflex coronary artery was exposed and fitted with an electromagnetic flow probe to measure blood flow. CFV were produced by controlled clamping of the artery and application of a fixed critical stenosis using an appropriately sized Lexan constrictor. CFV were monitored throughout the experiment, which involved a 60 min control period, a 60 min treatment infusion period and a 60 min recovery period. The drug treatments used were argatroban 30, 100 µg kg^{-1} min^{-1} i.v., heparin 5, 15 IU kg^{-1} min^{-1} or their vehicles (n = 6 - 8 per group). Blood samples were taken at intervals throughout the study to measure drug effects on blood coagulation parameters ex vivo, notably, the activated partial thromboplastin time (aPTT).

Argatroban reduced CFV amplitude overall and, in certain dogs, eliminated CFV entirely. Heparin had similar effects but these did not reach statistical significance. Mean reductions in the number of "large" CFV were: argatroban 30 µg kg^{-1} min^{-1} -29%, argatroban 100 µg kg^{-1} min^{-1} -63% ($p < 0.05$), heparin 5 IU kg^{-1} min^{-1} -28%, heparin 15 IU kg^{-1} min^{-1} -53%. At the highest doses used, both compounds produced large increases in minimum coronary blood flow; argatroban 2.1 ± 0.9 ml min^{-1} → 16.3 ± 4.5 ml min^{-1} ($p < 0.05$), heparin 6.8 ± 2.0 ml min^{-1} → 17.5 ± 4.6 ml min^{-1} ($p < 0.05$). Analysis of the coagulation parameters revealed differences between the two drugs. Argatroban produced modest dose-related increases in aPTT (10.7 ± 0.4 → 22.6 ± 1.0 sec after 30 µg kg^{-1} min^{-1}, 9.9 ± 0.3 → 33.4 ± 4.1 sec after 100 µg kg^{-1} min^{-1}, $p < 0.001$), whereas heparin caused a marked anticoagulant response (aPTT 10.3 ± 0.2 → 169.9 ± 42.0 sec after 5 IU kg^{-1} min^{-1}, 10.6 ± 0.4 → > 300 sec after 15 IU kg^{-1} min^{-1}, $p < 0.001$).

Hence, argatroban demonstrates significant antithrombotic activity in a canine model of unstable angina. This activity was achieved at doses which produced only modest anticoagulant effects. By contrast, doses of heparin necessary to produce equivalent antithrombotic effects resulted in marked systemic anticoagulation. The capacity of argatroban, but not heparin, to inhibit clot-bound thrombin in vitro (Berry et al., 1994) may provide a mechanistic explanation for its superiority in the present study.

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201P α-THROMBIN STIMULATES TNFα AND IL-8 RELEASE FROM THP-1 CELLS BY A NON-PROTEOLYTIC MECHANISM

A. M. Deakin, D. J. Longridge & A. N. Payne, Systems Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS

The serine protease, α-thrombin, in addition to having a pivotal role in blood coagulation, has chemotactic, mitogenic, and other properties consistent with it also being an important mediator of inflammation (Church & Hoffman, 1994). In particular, α-thrombin synergises with bacterial endotoxin in stimulating the secretion of the cytokines interleukin (IL)-1 and tumour necrosis factor alpha (TNFα; Jones & Geczy, 1990; Hoffman, 1993). In the present study we investigated the ability of α-thrombin to directly stimulate cytokine release in vitro from THP-1 cells, a human monocytic cell line.

Cells were incubated with human α-thrombin for 4 h at 37°C, following which the cell suspension was centrifuged (10,000g; 2 min) and the supernatant assayed for cytokines by ELISA. α-Thrombin (1-10 Uml^{-1}) stimulated a concentration-related release of IL-8 and TNFα but not IL-1β or IL-6. A significant increase in IL-8 (5.3 ±1.4 ng $2x10^6$ $cells^{-1}$, $P<0.05$, n=3) and TNFα release (0.37±0.08 ng $2x10^6$ $cells^{-1}$, $P<0.001$, n=5) was stimulated by thrombin (10 Uml^{-1}) compared to untreated cells where IL-8 was less than 0.4 ng $2x10^6$ $cells^{-1}$ and TNFα was below detection. The release of IL-8 and TNFα by α-thrombin was unaltered following pre-incubation with polymyxin B (10 μg ml^{-1}) demonstrating that contamination with bacterial endotoxin was not responsible for this effect.

The release of TNFα by α-thrombin was inhibited in a concentration-related manner by PGE_2 (0.1-100 nM, IC_{50} 10 nM), suggesting an effect of α-thrombin on cytokine gene transcription. Cytokine release was not dependent on the intrinsic proteolytic activity of α-thrombin, as inactivation of this property with phenylmethylsulphonyl fluoride, while abolishing the pro-aggregatory actions of α-thrombin (10 Uml^{-1}) on human washed platelets, was without effect on IL-8 and TNFα release. The proteolytic-independent release of IL-8 and TNFα by α-thrombin may reside in the RGD (Arg-Gly-Asp) sequence at residue 187-189 on the B chain. The RGD motif is a ligand for various integrins, including CD11b which is present on monocytic cells (Albeda & Buck, 1990). Fibrinogen (100 μg ml^{-1}), which also contains the RGD sequence at residues 95-97 and 527-574 on each of its 2 α-chains, also released IL-8 (9.7±3.6 ng $2x10^6$ $cells^{-1}$, n=3) and TNFα (1.39±0.142 ng $2x10^6$ $cells^{-1}$, $P<0.001$, n=3). Furthermore, RGDS peptide (3mM) also released IL-8 (1.2 ng $2x10^6$ $cells^{-1}$, n=2) and TNFα (0.21±0.06 ng $2x10^6$ $cells^{-1}$, n=3).

The present results support the concept that α-thrombin may be pro-inflammatory via a non-proteolytic mechanism, linked to an interaction of the RGD motif with glycoprotein integrins on the cell surface.

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202P LIPOPOLYSACCHARIDE PRETREATMENT ENHANCES EOSINOPHIL ACCUMULATION IN RESPONSE TO INFLAMMATORY MEDIATORS IN GUINEA-PIG SKIN

D.M.T. Macari, M.M. Teixeira & P.G. Hellewell, Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

Recent evidence has implicated bacterial endotoxins, by virtue of their presence on particulate allergen, with an enhancement of the allergic inflammatory response in diseases such as asthma (Michel *et al.*, 1991). Despite the well documented effects of endotoxins on neutrophils (PMN) and macrophages, effects of endotoxins on eosinophils (Eos) have only recently been investigated. *In vivo*, Eos accumulate to high doses of bacterial lipopolysaccharide (LPS) administered in both guinea pig skin (Weg *et al.*, 1995) and the rat pleural cavity (Bozza *et al.*, 1993). The aim of this study was to determine whether intradermal (i.d.) injection of low doses of LPS in guinea pig skin may modulate the inflammatory response to other mediators.

Eos, induced in the peritoneal cavity of guinea pigs by horse serum, were separated on a discontinuous Percoll gradient and labelled with [^{111}In] (Faccioli *et al.*, 1991). In some experiments, casein (5%) elicited peritoneal PMN were also studied. Doses of LPS were administered i.d. into the shaved dorsal skin of recipient guinea pigs up to 4 h prior to i.v. injection of [^{111}In]-labelled Eos or PMN (both 2-5 x 10^6 cells/animal) and [^{125}I]-human serum albumin. Inflammatory mediators were injected immediately i.d.. After a 1-2 h period, animals were killed and skin sites punched out and counted in a gamma-counter. Time course experiments showed that maximum accumulation of both [^{111}In]-Eos and [^{111}In]-PMN and local oedema formation occurred after 1 h pretreatment with LPS. This 1 h pretreatment was, therefore, adopted for all subsequent experiments.

LPS was approximately 30x less potent for Eos accumulation compared to PMN accumulation (threshold dose for significant increase above saline control injection being 1 ng/site for PMN accumulation, $P<0.05$, n=4, compared with 30-1000 ng/site for Eos accumulation, $P<0.05$, n=4). Thus, there was considerable variation in [^{111}In]-Eos number accumulating to LPS between Eos preparations, despite comparable responses to zymosan activated plasma (ZAP). Significant oedema formation above saline control occurred at 1 μg LPS/site ($P<0.001$, n=12). Injection of saline, 3, 10 and 30 ng LPS/site yielded, respectively, 336±67, 547±69, 759±148 and 1212±279 ($P<0.05$ cf saline) [^{111}In]-Eos/site (mean ± s.e.mean, n=11) and 4.5±0.3, 6.0±0.7, 7.0±0.7 and 8.2±1.1 μl plasma/site as an index of local oedema formation (n=11). Injection of 30% ZAP into saline pretreated sites induced 8180±1312 [^{111}In]-Eos/site (n=11) and 26±3 μl plasma/site (n=10). However, injection of 30% ZAP into skin sites pretreated with LPS for 1 h showed enhanced [^{111}In]-Eos accumulation and oedema formation. (Table).

	% increase in ZAP-induced responses		
	3ng LPS	10ng LPS	30ng LPS
[^{111}In]-Eos	22±16	36±17*	51±20*
oedema (μl plasma)	29±16	36±14*	41±16*

Values are mean±s.e.m for 10-11 experiments. *$P<0.05$.

In conclusion, our results suggest LPS pretreatment of skin sites *in vivo* can synergise with inflammatory mediators to enhance the inflammatory response. This may have implications for the pathogenesis of allergic inflammatory diseases such as asthma.

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203P LOCAL AND SYSTEMIC EFFECTS OF DEXAMETHASONE ON ACUTE INFLAMMATION IN GUINEA-PIG SKIN

M.M. Teixeira & P.G. Hellewell, Department Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY.

Steroids are the therapy of choice for allergic diseases, such as asthma and allergic dermatitis. The mechanism of action of steroids, which are beneficial in these diseases, is not entirely understood, but the ability of steroids to inhibit eosinophil accumulation *in vivo* may explain part of their efficacy. In this study, we have evaluated and compared the effects of local and systemic dexamethasone treatment on eosinophil accumulation and local oedema formation in guinea pig skin.

The accumulation of ^{111}In-eosinophils and ^{125}I-albumin leakage in response to i.d. injection of inflammatory agents, and in a passive cutaneous anaphylactic (PCA) reaction, were measured simultaneously in guinea pig skin (Teixeira *et al*, 1993). Dexamethasone was administered systemically (4 mg kg^{-1}, i.v.) or locally (10^{-10} to 10^{-8} mol per site, i.d.) 150 min prior to the i.d. injection of the inflammatory stimuli. Oedema formation (μl of plasma) and ^{111}In-eosinophil accumulation were assessed after 2 h.

When given locally, dexamethasone (10^{-8} mol per site) significantly inhibited oedema formation induced by histamine (2.5×10^{-8} mol per site: control, 46.7 $\pm$ 6.7 μl; treated, 31.9 $\pm$ 3.7 μl; $P < 0.05$, n= 5) or by PAF (10^{-9} mol per site: control, 73.6 $\pm$ 6.9 μl; treated, 52.7 $\pm$ 6.8 μl; $P < 0.05$, n= 5). Lower doses of dexamethasone (10^{-10} and 10^{-9} mol per site) had no significant effect on oedema formation. Oedema formation elicited in the PCA reaction was not significantly altered by local treatment with dexamethasone (control, 41.9 $\pm$ 3.9 μl; treated, 29.9 $\pm$ 4.2 μl; n= 4, P= 0.08). Similarly, ^{111}In-eosinophil accumulation induced by PAF, histamine or in the PCA reaction was not affected by local dexamethasone. In contrast, systemic treatment with dexamethasone (n= 5-6) significantly inhibited ^{111}In-eosinophil accumulation induced by PAF (10^{-10} and 10^{-9} mol per site), zymosan-activated plasma (ZAP, 10 to 100%) and in the PCA reaction (0.01 to 1.0 μg of antigen per site) by up to 70 % (e.g. PCA 0.01 μg antigen: control, 1514 $\pm$ 428 ; treated, 403 $\pm$ 99; PAF 10^{-9} mol per site: control, 3319 $\pm$ 905; treated, 1511 $\pm$ 350; ZAP 30%: control, 3362 $\pm$ 1003; treated, 1385 $\pm$ 398 ^{111}In-eosinophils per site; $P < 0.01$). Interestingly, oedema formation induced by PAF and ZAP was unaltered by systemic treatment with dexamethasone and oedema formation in the PCA reaction was inhibited by only up to 40% (e.g. PCA 1 μg antigen: control, 50.8 $\pm$ 9.9 μl; treated, 31.0 $\pm$ 5.2 μl; $P < 0.05$).

In guinea pig skin, locally administered dexamethasone preferentially inhibited oedema formation (possibly by inhibiting endothelial cell function) while systemic treatment had greater inhibitory effect on ^{111}In-eosinophil accumulation (possibly by inhibiting eosinophils).

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Teixeira, M.M., Williams, T.J. & Hellewell, P.G. (1993) *Br. J. Pharmacol.* **110**: 416-422.

204P SYNOVIAL FIBROBLAST CHEMOKINE PRODUCTION: A ROLE FOR PROTEIN KINASE C

N.J. Jordan, M.L. Watson & J. Westwick. Department of Pharmacology, University of Bath, Bath, U.K. BA2 7AY.

Cultured human synovial fibroblasts secrete several target cell specific chemotactic peptides in response to stimulation with IL-1 or TNFα. These chemotactic cytokines, now known as chemokines (Lindley *el al.*, 1993), which include IL-8, MCP-1 (monocyte chemotactic protein-1) and RANTES, provide one of the signals responsible for the accumulation and activation of selected populations of inflammatory cells in the synovial cavity in rheumatoid arthritis. We have investigated whether protein kinase C (PKC) plays a role in the regulation of chemokine expression in cultured synovial fibroblasts (passages 4-17) obtained from patients with rheumatoid or osteo-arthritis. IL-8 peptide in supernatants from ~1×10^4 cells cultured for 20h was measured by specific ELISA, untreated cells produced <0.5 ng/ml IL-8. mRNA was measured by Northern analysis using digoxigenin labelled oligonucleotide probes. None of the PKC inhibitors significantly affected cell viability as assessed by trypan blue staining.

The non-selective PKC inhibitor staurosporine (1-100nM), produced a bell-shaped enhancement of IL-1 (3ng/ml) induced IL-8 generation (n=3). 100nM staurosporine increased IL-1 induced IL-8 from 93$\pm$1.5ng/ml to 222$\pm$96ng/ml (mean $\pm$S.D.), concurrent with a 3x increase in IL-8 mRNA expression.

A specific PKC inhibitor, chelerythrine chloride (Herbert *et al.*, 1990) also caused a small concentration dependent (0.01-3μM) increase in IL-1 induced IL-8 production from 40.1$\pm$9.1 ng/ml (n=4) with IL-1 (3ng/ml) alone to 55.8$\pm$12.8 ng/ml with chelerythrine chloride (3μM). A 1.7x increase in IL-8 mRNA expression was also induced by 1μM chelerythrine chloride. In contrast, Ro 31-8220 (Ro) and GF 109203X (GF), two selective PKC inhibitors of the substituted bisindolylmaleimide family (Davies *et al.*, 1992; Toullec *et al.*, 1991) both inhibited IL-1 induced IL-8 peptide production in a concentration dependent manner. Ro was 10x more potent than GF, with an IC_{50} of 1μM (n=3). IL-1 (3ng/ml) induced the production of 50$\pm$5.8 ng/ml IL-8 (n=3) which was reduced to 0.43$\pm$0.4 ng/ml (n=3) by 10μM Ro. TNFα induced IL-8 production was similarly completely inhibited by 10μM Ro. 3μM Ro also inhibited IL-1 and TNFα induced RANTES, MCP-1 and IL-8 mRNA expression by 73$\pm$12%, although lower concentrations of Ro (0.3μM) increased the expression of IL-8, MCP-1 and RANTES mRNA.

We propose that a protein kinase, possibly an isoform of PKC or another unidentified kinase which is inhibited by the bisindolylmaleimides, may be important in the regulation of chemokine expression by IL-1 and TNFα. Hence bisindolylmaleimide compounds may also be useful in the design of novel anti-inflammatory agents.

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205P THE EFFECTS OF TRANEXAMIC ACID, AN INHIBITOR OR FIBRINOLYSIS, ON LIPOPOLYSACCHARIDE-INDUCED DISSEMINATED INTRAVASCULAR COAGULATION IN THE ANAESTHETISED RAT

A.J. Ford & D.J. Longridge. Systems Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS, U.K.

Disseminated intravascular coagulation (DIC), initiated by stimulation of the coagulation cascade, results in the formation of fibrin-based thrombi which may become trapped in the microvasculature, leading to tissue ischaemia and multiple organ failure. The present study now evaluates whether an anaesthetised rat model of lipopolysaccharide (LPS)-induced DIC, which expresses a heparin-susceptible primary thrombotic component (Ford & Longridge, 1993), also expresses the secondary fibrinolytic component of DIC. In addition the effects of a fibrinolytic inhibitor, (tranexamic acid, AMCA), on altered organ susceptibility or increased microthrombosis, was investigated.

DIC was induced by a 10 mg/kg/h i.v. infusion (1h) of LPS from E.coli. Blood samples were taken to determine total white blood cell and platelet counts. Four hours after LPS infusion, organs were removed and assessed for ^{125}I-labelled fibrin content. AMCA was given as a constant 100 mg/kg/h i.v. infusion throughout the protocol beginning 15 min prior to LPS. Control animals received a constant saline (0.9% w/v) infusion (0.5 ml/hr i.v.).LPS treatment caused significant fibrin deposition in spleen, liver and lung compared to saline-treated controls (Table 1). Co-infusion of AMCA with LPS increased the DR in these tissues and also caused significant deposition in heart and kidney, tissues which are not usually susceptible to fibrin accumulation. AMCA had no effect on fibrin deposition when infused with saline, compared to saline/saline controls.

Table 1. ^{125}I-Fibrin deposition ratio (compared to final blood counts)

Tissue	Saline/Saline	Saline/LPS	AMCA/Saline	AMCA/LPS
Spleen	0.17 ± 0.01	0.48 ± 0.18 [a]	0.18 ± 0.003	1.03 ± 0.35 [c]
Liver	0.18 ± 0.01	0.43 ± 0.07 [a]	0.19 ± 0.01	0.79 ± 0.12 [b,c]
Lung	0.21 ± 0.01	0.32 ± 0.03 [a]	0.25 ± 0.02	0.40 ± 0.03 [c]
Heart	0.15 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.23 ± 0.02 [b,c]
Kidney	0.14 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	1.80 ± 0.68 [b,c]

Data are mean ± sem, n=5-6 per group. a = P<0.05 vs sal/sal; b = P<0.05 vs sal/LPS; c = P<0.05 vs AMCA/sal; Mann-Whitney U test.

LPS produced a profound sustained leukopenia (-38 ± 7%) which was unaffected by co-infusion of AMCA (-44 ± 4%). Similarly, AMCA had no effect on the thrombocytopenia induced by LPS. AMCA alone had no significant effect on either white cell or platelet counts compared to saline/saline controls. Thus, altered fibrinolytic capacity did not significantly affect the cellular responses characteristic of LPS-induced DIC.

These results indicate that AMCA exacerbates the fibrin deposition induced by LPS in susceptible organs. That AMCA also increases the susceptibility of the kidney and heart which are usually unaffected by fibrin deposition indicates that secondary fibrinolysis is manifest in this model of DIC and is normally able to maintain microvascular patency in these organs.

Ford, A.J. & Longridge, D.J., (1993). Br. J. Pharmacol., **110**: 131P.

206P INVOLVEMENT OF PROTEIN KINASE G IN VASCULAR RELAXATION INDUCED BY THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IV INHIBITOR, ROLIPRAM

A.E. Eckly, J.-C. Stoclet & C. Lugnier, Université Louis Pasteur de Strasbourg, Laboratoire de Pharmacologie et Physiopathologie Cellulaires, CNRS URA 0600, B.P. 24, 67401 Illkirch, France.

The cyclic AMP specific phosphodiesterase IV (PDE IV) is involved in the regulation of vascular tone, for its selective inhibition by rolipram induces relaxation in the rat aorta with functional endothelium (Komas *et al.*, 1991). As expected from its selectivity for PDE IV, rolipram is able to enhance the cyclic AMP level in this tissue, without altering the cyclic GMP level (Eckly & Lugnier, 1994). The aim of the present work was to study the implication of protein kinases in rolipram-induced relaxation in the rat aorta.

Aortic rings with or without endothelium, from male Wistar rats (450g), were mounted in a myograph filled with a Krebs solution (in mM:NaCl 118, KCl 4.7, $CaCl_2$ 1.25, KH_2PO_4 1.14, $MgSO_4$ 1.19, glucose 10, $NaHCO_3$ 25) maintained at 37°C and oxygenated with 95% O_2 and 5% CO_2. The presence or absence of functional endothelium was verified by the relaxing effect or the lack effect of acetylcholine (1μM) in rings precontracted with noradrenaline (1μM). The role of protein kinase G (PKG), protein kinase A (PKA) and protein kinase C (PKC) was studied using selective inhibitors: Rp-8Br-cGMPS (10 μM), H-89 (1μM) and chelerythrine (10 μM), respectively (Butt *et al.*, 1990; Chijiwa *et al.*, 1990; Herbert *et al.*, 1990). Rp-8Br-cGMPS and H-89 were added 30 min and chelerythrine 1 hour before addition of 1μM noradrenaline. Then rolipram was added cumulatively in order to construct concentration-response curve. Results are mean ± s.e.mean of n experiments.

At the highest concentration which could be used because of its limited solubility (100 μM), rolipram decreased by 40.2 ± 6.0 % (n=22) the tone induced by noradrenaline in rings with endothelium. No significant relaxation was obtained in the absence of endothelium. In rings with endothelium, concentration-response curves to rolipram was neither significantly modified by chelerythrine nor by H-89. On the contrary, incubation with Rp-8Br-cGMPS induced a significant inhibition of the responses to all concentrations of rolipram used. Rp-8Br-cGMPS (10 μM) reduced the relaxing effect of rolipram (100 μM) to 16.8 ± 4.7 % of pre-contraction level (n=9, P< 0.05 compared by Student's t test to rolipram alone). No effect of protein kinase inhibitors was seen without endothelium.

These results show that PKG rather than PKA or PKC is involved in rolipram-induced vascular relaxation. Since rolipram only increased cyclic AMP content with no effect on cyclic GMP content, these data are in agreement with results showing a cross-activation of PKG by cyclic AMP (Lincoln *et al.*, 1990). Nevertheless, the endothelium-dependence of the relaxing effect of rolipram requires further investigations.

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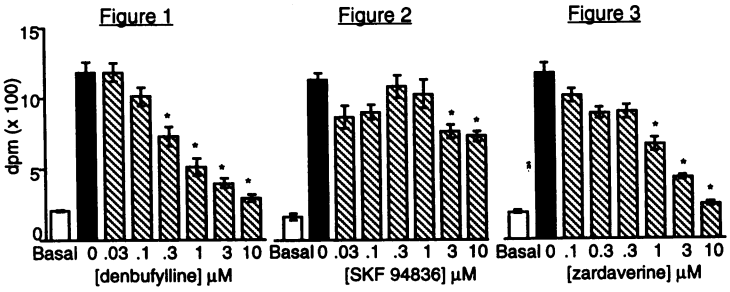
207P EFFECT OF ISOZYME SELECTIVE PHOSPHODIESTERASE (PDE) INHIBITORS ON PHYTOHAEMAGGLUTININ (PHA)-STIMULATED MURINE THYMUS CELL PROLIFERATION

Katharine H. Banner, Bernadette Bertin*, Indres Moodley*, Clive P. Page, Dept. of Pharmacology, King's College, Manresa Road, London SW3 6LX. * Institut de Recherche Jouveinal, Paris, France.

There is evidence to suggest that T lymphocytes play a key role in the pathogenesis of allergic and inflammatory diseases (Azzawi *et al.*, 1992). Since intracellular cAMP is an essential regulator of lymphocyte proliferation (Coffey, 1988) and it has been shown that type IV PDE isozyme inhibitors possess anti-inflammatory activity (Lowe & Cheng, 1992), the effect of selective PDE isozyme inhibitors on the PHA-stimulated proliferation of murine thymus cells was examined.

Thymus cell preparations were isolated (Kruisbeek, 1991) and resuspended in Spinner modified essential media containing glutamine (2mM), non-essential amino acids (1%v/v), sodium pyruvate (1mM) and penicillin (100Uml^{-1}) streptomycin (100μgml^{-1}), referred to as medium A. The cell concentration was adjusted to 10^5 cells per well in medium A containing 10% Ultroser (Jones Chromatography) and cells were stimulated with PHA (0.5-10μgml^{-1}). A dose of 1μgml^{-1} was selected since it produced a submaximal stimulation of proliferation. Cells were stimulated with PHA (1μgml^{-1}) in the presence of a PDE inhibitor (0.01-10μM) for 24h at 37°C in a 95% air, 5% CO_2 atmosphere. [^{3}H]-thymidine (0.1μCi per well) was then added and cells were incubated for a further 24h before being harvested onto glass fibre filters and counted in a scintillation counter. All assays were performed in triplicate and each drug was examined in three experiments. Results in Figures 1-3 are expressed as mean ± s.e.mean and data was analysed by Dunnett's test (*$P < 0.05$ vs. control).

The type IV PDE inhibitor, denbufylline (Figure 1) and the mixed III/IV inhibitor, zardaverine (Figure 3) produced a dose-related inhibition of [^{3}H]-thymidine incorporation. This inhibition attained statistical significance at concentrations of 0.3 and 1μM respectively. The type III PDE inhibitor, SK&F 94836 (Figure 2) inhibited [^{3}H]-thymidine incorporation at a dose of 3μM. Although PDE III and IV isozymes are present in lymphocytes, these results suggest that the type IV PDE isozyme is in the main involved in the control of murine thymus cell proliferation.



K.H.Banner is supported by the M.R.C.

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208P FURTHER INVESTIGATION OF THE MECHANISM OF ACTION OF GS-389: A THROMBOXANE A_2 ANTAGONISTIC ACTION IN PLATELET AND VASCULAR SMOOTH MUSCLE

K. C. Chang, W. S. Chong & H. K. Noh, Department of Pharmacology, College of Medicine, Cardiovascular Research Institute, Gyeongsang National University, Chinju, Korea (Introduced by W. C. Bowman)

Tetrahydroisoquinoline (THI) compounds have various pharmacological actions in the cardiovascular system. Trimetoquinol and its analogs, THI class, are competitive antagonists for endoperoxide/thromboxane A_2-mediated responses in human platelet and rat aorta (Shin et al., 1993). We reported that GS 389 (Fig. 1), an analog of trimetoquinol, showed vasodilating action without cardiac inotropic action in the rat due to Ca^{2+} inhibitory action (Chang et al., 1994). In the present study, further characterization of the pharmacological properties of GS 389 was assessed in isolated rat trachealis and thoracic aorta, in swine coronary artery and in human platelets. In isolated trachealis GS 389 inhibited carbachol (0.1 μM)-, and high K^+ (65.4 mM)-induced contractions in a dose-dependent manner. GS 389 noncompetitively inhibited muscarinic receptor-mediated contraction in rat trachealis. GS 389 inhibited Ca^{2+}-induced contraction on rat trachealis in Ca^{2+}-free media. Furthermore, 0.1 mM carbachol-induced phasic contraction was inhibited by pretreatment of GS 389 in Ca^{2+}-free media, suggesting that GS 389 can enter into the cytoplasm, where it may exerts secondary action on internal sites of the muscle, sarcoplasmic reticulum.

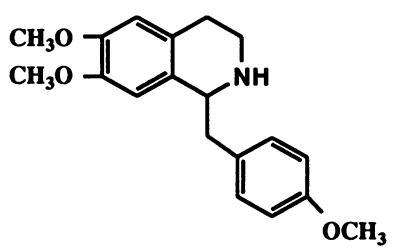


Fig.1. Chemical structure of GS 389

GS 389 blocked U46619-induced aggregatory responses in human platelets, and competitively inhibited U46619-induced contraction of rat aorta and swine coronary artery with pA_2 value of 5.23 ± 0.12 and 5.56 ± 0.14, respectively.

In rat thoracic aorta, phosphatidylinositide (PI) turnover by U46619 was inhibited by pretreatment of GS 389. The IC_{50} value for antagonistic potency of U46619-induced responses in rat aorta was 2.7 times less than that in human platelets.

It is concluded that GS 389 interferes with Ca^{2+}-influx and release of intracellulary stored Ca^{2+}, which may be, at least in part, responsible for bronchodilatation action, and blocking action of PI turnover by U46619 in rat aorta may account for thromboxane A_2 (U46619) antagonistic action in vascular smooth muscle.

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209P EFFECT OF FLORIFENINE ON ARACHIDONIC ACID METABOLISM AND SOME LEUKOCYTE FUNCTIONS

M.J. Sanz, G. Bustos, M.L. Ferrándiz, M. Payá & M.J. Alcaraz. Department of Pharmacology, University of Valencia, Avda. Vicent Andrés Estellés s/n, 46100 Burjasot, Valencia, Spain.

Florifenine, 2-(1-Pyrrolidinyl)ethyl *N*-[7-(trifluoromethyl)-4-quinolyl] anthranilate is an anti-inflammatory agent (Foguet *et al.*, 1982) which has recently undergone clinical trials (Llerena *et al.*, 1991). In order to establish its mechanism of action we have investigated the effect of florifenine on superoxide generation, degranulation and eicosanoid release by human neutrophils. We have also assessed the activity of florifenine on eicosanoid production in human whole blood *in vitro* and in the mouse air pouch.

Human neutrophils were purified by ficoll-hypaque sedimentation. More than 95% of the leukocytes were polymorphonuclear neutrophils and cell viability was greater than 95% (trypan blue exclusion test). Superoxide generation was estimated as the superoxide dismutase inhibitable reduction of cytochrome *c* at 550 nm (Ferrándiz *et al.*, 1994). For elastase release the cells ($2.5x10^6$/ml) were stimulated with 12-*O*-tetradecanoylphorbol acetate (TPA, 1 μM) and incubated for 10 min at 37°C. After centrifugation at 1200 g at 4°C, supernatants were incubated with N-tert-butoxy-carbonyl-L-alanine p-nitrophenyl ester (200 μM) for 10min at 37°C and absorbances were determined at 414 nm. Eicosanoids release: the cells were suspended at 10^7/ml in phosphate buffered saline (PBS) containing 3 μCi/ml [^{3}H]AA and were incubated with shaking for 1 h at 37 °C. The neutrophils were washed twice with PBS containing 0.05 % (w/v) bovine serum albumin. [^{3}H]AA-labeled cells (10^7/ml) were stimulated with ionophore A23187 (5 μM) for 10 min and the radioactivity in supernatants was quantified by liquid scintillation counting. Release of LTB_4: leukocytes ($5x10^6$/ml) were stimulated with ionophore A23187 (1μM) for 10 min and LTB_4 levels in supernatants were estimated by radioimmunoassay (RIA). Heparinized human whole blood (0.5 ml) was incubated with ionophore A23187 (5μM) for 60 min and LTB_4 and TXB_2 levels were determined by RIA. Airpouch inflammation was studied in Swiss mice (n=6; 25-30 g; Edwards *et al.* 1981). Leukocyte migration into exudates, PGE_2 and LTB_4 levels were determined 4 h after injection of zymosan.

Florifenine gave no significant inhibition of superoxide production but inhibited degranulation with an IC_{50} of 8.6 μM (95% confidence limits, C.L. 4.5-12.5; n=6), while a direct inhibition of elastase activity was discounted as in experiments with elastase purified from human neutrophils it was not inhibited. Indomethacin failed to decrease superoxide production and inhibited elastase release by 31.9±4.9% (means±s.e. mean, n=6, $P<0.05$) at 100 μM. Florifenine (50 μM) was able to decrease eicosanoid release by [^{3}H]AA-labeled human neutrophils (37.1±2.2 %, n=4, $P<0.01$). Florifenine showed a selectivity for inhibition of the cyclo-oxygenase pathway and a potency on eicosanoid production lower than indomethacin. In human neutrophils florifenine exerted a weak effect on LTB_4 release (19.0±3.5 % inhibition, n=6, 100 μM), while in human whole blood the compound inhibited TXB_2 and LTB_4 production (IC_{50}= 0.03μM, C.L. 0.02-0.06 and 11.4μM, C.L. 3.2-31.6, n=6, respectively). In this system, indomethacin only inhibited TXB_2 release (IC_{50}= 0.02 μM, C.L. 0.01-0.03, n=4). Oral administration of florifenine decreased neutrophil migration into the air pouch and exudate PGE_2 (but not LTB_4), with approximate ID_{50} of 16.2 and 4.2 mg/kg, respectively. Thus, florifenine inhibits the cyclo-oxygenase pathway and in addition, it may exert modulatory effects on neutrophil functions.

We thank Ferrer Internacional for financial support.

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210P POLYMORPHONUCLEAR LEUCOCYTES AND INTIMAL PROLIFERATION IN THE RABBIT CAROTID ARTERY

H. Bult, G.R.Y. De Meyer, D.J.M. Van Put, N. Van Osselaer, M.M. Kockx[2], L.J. Andries[1] & A.G. Herman, Divisions of Pharmacology (UIA) and [1]Physiology (RUCA), Univ. of Antwerp, and [2]Pathology, General Hospital Middelheim, Antwerp, Belgium.

Intimal thickening, a prerequisite for the development of atherosclerosis, can be evoked by surrounding the rabbit carotid artery with a flexible collar (Booth *et al.*, 1989). The intimal proliferation is preceded by an influx of polymorphonuclear leucocytes (PMNs) into the media on day one (Kockx *et al.*, 1992). We investigated whether the transient invasion of PMNs is essential for migration and proliferation of smooth muscle cells in the intima.

As a first approach New Zealand White rabbits (3 kg) continuously received granulocyte colony stimulating factor (G-CSF, 20 $\mu g\ kg^{-1}\ day^{-1}$, gift Dr. B. Agüero, Amgen, USA) or solvent (5% glucose) via osmotic minipumps from 3 days before collar placement until its removal. In a second approach rabbits were treated with a monoclonal antibody (R15.7, 1.5 mg kg^{-1} i.v., gift Dr. R. Rothlein, Boehringer Ingelheim, USA) against the CD18 subunit of β_2 integrins on leucocytes, or mouse IgG (controls) immediately prior to collaring. Silicone collars were placed around the left carotid artery under anaesthesia (Kockx *et al.*, 1992). The right artery was handled identically, but was not collared and served as sham-operated control. After 3 days (migration of smooth muscle cells) to 14 days (migration plus proliferation) the cross-sectional area of the intima was measured by planimetry (Kockx *et al.*, 1992). Subtraction of the area of the sham-operated artery gave the extent of thickening. The results are given as mean±s.e.mean.

G-CSF raised the basal number of circulating PMNs ($2900\pm500\ \mu l^{-1}$, n=12) five (day 0) to twelve (day 12) fold, without elevating intimal thickening on day 3 (control $10\pm3\ \mu m^2\ 10^3$, n=6; G-CSF $9\pm3\ \mu m^2\ 10^3$, n=6) or day 12 (control $20\pm8\ \mu m^2\ 10^3$, n=5; G-CSF $13\pm7\ \mu m^2\ 10^3$, n=6). The anti-CD18 treatment prevented collar-induced PMN adhesion to the endothelium and diapedesis as indicated by confocal laser microscopy on day one. However, intimal thickening on day 3 (control $12\pm5\ \mu m^2\ 10^3$, n=3; R15.7 $11\pm3\ \mu m^2\ 10^3$, n=3), or day 14 (control $20\pm5\ \mu m^2\ 10^3$, n=9; R15.7 $38\pm10\ \mu m^2\ 10^3$, n=7) was not suppressed by the CD18 blockade.

Since both elevation of the circulating PMN number and inhibition of the PMN-endothelial cell interaction failed to influence intimal thickening, the transient invasion of PMNs into the media is apparently without a stimulant effect on the subsequent migration of smooth muscle cells to the intima and their proliferation in the collar model.

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211P CHARACTERISATION OF MCP-1 AND ATP-INDUCED CHANGES IN CYTOSOLIC CALCIUM IN THE HUMAN MONOCYTIC CELL LINE, THP-1

Turner, S.J. and Westwick, J.
Department of Pharmacology, University of Bath, Claverton Down, Bath, Avon, UK.

Monocyte chemotactic peptide 1 (MCP-1) is a member of the chemokine family and a potent and specific monocyte agonist (Yoshimura *et al.*, 1989). We have characterised the MCP-1-induced elevation of cytosolic calcium ($[Ca^{++}]_i$) and compared it to that induced by ATP in the human monocytic cell line THP-1 (Charo *et al.*, 1994).

The cytosolic calcium concentration was determined in fura-2 loaded THP-1 cells as previously described (Ward & Westwick, 1988). MCP-1 (0.125-60 nM) induced a dose related elevation of $[Ca^{++}]_i$ which peaked at 5 secs, and returned to basal levels within 60 seconds. ATP (0.03-3 μM) induced a rapid elevation within 3 secs, which continued to rise over the next 30 sec, then declined over several minutes to a pre-agonist level. The second phase of the ATP-induced response was dose-dependently inhibited by the putative calcium influx inhibitor econazole (70 ± 8.3% at 30 μM, n=5), In contrast the MCP-1 response was not significantly inhibited by econazole (1-30 μM). Chelation of extracellular calcium by EGTA (1 mM) and subsequent calcium (1 mM) readdition had little effect on the MCP-1 $[Ca^{++}]_i$ response but the second phase of the ATP response was almost completely abrogated by EGTA and returned upon calcium readdition. The use of Mn^{++} (300 μM) as a surrogate Ca^{++} ion revealed that 12.5 nM MCP-1 did not induce a significant influx of Mn^{++} (10 ± 5%, $p>0.05$, n=4), compared to that induced by 3 μM ATP (50 ± 6%, n=4) with 100% influx induced by 10 μM ionomycin. The ATP-induced Mn^{++} influx was totally suppresssed by 1 mM Ni^{++}, but was without effect on the very small Mn^{++} influx induced by MCP-1 Furthermore, the MCP-1 induced elevation of $[Ca^{++}]_i$ was very sensitive to pertussis toxin (PT) pre-treatment (16 h). The IC_{50} against MCP-1 was 0.58 ± 0.05 ng/ml, (n=5) compared to ATP which was unaffected by PT pre treatment (0.25-250 ng/ml). In contrast the putative PLC inhibitor U73122 (Smith *et al.*, 1990), was an effective inhibitor of both MCP-1 (IC_{50} 3.0 ± 0.34 μM, n=4) and ATP (IC_{50} 8.2 ± 1.3 μM, n=4) induced elevations of $[Ca^{++}]_i$ Another difference between these elevations of $[Ca^{++}]_i$ was that Calyculin A, a serine phosphatase type II inhibitor (100 nM, n=5) was without effect on MCP-1 induced Ca^{++} elevations but was an effective inhibitor of the second phase of ATP-induced elevation of $[Ca^{++}]_i$.

These results suggest that the MCP-1 induced elevation of $[Ca^{++}]_i$ does not involve a major influx of extracellular calcium nor the activation of a type II serine phosphatase, but does involve pertussis toxin sensitive components and probably the activation of phospholipase C.

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212P THE ROLE OF TNF-α IN THE LOCALIZED SHWARTZMAN REACTION IN RABBIT SKIN

K.E. Norman, T.J. Williams, M. Feldmann[1] & A.G. Rossi[2]. Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London, SW3 6LY and [1]The Kennedy Institute of Rheumatology, London, W6 7DW.

Although the Shwartzman reaction is a poorly understood inflammatory, haemorrhagic and necrotic lesion there is evidence supporting a role for TNF-α in the pathology of the reaction (Brozna, 1990; Ozmen et al., 1994). Here we studied the effects of a soluble bivalent p55 TNF receptor IgG fusion protein (p55-sf2) and the protein synthesis inhibitor, cycloheximide, in the localised Shwartzman reaction (LSR).

New Zealand White rabbits were anaesthetized (ketamine, 30mgkg^{-1} and xylazine, 2mgkg^{-1}, i.m.), the dorsal skin shaved and marked out with 6 replicates of up to 12 treatment sites on a balanced site plan. The LSR was induced by giving i.d. priming injections of endotoxin (*Salmonella typhosa*) at t=0 followed by two i.v. challenge doses of endotoxin (100 μg) at t=20h and t=22h. The above endotoxin i.d. injections were either given alone, or in the presence of p55-sf2 (3μg/site) or cycloheximide (10μg/site). At t=20h the animals were re-anaesthetized (as above) and some sites that had been previously treated with endotoxin were injected i.d. with the above agents. Haemorrhage and oedema formation were determined by measuring the local accumulation of ^{51}Cr-erythrocytes and ^{125}I-albumin, respectively. At t=24h rabbits were killed and skin sites counted for radioactivity. Results are presented as μl/blood for haemorrhage and μl/plasma for oedema formation and are expressed as the mean ± s.e.mean.

In LSR challenged animals, sites that had received i.d. doses of endotoxin at t=0 were haemorrhagic and oedematous; for example the respective responses to saline were 4±1μl/blood and 11±2μl/plasma, to 10μg/site endotoxin they were 18±3 μl/blood and 44± 5μl/plasma and to 30μg/site endotoxin they were 41±12μl/blood and 72±6 μl/plasma (n=4). When p55-sf2 was co-injected with 10μg/site endotoxin, haemorrhage was reduced to 7±2 μl/blood ($p<0.05$) and oedema formation reduced to 21±6 μl/plasma ($p<0.05$) and with 30μg/site endotoxin the responses were reduced to 13±4 μl/blood ($p<0.05$) and 33±5μl/plasma ($p<0.05$). In separate experiments, when cycloheximide was co-injected with 30μg/site endotoxin, haemorrhage was reduced by 55±9% ($p<0.05$; n=6) whereas oedema formation was not significantly affected.

When p55-sf2 and cycloheximide were injected i.d. at t=20h into sites that had previously been treated with i.d. endotoxin neither haemorrhage or plasma leakage were affected. For example, the responses to 30μg/site endotoxin were 29±8 μl/blood and 48±10μl/plasma when injected alone or 32±9μl/blood and 49±8μl/plasma when co-injected with cycloheximide (n=6). In other LSR experiments, responses to 30μg/site endotoxin were 22±4 μl/blood and 41±9μl/plasma when injected alone or 26±3μl/blood and 42±8 μl/plasma when co-injected with p55-sf2.

These data indicate that haemorrhage and oedema formation are dependent on TNF-α and local protein synthesis during the priming phase but not during the challenge stage of the LSR.

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[2]Present address; Syntex Research Centre, Edinburgh, EH14 4AP.

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213P FEVER AND INTERLEUKIN-6 (IL-6) RESPONSES TO LOCALISED INFLAMMATION INDUCED IN AN AIR POUCH IN THE RAT

A.J.Miller, G.N.Luheshi, S.J.Hopkins[1] & N.J.Rothwell, School of Biological Sciences, 1.124, Stopford Building, University of Manchester, Manchester, M13 9PT and [1]University of Manchester Rheumatic Disease Centre, Hope Hospital, Salford, M6 8HD.

The subcutaneous (sc) air pouch was first described as a potential model for studying inflammation by Selyé in 1953. Since then, a wide variety of irritant materials have been injected into the pouch in order to study aspects of the local inflammatory process. However, few studies report the effects of these treatments on systemic responses, such as fever. We investigated the possibility of using turpentine, a potent pyrogen when injected intramuscularly (im), in the air pouch to study mechanisms of fever.

Male, Sprague-Dawley rats (275-325g) were anaesthetised with halothane and injected sc with 20ml of sterile air (via a 0.2μm non-pyrogenic hydrophobic filter) into the dorsum, just caudal to the scapulae. A further 10ml of air was injected three days later. On the sixth day, 100μl of turpentine oil (dose determined by preliminary studies) was injected directly into the pouch. Core temperatures were monitored using remote radio-telemetry via pre-implanted abdominal transmitters. Control animals were injected with 0.9% saline. To investigate the kinetics of the peripheral and central IL-6 activity, animals were killed at hourly time-points for 8h. Cerebrospinal fluid (CSF) was taken from the cisterna magna, and blood/plasma by cardiac puncture, for bioassay of interleukin-6. Data were analysed using ANOVA, with a probability of $<5\%$ being considered significant.

Injection of turpentine into the air pouch induced a rise in body temperature which commenced 3h post-injection and reached a peak of $39.9\pm0.1°C$ (mean$\pm$SEM) at 8h, compared to a basal temperature of $37.1\pm0.1°C$ ($p<0.001$) (n=5). These animals exhibited a reduced non-specific stress-induced hyperthermic response compared to animals injected with an equivalent volume of vehicle or turpentine intramuscularly. Plasma IL-6 concentration was elevated 1h after injection of turpentine into the air pouch, and rapidly increased until reaching a peak of 1808 ± 392U/ml, compared to a basal value of 6 ± 2U/ml, at 8h ($p<0.001$). CSF IL-6 concentration was also increased significantly, but exceeded the assay detection limit (3U/ml) only at 4h, before reaching a peak of 9 ± 1U/ml, compared to basal <3U/ml ($p<0.01$), at 7h. Correlation coefficients of $r=0.86$ ($p<0.001$), and $r=0.67$ ($p<0.001$) between core temperature and plasma IL-6 and CSF IL-6 concentrations respectively, suggest a positive relationship between these two variables.

These results support the hypothesis that IL-6 is involved in the development of fever (LeMay *et al* 1990). They also indicate that the sc air pouch may be useful for studying the systemic responses, such as fever, to locally induced inflammation. This model offers several advantages over more commonly used pyrogenic stimuli such as im turpentine or systemic endotoxin, since stress is attentuated in the air pouch model and the locus of action is clearly defined.

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214P INVOLVEMENT OF TUMOUR NECROSIS FACTOR-α IN FEBRILE RESPONSES TO TISSUE INJURY IN RATS: INTERACTIONS WITH INTERLEUKIN-6

A. Stefferl, S. J. Hopkins[1], S. Brouwer, N. J. Rothwell & G. N. Luheshi, School of Biological Sciences, 1.124 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT and [1]University of Manchester Rheumatic Diseases Centre, Hope Hospital, Salford M6 8HD

Interleukin (IL)-1, IL-6 and TNF-α have been proposed as key mediators of fever, which is an important element of the immune response to injury or infection. Injection of IL-6 into experimental animals causes fever (LeMay et al, 1990), plasma concentrations of IL-6 are elevated in febrile animals (LeMay et al, 1990) and the appearance of IL-6 in plasma precedes fever (Bate et al, 1994), indicating its role as a circulating pyrogen. There is also evidence that TNF-α participates in the early phase of the immune response leading to fever. However, the interaction of endogenous TNF-α with other pyrogens, such as IL-6, is not fully understood. In the present study we investigated the involvement of TNF-α in the development of fever resulting from localised inflammation in the rat, and studied IL-6 release during this response.

Male, Sprague Dawley rats weighing 250-350g were used in all experiments. Animals were intra-peritoneally (ip) implanted with radiotransmitters under halothane anaesthesia to allow subsequent measurement of body temperature by remote radio-telemetry (Datasciences, St. Paul, USA). Some animals were also implanted stereotaxically with guide cannulae for intracerebroventricular (icv) injections into the lateral ventricle of the brain under pentobarbitone anaesthesia (60mg/kg, ip). Animals (n=5-10) were injected with control serum or neutralising anti-TNF-α antiserum intravenously (iv, 0.4ml/rat) or icv (3μl/rat) 24h before the beginning of the experiment. Inflammation was induced by intramuscular (im) injection of 10μl turpentine (TRP) into the hindlimb. Control animals were injected with an equal volume of either non-immune serum or 0.9% sodium chloride. Blood was collected by cardiac puncture under terminal halothane anaesthesia, either 6h or 8h after injection of TRP. Plasma was prepared by centrifugation, and the samples analysed for IL-6 by bioassay using B9 hybridoma cells. Results are presented as mean$\pm$sem, and statistical analysis was performed using Student's t-test for IL-6 concentrations and MANOVA for temperature data.

Injection of TRP resulted in fever that commenced 4.5h later and peaked by 8h (38.8 ± 0.2°C compared to 37.6 ± 0.1°C in controls, P=0.01). Treatment with TNF-α antiserum significantly reduced this febrile response (38.1 ± 0.2°C P=0.014 vs TRP). Plasma IL-6 concentrations were significantly increased in animals injected with TRP (103 ± 8 U/ml compared to 32 ± 8 U/ml in controls, P=0.001), but were not reduced by treatment with TNF-α antiserum (132 ± 43 U/ml) at 8h. Animals killed 6h after TRP exhibited only slight fever (37.6 ± 0.2°C vs 36.9 ± 0.1°C in controls $P>0.05$), and this was not significantly attenuated by treatment with antiserum (37.3 ± 0.4°C). Plasma IL-6 concentrations were elevated 6h after TRP (265 ± 44 U/ml vs 13 ± 1 U/ml in controls, P=0.002), and significantly higher than at 8h (P=0.015). IL-6 concentrations at this time were significantly attenuated by treatment with TNF-α antiserum (47 ± 8 U/ml, P=0.004). In a further set of experiments, the role of brain TNF-α was investigated by icv injection of anti TNF-α antiserum. Animals injected with TRP developed fever (38.7 ± 0.2°C compared to 37.3 ± 0.1°C in controls at 8h, $P<0.001$) which was significantly attenuated by icv injection of TNF-α antiserum (37.8 ± 0.2°C, P=0.018).

These results suggest, that endogenous TNF-α in the brain and the periphery is involved in fever induced by tissue injury in the rat, and that these effects may be related to release of IL-6.

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215P DEXAMETHASONE INDUCES AN INCREASE IN INTRACELLULAR- AND MEMBRANE-ASSOCIATED LIPOCORTIN-1 (ANNEXIN-1) IN RAT ASTROCYTE PRIMARY CULTURES

J.D. McLeod & C. Bolton, Pharmacology Department, School of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY

Glucocorticoid steroids have profound anti-inflammatory effects and influence cell differentiation and proliferation (Flower & Rothwell, 1994). A putative mediator of these actions is Lipocortin-1 (LC-1). In this study we investigated the influence of the synthetic glucocorticoid, dexamethasone (Dex.), on the cellular localisation of LC-1 in enriched astrocyte cultures.

Steroid-induced changes in cellular levels of LC-1 were determined by electrotransfer and immunoblotting techniques (Peers *et al.*, 1993). Densitometric quantitation of the staining intensity of LC-1 bands was undertaken and results expressed as % change from control level. Firstly, the effect of culture state on LC-1 expression was studied in medium depleted of steroids. Cells cultured without a medium change for 1-5 days showed a decrease in intracellular LC-1 (65 $\pm$ 9% of day 1 value; mean $\pm$ s.e.mean of daily changes, n=3). However, upon medium replenishment on culture day 5 a dramatic increase in intracellular LC-1 occurred (262 $\pm$ 62% of day 1 value) in conjunction with an increase in proliferation, as measured by [^{3}H]-thymidine uptake (200 $\pm$ 25% of day 1 value). The mitogenic effect of medium replenishment was also found to facilitate the induction of intracellular LC-1 by Dex.(0.1μM) (32 $\pm$ 30% of control compared with 96 $\pm$ 45% after a medium change, n=5). All subsequent experiments were determined one day after medium (steroid-depleted) change. The effect of Dex. on three separate cell fractions; extracellular, intracellular and membrane, was investigated as described previously (Peers *et al.*, 1993).

Dex. (0.1μM) was added for 30min, 2h and 16h. At 30min no change in the levels of LC-1 in the intracellular or membrane fractions (15 $\pm$ 33% and 12 $\pm$ 54% from control, respectively, n=3) was apparent when compared to vehicle control levels. By 2h Dex. had caused an appreciable increase in LC-1 associated with both the intracellular and membrane fractions (35 $\pm$ 10% and 154 $\pm$ 91% of control respectively, n=6). The LC-1 content present in the extracellular fraction did not change (-34 $\pm$ 24%, n=4) from control. Cells in the presence of Dex. for 16h showed no change in extracellular LC-1 (18 $\pm$ 56% from control, n=4), although there was an augmented level present in the intracellular and membrane fractions (17 $\pm$ 7% and 94 $\pm$ 45% from control respectively, n=6).

These results indicate that rat astrocytes, under specific culture conditions, respond to Dex. by raising the content of LC-1 within separate cellular fractions and facilitating the translocation of LC-1 to the membrane.

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216P EFFECT OF A LIPOCORTIN 1 N-TERMINUS PEPTIDE ON NEUTROPHIL KINETICS IN COMPARISON WITH SIALIDASE, FUCOIDIN AND AN ANTIBODY TO CD11b

Jeanette G Harris, Roderick J Flower and Mauro Perretti
Department of Biochemical Pharmacology, The Medical College of Saint Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ.

There is evidence to suggest that the neutrophil (PMN) is an important target for both lipocortin 1 and its N-terminus-derived peptide acetyl 2-26 (Ac2-26). As the activity of the peptide parallels that of a monoclonal antibody to murine CD11b (mAbCD11b) in several *in vivo* models (Perretti *et al.*, 1993), we have now compared its effect to that of compounds known to alter specific PMN functions.

Male Swiss Albino mice (28-32g Tuck, UK) were treated i.v. with bovine serum albumin (BSA) 0.1% in sterile phosphate-buffered solution (PBS; 100 μl), sialidase (0.01 mU), fucoidin (0.3 mg), mAbCD11b (clone 5C6; 500 μg), human Ac2-26 (200 μg) or platelet activating factor (PAF, 30-300 ng). Mice were bled by cardiac puncture 1 h or 2 h after sialidase, fucoidin, mAbCD11b and Ac2-26, or 2 min after PAF. In some mice pharmacological treatments were performed prior to giving PAF. Total white cell counts were determined using a Coulter counter and differential counts were obtained from blood smears stained with Turk's solution. CD11b antigen on PMN surface was determined *in vitro* and *ex vivo* using a whole blood technique. *In vitro*, blood aliquots (200 μl) were incubated in the absence or presence of Ac2-26 (100 ngml^{-1}) for 10 min prior to incubation with PAF for 15 min at room temperature (RT). After 2 washes, samples were stained with the mAbCD11b (60 μgml^{-1}) for 60 min (4°C) and labelled with FITC-conjugated anti-rat IgG Ab (5 min, RT) prior to erythrocyte lysis. The number of CD11b molecules per PMN was determined by flow cytometry. *Ex vivo*, blood samples were prepared from mice treated with i.v. PAF 100 ng and stained with mAbCD11b before flow cytometry. Statistical analysis on original values was performed by ANOVA followed by Bonferroni test for inter-group comparisons or unpaired Student's *t* test when two groups were compared.

PMN circulating levels in untreated mice were 0.65$\pm$0.05 x10^6 ml^{-1} (n=40). Sialidase and fucoidin caused a significant neutrophilia at 2 h (+173% and +261%, respectively, n=8, $p<0.01$). An increase in circulating PMN was also observed by 1h for the mAbCD11b (+137%, n=5, $p<0.05$)., whereas a 2 h neutrophilia was measured for peptide Ac2-26: 2.35$\pm$0.25 x10^6 ml^{-1} PMN, n=6, vs. 0.84$\pm$0.06 x10^6 ml^{-1} PMN, n=10, $p<0.05$. PAF induced a dose-dependent (30-300 ng) neutropenia at 2 min with an ED_{50} of 100 ng (n=16, $p< 0.01$). This effect was inhibited by sialidase and fucoidin (circulating PMN number fell from 70% to 25% and 45%, respectively, n=10, $p<0.05$). This neutropenic response was also attenuated by mAbCD11b (from 68% to 29%, n=10, $p<0.05$) and abrogated by pretreatment with peptide Ac2-26 (n=7, $p<0.05$). *Ex vivo* CD11b expression on PMN was maximal at 2 min after PAF 100 ng i.v. (from 8,522$\pm$1,051 to 14,819$\pm$1,940 CD11b molecules per cell, n=7, $p<0.05$). *In vitro*, PAF induced a dose-dependent (10^{-9}M-10^{-6}M) increase in CD11b expression on mouse PMN, but incubation with peptide Ac2-26 did not alter this up-regulation.

In conclusion, peptide Ac2-26 mimics the action of agents known to affect leucocyte rolling and adhesion *in vivo*. The peptide, however, does not affect stimulated neutrophil CD11b up-regulation *in vitro* suggesting other phenomena are important for its anti-neutropenic and anti-inflammatory action.

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217P LIPOPOLYSACCHARIDE REDUCES LIPOCORTIN 1 BINDING TO RAT PERITONEAL MACROPHAGES

D Hague, RJ Flower, #NJ Goulding & M Perretti
Department of Biochemical Pharmacology and #Department of Rheumatology, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ, U.K.

Lipocortin 1 (LC1) binds to human and murine leucocytes, but this binding capacity is greatly reduced when cells are recovered from an inflammatory site [1,2]. Here, we have studied LC1 binding to rat peritoneal macrophages (Mø) and its modulation by cell activation.

Rat peritoneal cells (>80% Mø) were sedeed in 24-well plates at a concentration of $2x10^6$ Mø per well in RPMI supplemented with 20% foetal calf serum (FCS). After 3h at 37°C, 95% O_2-5% CO_2, adherent cells (>90% Mø) were incubated with or without different concentrations of lipopolysaccharide (LPS; serotype *E. coli* 055:B5; Sigma) in RPMI 5% FCS for a variable length of time (usually 20h). In some cases indomethacin, dexamethasone, BWA4C [3], WEB2086 [4] were added together with LPS. At the end of the incubation, Mø were removed by addition of PBS+3mM EDTA, aliquoted (0.5-1 $x10^6$) in 96-well plates and incubated with graded concentrations of hrLC1 for 1h at 4°C in PBS supplemented with 0.2 % bovine albumin and 1.8 mM calcium (PBC). LC1 molecules bound were detected by incubation with a specific monoclonal antibody (mAb 1B; 20 μgml^{-1}; Biogen Corp. Cambridge, MA) followed by another step with a $F(ab')_2$ fragment anti-mouse IgG conjugated to FITC. Flow cytometry was performed using a FACScan with Lysis II software (Becton Dickinson, UK). The number of LC1 binding sites per cell was estimated by median fluorescence intensity in the FL1 channel with reference to microbeads labelled with standard molecules of FITC. Results are reported as mean±s.e.mean of n experiments. Statistics were applied to raw data.

Dose-dependent (0.01-1 μM) LC1 binding to cultured Mø was observed with an estimate K_d of 100±10 nM (n=4). A total binding site number of 51,747±5,787 (n=25) was measured with 1 μM LC1 versus a non-specific binding for mAb 1B alone of 1,560±235 (n=25). LC1 binding to adherent Mø was not significantly different from the figure obtained with freshly prepared cells: 26,423±7,500 and 17,678±3,160 LC1 molecules bound, respectively (n=3, not significant). Mø activation by incubation with LPS resulted in a concentration-dependent reduction in LC1 binding with an approximate EC_{50} of 1 $ngml^{-1}$ (49.5±3% inhibition, n=11, $p<0.01$) with a maximal inhibition of 79.5±1.1% at 10 μgml^{-1} (n=3, $p<0.05$) This response was not attributable to competition between LC1 and LPS for the putative LC1 binding site, with only 2.5±2% inhibition of LC1 binding with LPS (1 $ngml^{-1}$) when incubations were carried out at 4°C. Mø had to be incubated with LPS (1 $ngml^{-1}$) for at least 5h to observe a significant inhibition in subsequent LC1 (1μM) binding: 35±10% (n=3, $p<0.05$). The LPS (1 $ngml^{-1}$)-induced reduction in LC1 (1 μM) binding capacity was not prevented by Mø incubation with BWA4C (1 μM), indomethacin (1 μM), WEB2086 (5 μM) or dexamethasone (0.1 μM) (data not shown).

In conclusion, LPS down regulates LC1 binding capacity of rat Mø by a mechanism related to cell activation and is a specific and sensitive property of the endotoxin. Further studies are required to better understand the underlying mechanisms.

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218P LIPOCORTIN 1 MEDIATES THE EFFECT OF DEXAMETHASONE ON LEUCOCYTE BEHAVIOUR IN THE HAMSTER CHEEK POUCH POST-CAPILLARY VENULE

Franca Mancuso, Roderick J Flower & Mauro Perretti
Department of Biochemical Pharmacology, The Medical College of Saint Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ. U.K.

Acute systemic treatment of hamsters with an anti-inflammatory dose of dexamethasone (Dex) selectively affects leucocyte emigration, but not rolling and adhesion, at the level of the post-capillary venule (Mancuso et al., 1994). Since passive immunisation of mice with anti-lipocortin 1 (αLC1) antibodies abrogated Dex inhibition of neutrophil accumulation (Perretti & Flower, 1993), we have now investigated whether LC1 also mediates the Dex effect in the hamster cheek pouch preparation.

A mixed population of hamster blood cells (prepared by centrifugation at 400 *g* 30 min on ficoll-metrizoate gradient and two washes in phosphate-buffered saline) was prepared from saline and Dex (1 $mgkg^{-1}$ s.c.) treated animals. Leucocytes were stained with two specific αLC1 antibodies prior to addition of the FITC-bound detecting antibody. Flow cytometry was performed using a FACScan, and distinct cell populations were discriminated using forward and side scatter characteristics. Post-capillary venules of the hamster cheek pouch with a diameter of 20-30 μm and a segment of at least 100 μm length were prepared according to Duling (1973) and superfused with bicarbonate-buffered solution (5 $mlmin^{-1}$). Formyl-Met-Leu-Phe (FMLP) was topically applied at the dose of 10 nmol (in 50 μl) and images recorded for 30 min. A Zeiss Axioskop microscope with a water immersion 40X objective was used. A videocamera (Panasonic) connected to a recorder (Panasonic) and a colour monitor (Sony) were used for recording and off-line analysis. The time necessary for leucocytes to begin migration was calculated. Animals received either saline or Dex 1 $mgkg^{-1}$ s.c. 2h prior to application of FMLP. Either non immune or specific αLC1 sheep sera (1 $mlkg^{-1}$ s.c.) were given 24h prior to Dex. Statistical differences were evaluated by analysis of variance followed by the Bonferroni test or by Fisher's exact test.

Treatment with Dex increased cell-associated LC1 in circulating leucocytes with a consistent effect upon neutrophils (molecules per cell: 93,512±13,310 for saline- and 247,689± 71,511 for Dex-treated hamsters, n=3 experiments, $p< 0.05$). Following FMLP application at the level of the post-capillary venule, adherent leucocytes began to migrate (as evaluated by pseudopodia emission) within 3.4±0.1 min (n=36 cells of 8 hamsters). Treatment with Dex delayed this process to 13.2±0.9 min (n=13 cells of 8 hamsters, $p<0.01$). Whereas pretreatment with normal sheep serum did not modify this figure (3.1±0.1 min and 10.6±0.3 min in saline- Dex-treated hamsters, $p<0.01$), pretreatment with the αLC1 antibody abrogated the effect of the steroid: 3.0±0.1 min vs. 4.2±0.2 min, n=8 animals, not significant. Similarly, Dex increased the rate of cell detachement from the endothelial wall (from 20% to 69%, $p<0.01$), this phenomenon being unmodified by the control serum (from 17% to 47%, $p<0.01$) but abrogated after treatment with the αLC1 serum (from 11% to 25%, n.s.).

In conclusion, the specific action of Dex on the emigration of adherent leucocytes correlates strongly with the induction of the inhibitory protein LC1. Further studies will seek a better definition of the molecular mechanisms responsible for these effects.

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219P CHOLERA TOXIN PRODUCES FLUID SECRETION IN THE RAT SMALL INTESTINE *IN VIVO* BUT DOES NOT INCREASE SHORT-CIRCUIT CURRENT *IN VITRO*

R.A. Borman, D.E. Burleigh, F.H. Mourad[1] & M.J.G. Farthing[1], Department of Pharmacology, Queen Mary & Westfield College, London E1 4NS and [1]Digestive Diseases Research Centre, Medical College of St. Bartholomew's Hospital, London EC1M 6BQ.

Cholera toxin (CT) has previously been shown to produce fluid secretion in rat jejunum *in vivo* (Cassuto *et al.*, 1982), although it remains controversial as to whether this effect occurs in isolated small intestine *in vitro*. In the present study, we have compared the effects of CT in the small intestine of the rat *in vivo* and *in vitro*.

In vivo: Male adult Wistar rats (180-220g) were anaesthetised with pentobarbitone (60mg/kg i.p.), the abdomen opened and the small intestine isolated between two cannulae. After flushing with isotonic saline, 75µg CT in 6ml of saline, or 6ml of saline alone (control) were injected into the isolated intestine before clamping proximal and distal cannulae. After 2h, the segment was perfused *in situ* ($0.5 ml min^{-1}$) with plasma electrolyte solution containing [^{14}C]-polyethylene glycol 4000 as a non-absorbable marker. After 30 min, 3 x 10min collections of effluent were made, to assess water and electrolyte movement. All animals receiving CT showed a net secretion of water (median -39.1µl/min/g dry intestinal weight [interquartile range -49.5 to -33.8]; n=10). Chloride and sodium movement paralleled that of water (-4.2µmol/min/g dry weight [-6.1 to -2] and -7.0 [-8.5 to -3.5] respectively). Net water and electrolyte absorption was observed in controls (n=8, $p<0.05$ compared to CT; Wilcoxon rank sum test).

In vitro: Rats were sacrificed and sections of proximal (within 20cm of duodenojejunal junction) or distal (within 30cm of ileocaecal junction) small intestine were removed and mounted as full thickness or muscle-stripped preparations in Ussing chambers, bathed either side by gassed (95%O_2 in CO_2) Krebs solution at 37°C. Mannitol replaced glucose in Krebs bathing the mucosal side of the tissue. After 1h equilibration, CT ($10 µg ml^{-1}$) or control vehicle (H_2O) were applied to the solution bathing the mucosal aspect, and changes in short-circuit current (SCC) were monitored for a further 3h. In parallel experiments, forskolin (25µM) or dibutyryl cAMP (300µM) were applied to tissues at either 1 or 4h in order to detect changes in tissue viability. There was no significant difference between responses in proximal and distal small intestine; the two sets of results have therefore been combined. After 1h equilibration, basal SCC (mean±s.e.mean) was $54.4±3.3 µAcm^{-2}$ in stripped and $15.3±1.7 µAcm^{-2}$ in full thickness preparations (n=24). Application of CT had no significant effect on SCC in muscle-stripped or full thickness preparations ($p>0.05$, Table 1).

	Change in SCC ($µAcm^{-2}$) after 3h	
	Muscle-stripped	Full thickness
Control	+27.3±4.3	+12.9±2.3
CT-treated	+26.9±6.8	+11.2±2.0

Table 1. Effect of CT on the change in SCC 3h following application of CT ($10 µg ml^{-1}$) or control vehicle. Data are mean±s.e.mean, n=12.

In viability studies, application of forskolin or dibutyryl cAMP produced a rapid rise in SCC, in both muscle-stripped and full thickness preparations. After 1h, forskolin caused a rise of 57.7±17.6 and 14.4±1.9 $µAcm^{-2}$ (stripped and full thickness), whereas dibutyryl cAMP increased SCC by 30.0±7.7 and $9.2±1.9 µAcm^{-2}$ respectively. These values were not significantly different in tissues treated at 4h instead of 1h (n=8-10, $p>0.05$), indicating tissues retain viability for the duration of the experiment.

In conclusion, application of CT produced fluid secretion in rat small intestine *in vivo* but did not increase SCC *in vitro*, in either stripped or full thickness preparations. Although the possibility of electroneutral secretion has not been excluded, these findings may imply a pivotal role for extrinsic innervation and/or intestinal blood flow in the secretory process.

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220P ANTAGONISM OF PENTAGASTRIN RESPONSES BY L-365,260 IN RAT, GUINEA-PIG AND MOUSE ISOLATED GASTRIC TISSUE ASSAYS: EVIDENCE FOR VARIABLE EXPRESSION OF TWO CCK/GASTRIN RECEPTORS

S.P. Roberts, G.F. Watt, V.P. Gerskowitch, R.A.D. Hull, N.P. Shankley & J.W. Black, James Black Foundation, 68 Half Moon Lane, London SE24 9JE.

Initial studies on the selective antagonist L-365,260 indicated that CCK/gastrin receptors in CNS and peripheral tissues were indistinguishable (Chang *et al.*, 1989). However, Spraggs & Patel (1992), estimated different affinities for L-365,260 in guinea-pig ileum (pA_2=8.8) and rat gastric mucosa (pA_2=7.6) which, at least, suggested species differences. We now present several, replicate, competitive analyses of the interaction between pentagastrin and L-365,260 using established methods and protocols on isolated, perfused, stomach preparations from mouse and immature rat (Welsh *et al.*, 1994) and guinea-pig gastric muscle (Bishop *et al.*, 1994). In all experiments the pentagastrin concentration-effect curves were shifted in parallel with the maxima unchanged. However, dose-ratio analysis indicated that the data were not consistent with expectations for competition at a single receptor between assays or, with the possible exception of the rat, within assays (Table 1). An attempt was then made to simulate the data with the simplest (addition of stimuli) 2-receptor model. In the model, pentagastrin was non-selective for the 2 receptors, whereas L-365,260 expressed pK_B values of 7.5 and 8.8. The data were simulated by varying the absolute and relative density of the two receptors between, but not within, experiments particularly to account for the high variation in the mouse pA_2 and b values. The affinity values were similar to those obtained by analysis of CCK/gastrin radioligand data in other tissues from the same species (Harper *et al.*, 1995). However, as yet, we do not know whether the apparent expression of two sites can be related to the human CCK/gastrin receptor mRNA isoforms (Song *et al.*, 1993).

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Table 1

rat stomach (acid secretion)		mouse stomach (secretion)		guinea-pig gastric muscle (length)		
pK_B±s.e.	b±s.e. (d.f.)	pK_B±s.e.	b±s.e. (d.f.)	pK_B±s.e.	b±s.e. (d.f.)	NB: if Schild slope parameter (b) is significantly different from unity(*) then, $pK_B=pA_2$
7.61±0.12	1.07±0.16 (30)	8.70±0.34	0.20±0.15*(31)	8.97±0.19	0.68±0.12*(21)	
7.72±0.11	0.62±0.15*(40)	7.95±0.30	0.96±0.15 (44)	8.39±0.11	0.86±0.15 (26)	
7.41±0.12	1.11±0.15 (22)	8.31±0.20	0.14±0.15*(33)	8.39±0.10	0.80±0.14 (25)	
7.80±0.12	0.88±0.10 (25)	8.47±0.16	1.23±0.23 (25)	8.78±0.16	0.77±0.06*(52)	
		8.67±0.27	0.31±0.14*(38)			

221P COMPETITION BETWEEN L-365,260 AND [^{125}I]BH-CCK8S IN GUINEA-PIG GASTRIC GLANDS AND MOUSE AND RAT CORTEX ASSAYS: EVIDENCE FOR VARIABLE EXPRESSION OF TWO CCK/GASTRIN RECEPTORS

E.A. Harper, N.P. Shankley & J.W. Black, James Black Foundation, 68 Half Moon Lane, Dulwich, London SE24 9JE.

Radioligand binding studies performed on cloned gastric and CNS CCK/gastrin receptors from a number of species have indicated that amino acid sequence variations result in differences in the affinity of the antagonist L-365,260 (e.g. Beinborn *et al.*, 1993). However, as far as we are aware, no affinity differences have been reported for L-365,260 between or within tissues from a single species (e.g. Hunter *et al.*, 1993). In this study, we have analysed replicate data sets obtained with L-365,260 in CCK/gastrin receptor radioligand binding assays over a 3 year period. Mouse and rat cerebral cortical cells (MC and RC) were prepared according to Clarke *et al.* (1986) and guinea-pig gastric glands (GPGG) were prepared from gastric mucosa using a Griffith's homogeniser and a buffer based on Praissman & Walden (1984). Competition curves were obtained by incubating (21°C) tissue at 5, 2 and 4mg/ml concentrations (original wet weight) with 20pM [^{125}I]-BH-CCK8S for 150, 150 and 100min for MC, RC and GPGG, respectively. No significant labelling of CCK_A binding sites occurred in the assays. Competition curves for L-365,260 (Table 1) in the mouse cortex were not different from rectangular hyperbolae implying the presence of a single class of binding sites (pK_I ~8.5). In RC and GPGG the mean slope was significantly less than one and the mean pK_I lower than that obtained in the MC. The distribution of the individual pK_I and slope estimates of the competition curves in these two assays was consistent with simulated expectations for the variable expression (in terms of absolute concentrations and ratios between experiments) of two binding sites. Thus, pK_I values approaching either 8.5 or 7.3 were associated with slopes approaching unit value. These values were indistinguishable from those obtained from analysis of L-365,260 antagonism of pentagastrin stimulated responses in mouse and rat stomach (acid secretion) and guinea-pig gastric muscle (length) assays (Roberts *et al.*, 1995). However, as yet, we do not know if the apparent expression of two sites in these assays is related to the two human CCK/gastrin receptor mRNA isoforms reported by Song *et al.* (1993).

Table 1 L-365,260 competition curve data

	n	$pK_I \pm$ s.e.m.	slope ± s.e.m.
mouse cortex	47	8.46±0.03	1.05±0.03
rat cortex	50	7.85±0.05	0.79±0.03*
guinea-pig gastric glands	46	8.26±0.06	0.87±0.03*

(* slope significantly different from unity)

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222P COMPARISONS OF HUMAN CCK_A RECEPTOR IN A TRANSFECTED CELL LINE AND NATIVE GALL-BLADDER TISSUE

P.A. Howson, J. Denyer and C. F. Spraggs, Pharmacology 1, Glaxo Research and Development Ltd., Park Road, Ware, Herts. SG12 0DP.

CCK_A receptors have been widely characterised in animal tissues including gall-bladder (Maubach *et al*, 1992). In the present study, we have compared the pharmacology of the recombinant human gall-bladder CCK_A receptor stably expressed in CHO cells (Ulrich *et al*, 1993) with the CCK receptor present in human isolated gall-bladder.

The gene encoding the human gall-bladder CCK_A receptor was stably transfected into CHO cells. Cells were seeded into transwells for use in a microphysiometer (Cytosensor, Molecular Devices Corporation, USA) which measures metabolic extracellular acidification (Denyer *et al*, 1994). Cumulative concentration response curves (CRC) were constructed by superfusing agonists and were repeatable. Antagonists were superfused for 30 min prior to construction of a second CRC. Human gall-bladders were obtained from patients undergoing surgery for gall-bladder disease. Gall-bladder strips (15x3 mm) were suspended in Krebs solution, gassed with 95% O_2/5% CO_2 and maintained at 37°C. A single CRC to agonists was constructed in each tissue. Antagonists were equilibrated for 30 min prior to CRC for sCCK-8 and affinity estimates (pK_B) were determined by comparison of EC_{50} values in paired tissues.

In the microphysiometer, sCCK-8 (0.01-1 nM) stimulated increases in extracellular acidification by CHO-hCCK_A cells. The CCK peptides, sCCK-8, A72962 and nsG-17 produced similar maximum responses with a rank order of potency consistent with CCK_A receptor activation (EC_{50} values of 0.32 (0.17-0.58), 6.5 (2.1-20.6) and 1534 (339-7046) nM respectively, n=5). The CCK antagonists devazepide (30 nM) and L-365,260 (3 μM) did not modify basal acidification rates but did competitively inhibit responses to sCCK-8 with pK_B values of 9.7 (n=4) and 6.9 (n=6). In human gall-bladder, sCCK-8 (1-10000 nM) produced sustained contractions with a CRC that extended more than 4 orders of magnitude of concentration. The CCK peptides produced similar maximum responses, again with sCCK-8 and A72962 being more potent than nsG-17 (EC_{50} values of 86 (39-192), 135 (89-204) and 1507 (939-2418) nM respectively, n=4). Devazepide (100 nM) and L-365,260 (3 μM) did not modify basal tone and were weaker antagonists with pK_B values of 7.5 and <5.5 (n=4).

These limited studies utilising human gall-bladder tissue are consistent with the view that CCK peptides mediate contraction via a CCK_A receptor. Whilst the rank order of agonist potency in human gall-bladder and CHO-hCCK_A cells was similar, it was surprising to observe that functional antagonist affinity estimates were different. These differences may be due to i) differences in antagonist equilibration ii) receptor changes in diseased gall-bladder tissue iii) receptor transfection into a non-human host cell. Further studies are warranted to determine the properties of this receptor when expressed in different cell types.

We are grateful to Mr S Chadwick (Northwick Park Hospital) for supplying human gall-bladder tissue.

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223P EFFECTS OF BRADYKININ ON THE ACTIVITY OF C-FIBRE AFFERENTS INNERVATING THE RAT KNEE JOINT: STUDIES *IN VIVO* AND *IN VITRO*

D.C. Kelly, A.U.R. Asghar, C.G. Marr, D.S. McQueen & M.N. Perkins[1], Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ and [1]Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN.

Bradykinin (BK) is one of the most potent algogenic substances known and has been shown to activate C-fibre afferents innervating the cat knee joint (Kanaka *et al.*, 1985). The aim of this study was to investigate and compare the effects of bradykinin on afferent neural activity in both the *in-vivo* and *in-vitro* rat knee joint.

Male wistar rats were anaesthetised using urethane (25% w/v, 0.6ml $100g^{-1}$ weight i.p.). For *in-vivo* studies the trachea, right carotid artery (blood pressure monitoring) and right femoral artery (drug administration) were cannulated. For *in-vitro* studies the abdominal aorta and vena cava were cannulated - the catheters were advanced past the iliac bifurcation to allow perfusion of the left hind limb with modified Krebs solution; the rat was then killed by cardiac puncture. Recording of afferent neural activity from high threshold mechanonociceptors was similar to that described elsewhere (Birrell *et al.*, 1993) except the medial articular nerve was dissected from the saphenous nerve.

All units studied were activated by injection of capsaicin (3μg i.a.) and had conduction velocities within the C-fibre range (*in-vivo*: $1.1 \pm 0.1 m\ s^{-1}$, *in vitro*: $0.63 \pm 0.2 m\ s^{-1}$). BK (3-30μg) produced a dose-dependent excitation of knee joint afferents both *in vivo* and *in-vitro* (Table 1). This excitation was completely blocked by the BK B_2 receptor antagonist, HOE 140 ($10\mu g\ kg^{-1}$). The B_1 receptor agonist, desArg^9-BK (30μg i.a.), had no effect on afferent activity.

These data show that bradykinin acting at B_2 receptors excites C-fibre afferents innervating the rat knee joint. As the results obtained for both *in-vivo* and *in-vitro* were comparable (apart from the longer delay observed *in-vitro*), the *in-vitro* model should prove to be useful for investigating the effects of bradykinin and other inflammatory mediators on C-fibre afferents innervating the rat knee joint.

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Table 1. Effects of bradykinin on knee joint afferents both *in-vivo* and *in-vitro* (values shown as mean ± s.e. mean).

	IN-VIVO (n=4)			*IN-VITRO* (n=3-9)		
	peak response (i.p.s.)	*delay to onset(s)*	*duration(s)*	*peak response (i.p.s.)*	*delay to onset(s)*	*duration(s)*
SALINE	0.0 ± 0.1	-	-	0.1 ± 0.1	-	-
BK 3μg	4.9 ± 2.6	7.0 ± 2.7	74.4 ± 45.9	3.6 ± 1.2	22.5 ± 5.9 *	72.4 ± 14.9
BK 10μg	9.3 ± 2.8	5.3 ± 3.4	73.0 ± 22.1	6.7 ± 1.7	24.8 ± 8.9 *	91.2 ± 12.3
BK 30μg	18.7 ± 8.6	5.2 ± 4.2	92.6 ± 38.5	8.3 ± 2.4	19.9 ± 4.5 *	155.8 ± 49.2
BK 30μg(HOE140)	2.2 ± 0.3 †	11.3 ± 4.3	105 ± 28.9	0.8 ± 0.1 †	22.0 ± 3.0	84.5 ± 6.5

*p < 0.05 Mann Whitney U-test; compared to *in-vivo*. † p < 0.05 paired t-test, compared to BK 30μg.

224P BRADYKININ CONTRIBUTES TO THE MYOCARDIAL RELAXANT EFFECT OF CAPTOPRIL

P. B. Anning, *R. M. Grocott-Mason, M. J. Lewis, *A. M. Shah, Depts of Pharmacology & *Cardiology, Univ Wales College of Medicine, Cardiff, CF4 4XN

The mechanism underlying the beneficial effect of angiotensin converting enzyme (ACE) inhibitors in heart failure and left ventricular (LV) hypertrophy remains unresolved. ACE increases angiotensin II, and inactivates bradykinin (BK) which releases nitric oxide (NO) from endothelial cells. We have previously demonstrated that exogenous NO enhances LV relaxation (Grocott-Mason *et al.*, 1994). In the present study we report the effects of BK and the ACE inhibitor captopril (CAP) on LV function in isolated ejecting guinea-pig hearts (constant loading and rate; Krebs-Henseleit buffer; 37°C). LV pressure (P) was measured using a 2F Millar catheter, and LV relaxation assessed by a mono-exponential time constant (T_E, ms) as reported previously (Grocott-Mason *et al.*, 1994). Coronary flow (CF, ml min^{-1}) was measured by timed collections of pulmonary effluent. We compared (1) BK (0.1-1nM); (2) CAP (1μM); (3) BK (0.1nM) after 30min pretreatment with CAP; (4) BK (1nM) in the presence of the NO-scavenger haemoglobin (Hb, 1μM); (5) CAP in the presence of the B_2-kinin receptor antagonist HOE 140 (HOE 10nM). After a 12 min equilibration period, the study drug was added to the buffer and repeat measurements were taken over the next 16 min. Hb and HOE were added during the equilibration period. Data (absolute values) were analysed using Student's *t* test (unpaired). BK caused enhancement of LV relaxation (reduced T_E), and a transient (≤4 min) increase in CF.

No correlation was found between the changes in T_E and CF. CAP also enhanced LV relaxation, but did not change CF. In the presence of CAP, the relaxant effect of BK (0.1nM) was enhanced and the increase in CF prolonged (up to 16 min). CAP effects were blocked by HOE and BK effects on T_E (but not peak CF (pCF)) blocked by Hb (see Table 1)

%	0.1nM BK	1nM BK	CAP	CAP+ BK	CAP+ HOE	BK+ Hb
T_E	-9±1.5★	-9±1.5★	-15±1.2★	-16±1.5☆	-7±1.5†	0.5±7.6
pCF	2.6±0.5	15.5±3★	2±2.8	9±2.8☆	-2±1.0	26±7.1★

Table 1. Data expressed as mean ± s.e.mean (percentage change). ★p<0.05 cf control; ☆ effect of BK in CAP pretreated group p<0.05 cf 0.1nM BK alone. †p<0.05 cf CAP alone; n ≥ 6. There were no changes in LVP or rate of change of pressure (dP/dt).

Conclusions: (1) BK and CAP exert selective myocardial relaxant effects, with attenuation by Hb and HOE respectively suggesting involvement of endogenous BK, B_2-kinin receptors and NO. (2) The enhancement of BK effects by CAP probably reflects decreased breakdown of BK by ACE. (3) The lack of vasodilator activity of CAP may reflect the site of BK/NO release. (4) The relaxant actions of CAP may be beneficial in "diastolic dysfunction".

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225P CHARACTERISATION OF THE BRADYKININ RECEPTORS THAT MEDIATE CONTRACTION OF THE RAT OESOPHAGUS *IN VITRO*

S.J. Boxall, A. Wheeldon and P.J. Birch, Pharmacology 1, Glaxo Research and Development Ltd, Ware, Herts, SG12 0DP, UK.

The rat appears to be the species of choice for evaluating the role of bradykinin (BK) B_1 receptors *in vivo* (Perkins *et al*, 1993). With this in mind, we have characterised the BK receptors on the rat isolated oesophagus.

The oesophagus was removed from male (Glaxo bred) random hooded rats (120-200g). The tunica muscularis was dissected free and suspended under a resting tension of 0.5g in Krebs' solution at 37°C, gassed with 95% O_2/5% CO_2. Tissues were sensitized to carbachol (1μM), followed by either BK or des-Arg^9-BK (1μM) the B_2 and B_1 receptor-selective agonists respectively, with three wash cycles and a 30 minute recovery period between each contraction. Cumulative concentration-response curves to either BK or des-Arg^9-BK (0.001-30μM) were constructed until reproducible and compared with curves constructed in the presence of an antagonist (pretreatment 30 minute).

The concentrations (geometric means ± s.e.m.; n=7) of des-Arg^9-BK and BK required to produce 50% of each maximum response (EC_{50}) were 27.8±6.5 and 50.4±10.2 nM, respectively. Bestatin, phosphoramidon and captopril, (all at 1μM) inhibitors of potential degradative enzymes, had no significant effect on contractions to either agonist. In addition, the kininase I inhibitor, MERGETPA (10μM) did not affect the potency of BK, a prime substrate for this enzyme. In contrast, 10nM, HOE140 the B_2 receptor-selective antagonist caused parallel, concentration-related (1-100nM) rightward displacements of BK curves to produce a mean pK_b value of 9.2 ±0.08 (n=3). HOE140 (10nM) did not however affect contractions to des-Arg^9-BK. Conversely, des-Arg^{10}-HOE140, the putative B_1 receptor antagonist caused parallel, concentration-related (0.1-1μM) rightward displacement of des-Arg^9-BK curves to produce a mean pK_b value of 6.4±0.2 (n=4). In addition, the B_1-receptor antagonist des-Arg^9-Leu^8-BK caused a concentration-related rightward displacement of des-Arg^9-BK and a marked suppression of the maximum contraction. Neither B_1 receptor antagonist had any significant effect on BK-induced contraction response curves. Contractions to des-Arg^9-BK on the rat oesophagus were immediately apparent and did not significantly change with time, up to 150 min.

In conclusion, contraction of the rat isolated oesophagus tunica muscularis can be mediated by both BK and des-Arg^9-BK through activation of B_2 and B_1 receptors respectively. Increased sensitivity to des-Arg^9-BK, as seen in rabbit vasculature, and attributed to de novo synthesis of B_1 receptors (Bouthillier *et al*, 1987) or B_2 receptors was not apparent. The rat oesophagus should prove a practical and robust preparation in the investigation of B_1 receptor pharmacology *in vitro*.

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226P BRADYKININ DEPOLARISES THE RAT ISOLATED SUPERIOR CERVICAL GANGLION VIA B_2 RECEPTOR ACTIVATION

Rachel Babbedge, Andy Dray & Laszlo Urban. Sandoz Institute for Medical Research, 5 Gower Place, London, WC1E 6BN

Bradykinin (BK) is an important mediator of inflammatory processes (Dray & Perkins, 1993). It has been postulated that at least part of the inflammatory effects of BK, in particular the production of hyperalgesia and oedema, are secondary to an activation of sympathetic nerves (Levine et al 1986). The following experiments were undertaken to characterise the action of kinins on sympathetic neurones of the rat superior cervical ganglion (SCG) by using desArg^{9}BK and BK as agonists for B_1 and B_2 receptors respectively.

Male Sprague Dawley rats (180-300g) were killed by exposure to CO_2 and SCG with associated pre- and postganglionic nerve trunks were removed, desheathed and placed in a chamber arranged for grease-gap extracellular recording from the ganglion according to the method of Brown and Marsh (1978). Ganglia were superfused (5-6 ml min^{-1}; 20-25°C) with 95% O_2 and 5% CO_2 saturated Krebs solution. Agonists and antagonists were added to the superfusate for 1 min and 30 min respectively. All tissues were exposed to the nicotinic agonist 1,1-dimethyl phenylpiperazinium (DMPP, 10μM) and depolarising responses employed as an internal standard for each preparation.

BK (10-1000nM), perfused at 45 min intervals to prevent tachyphylaxis, produced concentration-dependent surface depolarisation of the ganglion (pD_2 7.02 ± 0.04, n=7) with a maximum depolarisation of approximately 160 μV in response to 300 nM BK. In contrast desArg^{9}BK (10-1000nM) had no effect on surface potential up to 6h after the preparations were set up. The selective B_2 receptor antagonist HOE 140 (Hock et al., 1991) significantly reduced BK-induced depolarisations such that, in the presence of HOE 140 (100 nM) responses to BK (300nM) (previously maximal) were reduced by approximately 70% (n=5, P<0.05). The B_1 receptor antagonist Leu^{8}desArg^{9}BK (1μM) had no effect on depolarisations to BK. Endogenously generated NO or prostaglandins did not appear to be required for BK-depolarisations as neither L-NAME (300 μM) nor indomethacin (7 μM) each superfused for 30 min, influenced the action of BK (BK+ indo., pD_2 7.15 ± 0.08, n=4,P>0.05; BK+ L-NAME, pD_2 6.99 ± 0.07, n=4, P>0.05, with no change in E_{max}).

Systemic expression of B_1 receptors may be induced by inflammatory stimuli such as bacterial lipopolysaccharide (LPS; Regoli et al 1981). In in further experiments LPS (3 mg kg^{-1}; i.v.) was administered to rats 5h and 18h before SCG were removed for recording as before. In these preparations, desArg^{9}BK (10-1000nM) failed to produce depolarisation either 5 or 18h following LPS administration (P>0.05, n=4 animals per group) whereas responses to a sub-maximal concentration of BK (100nM) were unchanged (79.9 ± 7.8 μV and 72.6 ± 6.5 μV, 5 and 18h post LPS respectively, both n=4 and P>0.05).

In conclusion, BK depolarises rat SCG via B_2 receptor activation but not via the generation of NO or prostaglandins. This action of BK may relate to its role in hyperalgesia, plasma extravasation and other inflammatory processes.

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227P HAEMODYNAMIC EFFECTS OF BRADYKININ B_1 AND B_2 RECEPTOR ACTIVATION IN THE ANAESTHETISED RAT

M.P. Cunoosamy and A. Wheeldon (introduced by P.J. Birch), Pharmacology 1, Glaxo Research and Development Ltd., Ware, Herts SG12 ODP, U.K.

It has been proposed that endogenous bradykinin (BK) might be an important regulator of blood pressure (BP), particularly in conditions of hypertension (Waeber *et al.*, 1986). The aim of this study was to investigate the potential role of BK B_1 receptors in modulating BP in the anaesthetised rat.

Male Sprague Dawley rats (450-550g) were anaesthetised and allowed to breathe spontaneously following a tracheotomy. A carotid artery was cannulated for monitoring changes in diastolic BP and a jugular vein was cannulated for administration of test compounds. In rats with a mean resting BP of 95±3 mmHg (n=10), mean dose-response (DR) curves to bolus injections of BK, des-Arg^9-BK (a B_1 receptor-selective agonist) or angiotensin II (AII) were constructed until reproducible. Antagonists were administered i.v. 30min before a final DR curve to an agonist.

BK (0.3-30 nmol kg^{-1}, n=3) produced a dose-related depressor response, while des-Arg^9-BK (0.01-3 μmol kg^{-1}, n=3) produced a dose-related pressor response, achieving a maximum of 60% decrease or 30% increase in diastolic BP respectively. HOE140 , a B_2 receptor-selective antagonist, at 0.01 and 0.1 μmol kg^{-1} (n=3-8), produced dose-dependent rightward shifts of the BK DR curves with mean dose-ratios of 9 (1-74) (95% C.L.) and 182 (68-486), respectively. At a higher dose of HOE140 (1 μmol kg^{-1}, n=3-8), BK evoked a 30% increase in diastolic BP, but HOE140 had no effect on an equivalent pressor response to des-Arg^9-BK (n=3-4). Des-Arg^{10}HOE140, a putative B_1 receptor-selective antagonist, at 1.1 and 3.4 μmol kg^{-1} (n=3-5), produced rightward shifts of the des-Arg^9-BK DR curves with dose-ratios of 5.1 (1.3-19) and 5.1 (3-9) respectively. At these doses, des-Arg^{10}-HOE140 had no effect on the BK DR curve (n=3-6). When tested against AII-induced pressor responses, des-Arg^{10}-HOE140 (3.4 μmol kg^{-1}, n=3) had no effect at the lower doses (1-30 nmol kg^{-1}) to AII, but significantly reduced the pressor response of 100 nmol kg^{-1} of AII by 27.6% ± 4.6. At the doses used neither HOE140 nor des-Arg^{10}-HOE140 had any effect on resting diastolic BP.

In conclusion, BK-induced depressor responses are mediated by activation of B_2 receptors. A significant observation was the pressor response to BK which was unmasked by the highest dose of HOE140 (1 μmol kg^{-1}). The observation that dose-related pressor responses could also be produced to des-Arg^9-BK, a closely related BK metabolite, implies that B_1 receptors could be involved. The antagonism of des-Arg^9-BK by des-Arg^{10}-HOE140 supports the involvement of B_1 receptors, however, the inability of des-Arg^{10}-HOE140 to produce complete antagonism implies that high doses of des-Arg^9-BK could also activate other, non B_1 receptors. Further work is required to ascertain the nature of these receptors.

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228P THE INDUCTION OF BRADYKININ B_1 RECEPTORS IN THE NON-INFLAMED AND INFLAMED RAT URINARY BLADDER

R. Roslan, E.A. Campbell and A. Dray, Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN

The development of responsiveness to the B_1 agonist desArg^{9}bradykinin (desArg^{9}BK) *in vitro* was first seen in the isolated anterior mesenteric vein of the rabbit in which a contractile response increased to a maximum level after 6h (Regoli *et al.*, 1977). In the urinary bladder, Marceau *et al.*, 1980 have shown that kinin induced contractions were mediated by bradykinin B_1 and B_2 receptors, and Maggi *et al.*,1989 & 1993 have implicated bradykinin B2 receptors in inflammatory responses. In our studies, we have examined *in vitro* the contractions mediated by B_1 receptors in non-inflamed versus inflamed bladders (a model of cystitis).

Inflammation was induced under general anaesthetic (sodium pentobarbitone, 50mg.kg^{-1}) by local instillation of 25% turpentine in olive oil (0.4ml). Animals were allowed to recover and 2 days later bladders were removed and strips including the dome incubated in aerated Krebs solution (37°C).

In non-inflamed rat bladder at 1h, there was no response to desArg^{9}BK (1μM) however, an increase in responses to desArg^{9}BK and similarly to desArg^{10}Kallidin (desArg^{10}KD) (1μM) continued beyond 7h, therefore limiting further quantitative antagonist studies in this tissue. This induction of B_1 responses was inhibited by cycloheximide (70μM) and enhanced by IL-1ß (25units/ml) but unaffected by pre-treatment of animals with capsaicin (50mg/kg s.c.), 6-OH dopamine (2 injections 24h apart), or incubation with NGF (100ng/ml).

Inflamed bladders, showed an immediate and progressively increasing responsiveness to the B_1 agonists reaching a plateau at 5h. Therefore subsequent experiments were carried out at this time.

Concentration response curves revealed similar EC_{50} values for both the B_1 agonists desArg^{9}BK (0.0168 ± 0.0055 μM) (n=3) and desArg^{9}KD (0.015 ± 0.006 μM) (n=6). These responses were antagonised by the B_1 antagonists desArg^{9}Leu^{8}BK and desArg^{10}HOE140. The IC_{50} value for desArg^{10}HOE140 against desArg^{9}BK in inflamed rat bladder was 0.103 ± 0.014 μM (n=6) and the IC_{50} value for desArg^{9}Leu^{8}BK was 0.083 ± 0.010 μM (n=7). Similar IC_{50} values were observed for the B_1 antagonists when used against desArg^{10}KD [desArg^{9}Leu^{8}BK 0.039 ± 0.008 μM (n=8); desArg^{10}HOE140 0.065 ± 0.011 μM (n=7)].

These data show that B_1 receptors are not present in normal rat bladders however, there is a definite induction of B_1 responses during *in vitro* incubation and following bladder inflammation. The mechanism of this induction is still unclear but inflammatory mediators as well as protein synthesis appear to be involved.

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229P BRADYKININ FACILITATES NORADRENALINE RELEASE FROM HUMAN RENAL CORTEX IN THE PRESENCE OF ANGIOTENSIN CONVERTING ENZYME INHIBITION

L.C. Rump, U. Schaible, C. Bohmann, A. Frankenschmidt* & P. Schollmeyer, Department of Internal Medicine IV and Urology*, University of Freiburg, Hugstetter Str. 55, D-79106 Freiburg, FRG

Bradykinin has been shown to inhibit noradrenaline release in rabbit pulmonary artery and heart due to the formation of prostaglandins (Starke et al. 1977). In contrast, there is indirect evidence that the positive inotropic effect of bradykinin in rat atria and ventricle may be due to prejunctional facilitation of sympathetic neurotransmitter release, an effect, which is more pronounced in the presence of the angiotensin converting enzyme/kininase II (ACE) inhibitor ramiprilat (Minshall et al. 1994). The aim of the present study was to investigate the effect of bradykinin on noradrenaline release from human renal sympathetic nerves. Human kidney cortex slices (10-20 mg) were incubated with ^{3}H-noradrenaline (0.5 μM), placed into superfusion chambers, held between two platinum electrodes and superfused with Krebs-Henseleit solution. After a washing procedure (2.0 ml/min) of 110 min, there were four electrical field stimulations (S_1-S_4, pulse width 1 ms, 20 mA current strength, each at 2.5 Hz for 60 s). Under these conditions the stimulation induced (S-I) outflow of radioactivity can be taken as an index of endogenous noradrenaline release (Rump et al. 1993). Bradykinin (0.01-1 μM) alone did not influence the S-I outflow of radioactivity, but in the presence of the ACE inhibitor captopril (3 μM) bradykinin (0.01-1 μM) concentration dependently enhanced the S-I outflow of radioactivity to a maximum of 136.5 % of control (SEM=6.4, n=6). Captopril (0.03-3 μM) alone had no effect on the S-I outflow of radioactivity. The facilitatory effect of bradykinin in the presence of captopril was abolished by addition of the selective bradykinin B_2-receptor antagonist HOE 140 (0.3 μM). The present results suggest that bradykinin activates prejunctional bradykinin receptors of the B_2-subtype to enhance noradrenaline release in human kidney cortex. This facilitatory effect of exogenous bradykinin can only be demonstrated, when the enzymatic degradation of bradykinin is inhibited in the presence of ACE inhibition.

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230P EFFECT OF PHORBOL ESTER ON BRADYKININ-INDUCED PHOSPHO-INOSITIDE HYDROLYSIS AND CALCIUM MOBILIZATION IN CULTURED CANINE TRACHEAL SMOOTH MUSCLE CELLS

Yang, C.M., Department of Pharmacology, Chang Gung College of Medicine and Technology, Tao-Yuan, Taiwan. (Introduced by S J Hill).

It is established that many agonists cause contraction of tracheal smooth muscle through the activation of phosphoinositide (PI) hydrolysis to generate inositol 1, 4, 5,-trisphosphate (IP_3) and subsequently an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Coburn & Baron, 1990). In canine tracheal smooth muscle cells (TSMC), we have previously reported that bradykinin (BK) is a potent stimulant for PI hydrolysis (Yang *et al.*, 1994a) and Ca^{2+} mobilization (Yang *et al.*, 1994b), which are mediated through the activation of BK B_2 receptors. The other product of PI hydrolysis, diacylglycerol, has been shown to activate protein kinase C (PKC) and, in the present study, the effect of PKC activation on BK-induced IP production and increase in $[Ca^{2+}]_i$ has been investigated.

Canine TSMCs were isolated and cultured according to methods described previously (Yang *et al.*, 1994a). IP_3 accumulation and $[Ca^{2+}]_i$ measurements were performed as described (Yang *et al.*, 1994a, b). BK receptor-binding was determined using [^{3}H]BK as a radioligand and non-specific defined with 10 μM unlabelled BK. Protein Kinase C activity was assayed as described by Barthelson *et al.* (1987).

Stimulation of TSMCs by BK led to IP_3 formation and caused an initial transient peak of Ca^{2+}, followed by a sustained elevation in a concentration-dependent manner. Pretreatment of TSMCs with phorbol 12-myristate 13-acetate (PMA, 1 μM) for 30 min blocked the BK-induced IP_3formation and Ca^{2+} mobilization. This inhibition was reduced after incubating the cells with PMA for 4 h, but within 12 h the BK-induced Ca^{2+} mobilization reached the same extent of control cells. The concentrations of PMA (30 min) that gave half-maximal inhibition of BK-induced IP_3formation and increase in $[Ca^{2+}]_i$ were 0.5 ± 0.2 nM (n = 4) and 2.8 ± 0.5 nM (n = 8), respectively. Inactive phorbol ester, 4α-phorbol 12,13-didecanoate at 1 μM, did not inhibit these responses of BK. Prior treatment of TSMCs with staurosporine (1 μM), a PKC inhibitor, inhibited the ability of PMA to attenuate the BK-induced response. In parallel with the effect of PMA on the BK-induced IP_3 formation and Ca^{2+} mobilization, a change of PKC activity was observed in TSMCs. PMA (1 μM) rapidly decreased PKC activity in the cytosol of TSMCs, while increasing it transiently from 0.78 ± 0.10 to a maximum of 2.05 ± 0.04 pmol/min/mg protein (n=4, $p<0.001$) in the cell membranes at 30 min. Thereafter, membrane-associated PKC activity decreased to 0.94 ± 0.02 pmol/min/mg protein after 2 h and persisted for at least 24 h of PMA treatment. Treatment with 1 μM PMA for 2 and 24 h did not significantly change the K_D and B_{max} of the BK receptor for [^{3}H]BK binding compared with the control (control: K_D = 2.3 ± 0.3 nM, B_{max} = 25.2 ± 1.4 fmol/mg protein, n = 4; 2 h PMA treatment: K_D = 2.6 ± 0.4 nM, B_{max} = 26.3 ± 1.5 fmol/mg protein, n = 3).

These results suggest that activation of PKC inhibits the phosphoinositide hydrolysis and consequently attenuates $[Ca^{2+}]_i$ increase or inhibits independently both responses. The PMA-induced inhibition of responses to bradykinin was associated with an increase in membranous PKC activity.

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231P IDENTIFICATION OF SOMATOSTATIN (SRIF) RECEPTOR TYPES IN RAT COLONIC MUCOSA USING [I^{125}]Tyr^{11} SRIF AND THE NOVEL RADIOLIGAND [I^{125}]BIM23027

E. McKeen, W. Feniuk and P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ.

The SRIF analogue, BIM23027, is a highly selective ligand at the recombinant SSTR2 receptor (Raynor et al., 1993; Castro et al., 1994) and is a potent antisecretory agent in rat colonic mucosa (McKeen et al., 1994). Preliminary binding studies using [I^{125}]-Tyr^{11} SRIF in rat colonic mucosal membranes showed that BIM23027 and seglitide (both 1μM) maximally inhibited SRIF binding by only 43±3 and 41±2%, respectively. These sites have now been studied in more detail using [I^{125}]-BIM23027 (specific activity 2000Ci/mmol; Amersham).

Membranes of rat distal colonic mucosa were prepared in 50mM Tris HCl buffer (pH 7.4 at 25°C) containing 5mM $MgCl_2$, soyabean trypsin inhibitor (1μg/ml), leupeptin (10μg/ml) and bacitracin (0.2mg/ml). In association studies, membranes (2mg/ml) were incubated with [I^{125}]-BIM23027 (0.1nM) at 25°C for 2-240 min. In dissociation studies, membranes were incubated with [I^{125}]-BIM23027 for 60 min at 25°C and dissociation (0-360 min) measured by isotopic dilution with SRIF (1μM). In competition and saturation studies, membranes were incubated with [I^{125}]-BIM23027 (approx. 0.1nM) for 60min with various concentrations of competing drug. Binding was terminated by vacuum filtration and non-specific binding (25-30%) defined by SRIF (1μM). Binding was performed in triplicate and parameters were determined by iterative curve fitting (Sigma plot). Values quoted are mean ± s.e. mean from at least 3 experiments.

Binding of [I^{125}]-BIM23027 reached steady state within 60min and was stable for up to 6h. The observed association rate constant (K_{obs}) was 0.034±0.001 min^{-1}. Dissociation of [I^{125}]-BIM23027 was biphasic consisting of a fast component (K_{-1} of 0.26±0.16 min^{-1}) and a slow component (K_{-1} of 0.003±0.001 min^{-1}). Specific binding of [I^{125}]-BIM23027 was saturable and consistent with binding to a single site (B_{max} = 25±2 fmol/mg protein, K_d 0.25±0.05 nM). With the exception of BIM23056, several SRIF analogues (Raynor et al., 1993) completely inhibited [I^{125}]-BIM23027 binding with Hill slopes close to unity (Table 1).

Table 1

Competition binding data for SRIF analogues to inhibit [I^{125}]-BIM23027 binding in rat colonic mucosal membranes

	pIC_{50}	n_H		pIC_{50}	n_H
SRIF	9.32±0.06	0.92±0.10	**BIM23027**	9.61±0.05	1.10±0.18
$SRIF_{28}$	9.11±0.07	0.89±0.01	**I-BIM23027**	9.51±0.04	0.88±0.13
Seglitide	9.78±0.05	0.99±0.06	**BIM23056**	<6	-
D-Trp^8SRIF	9.62±0.05	1.02±0.07	**L362855**	8.17±0.07	1.07±0.04
Octreotide	8.84±0.06	0.90±0.06	**CGP23996**	8.40±0.04	0.90±0.06

The results suggest that [I^{125}]-Tyr^{11} SRIF labels two sites in the rat colonic mucosal membranes. One of these sites exhibits high affinity for BIM23027 and seglitide. The pIC_{50} values of the SRIF agonists at inhibiting [I^{125}]-BIM23027 binding correlates well with the potencies reported for these agonists as antisecretory agents (McKeen et al., 1994) in the rat colonic mucosa (r = 0.92 slope = 1.06). The binding sites labelled by [I^{125}]-BIM23027 are therefore functionally important. The rank order of pIC_{50} values (BIM23027 > SRIF > L362855 >> BIM23056) most closely resembles that seen for the recombinant SSTR2 receptor. The functional significance of the BIM23027- and seglitide-insensitive SRIF binding sites remains to be established.

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232P VASODILATOR EFFECTS OF LIVIAL, ITS METABOLITES AND SELECTIVE STEROID HORMONES: INFLUENCE OF THE ENDOTHELIUM

M. Beaughard, G. Dureng & E. Winslow, Department of Pharmacology, RL Cerm, Rue Henri Goudier, 63200 Riom Cedex, France.

Controversy exists concerning the acute vasodilator actions of oestrogens and the possible role played by the endothelium (Chester et al., 1993; Miller and Vanhoutte, 1990). Livial (L), which combines weak oestrogenic, progestagenic and androgenic activity induces vasodilator effects in post-menopausal women (Hardiman et al., 1991). The present study aimed to compare the vasodilator activity of L, its two major metabolites (OM38 and Org 4094) and 17-β-oestradiol (O) with a selective progestagen (16α-16-ethyl-21-OH-19-norpreg-4-ene-3,20-dione (PR) and a selective androgen ((5α,17β)-17-OH androstan-3-one) (A).
Female rat thoracic aortic rings were mounted in organ baths containing physiological salt solution at 37°C gassed with carbogen. Cumulative concentration response curves for relaxation of K^+ (40 mM)-induced contractions were obtained. After correction for vehicle (DMSO)-induced small relaxations, IC values were calculated. L, its metabolites and O all induced non-endothelial dependent relaxation (see Table 1) with maximal vasodilatation of between 70-85% of the K^+ response.

Table 1: Mean (95% confidence interval)

	n	endothelium present		endothelium absent	
		IC_{25}	IC_{50}	IC_{25}	IC_{50} μM
Livial	6	0.10 (0.03-0.30)	4.7 (1.7-13.1)	0.17 (0.02-1.2)	2.3 (0.63-8.3)
OM 38	6	2.5 (1.2-5.1)	20.4 (11.1-37.5)	3.6 (1.1-11.8)	33.6 (9.8-116)
Org 4094	6	0.15 (0.07-0.36)	4.6 (2.1-10.5)	0.18 (0.03-1.1)	2.6 (0.76-8.8)
17-β-oestradiol	6	0.11 (0.03-0.41)	3.6 (1.1-11.7)	0.29 (0.06-1.4)	3.4 (1.1-10.6)
PR	6	0.05 (0.01-1.30)	17.4 (0.7-415)	1.3 (0.38-4.4)	28.0 (6.2-127)
A	6	0.08 (0.01-0.55)	6.5 (1.1-37.5)	2.0 (0.41-9.5)	21.3 (5.9-76.8)

A small endothelial dependent component to the vasorelaxant actions of PR and A appeared to be present at low concentrations (<1 μM, reflected by the IC_{25} values) although statistical significance was not attained.
It was concluded that L, its metabolites and O all induce non-endothelial dependent relaxations perhaps via a direct effect on smooth muscle oestrogen receptors. However, since similar concentrations of the selective androgen and progestagen also induced relaxation, notably when the endothelium was present, these results could also equally well be explained, not by hormonal actions, but by a common moiety in the molecular structures of these streroids (e.g. the OH group on the D ring) which influences another receptor system. The vasodilator effects of L cannot therefore be solely attributed to any one, if any, of its hormonal actions and may possibly reside in its chemical structure.

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233P INHIBITION BY DEXAMETHASONE, ENALAPRIL AND HEPARIN OF THE LESION INDUCED BY COLLAR IMPLANTATION IN THE RABBIT

L.C. Tilling, G. Fleetwood & A.C. Honey, Biology Division, Wellcome Research Labs., Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS

Balloon injury to the lumenal surface of an artery causes removal of the endothelium, medial damage and subsequent intimal hyperplasia (Clowes *et al.*, 1983). Neointimal growth can also be induced after adventitial manipulation by enclosing the artery within a silastic collar (Booth *et al.*, 1989). We have investigated whether agents shown to be effective in reducing lesion size after balloon injury (Ferns *et al.*, 1992) are also effective in reducing the lesion induced by the silastic collar.

Collars (length, 25mm; inlet diameter, 1.75mm) were implanted (under Hypnorm/Hypnovel anaesthesia) around the carotid artery of NZW rabbits. After 14 days arteries were perfusion fixed with neutral buffered formalin and embedded in wax. Transverse sections were stained with haematoxalin/ eosin-phloxin. Intima and media areas and cell numbers were determined by image analysis (Sight Systems). Statistical comparisons were performed by unpaired Student's t-test.

Table 1 shows that the angiotensin converting enzyme inhibitor, enalapril (3 $mgkg^{-1}$, p.o. daily), the steroid anti-inflammatory agent dexamethasone (3 $mgkg^{-1}$, i.m. daily) and heparin (local delivery, 0.15 μghr^{-1} into collar) significantly reduced intimal area (I/M ratio) and the number of cells in the intima (nuclei count). Enalapril and dexamethasone also significantly reduced the total number of cells in the vessel wall, whilst this was unaffected by heparin. The calcium antagonist, flunarazine (25 $mgkg^{-1}$, p.o. daily) had no significant effect on lesion size. These results suggest that enalapril and dexamethasone act by inhibiting cell proliferation, whilst heparin inhibits cell migration into the intima.

Table 1.

	ENALAPRIL	DEXAMETHASONE	HEPARIN
I/M ratio:		geometric mean (95% confidence limits):	
vehicle	0.13 (0.10-0.16)	0.26 (0.21-0.32)	0.26 (0.13-0.53)
drug	0.05* (0.03-0.10)	0.07* (0.05-0.09)	0.09* (0.07-0.12
intimal nuclei count:		mean ± s.e.mean	
vehicle	351±50	507±57	469±68
drug	120±40*	146±17*	281±43*
total nuclei count:			
vehicle	1124±115	1017± 64	1088±192
drug	328±119*	457±54*	1331±126

* $P < 0.05$ vs. vehicle group

We conclude that agents able to attenuate neointimal lesion formation after balloon injury are also able to reduce the size of the lesion caused by enclosing the artery within a silastic collar.

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234P INFLUENCE OF COLLAR GEOMETRY ON INTIMAL HYPERPLASIA INDUCED BY ENCLOSING THE CAROTID ARTERY OF THE RABBIT WITHIN A SILASTIC COLLAR

G. Fleetwood & A.C. Honey, Biology Division, Wellcome Research Labs., Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS

Enclosing the carotid artery of the rabbit within a saline filled silastic collar results in intimal hyperplasia which becomes maximal after 14 days (Booth *et al.*, 1989). To determine whether the dimensions of the collar affect the characteristics of this lesion we have carried out experiments with four different types of collar.

All collars were made from 'Silastic-382 Medical Grade Elastomer' (Dow Corning) and were implanted (under Hypnorm/Hypnovel anaesthesia) around the left carotid artery of male New Zealand White rabbits (2.5-3.5 kg) for 14 days. The right carotid was sham operated. Arteries were perfusion fixed *in situ* with neutral buffered formalin and embedded in wax. Transverse sections (TS) were stained with haematoxalin/ eosin-phloxin. Lesion sizes, measured as intima/media ratio (I/M ratio) at the centre of the collared segment, followed a log normal distribution and are expressed as geometric means [95% confidence limit]. Cell numbers, measured by counting nuclear profiles, were normally distributed and are expressed as mean±s.e.mean cells/TS. Student's unpaired t-test was used for statistical analysis.

Standard collars (length 15mm, inlet diameter 2mm, touching artery for 2.5mm at each end and enclosing a volume of 0.126cm^3) produced a lesion with an I/M ratio of 0.177 [0.128-0.246]. The I/M ratio of the sham operated contralateral was 0.058 [0.037-0.090]. Intimal cell number increased significantly from 124±38 in sham to 327±46 in the collared artery ($P<0.05$). Media cell number was significantly reduced from 791±78 to 475±81 ($P<0.05$). There was no significant change in total cell number (915±85 in sham, 892±155 in collar).

The size of the lesion could be significantly increased by reducing the diameter of the inlet of the collar to 1.75mm (I/M ratio 0.395 [0.277-0.564]) ($P<0.05$) or by increasing the length to 25mm (0.385 [0.279-0.531]) ($P<0.05$). Combining these two modifications had no further effect on lesion size (I/M ratio 0.394 [0.269-0.577]). Cell number in the intima tended to be increased by changes in collar length and inlet diameter (520±81 with the combined modifications). This was probably due to increased cell migration since total cell number was not significantly altered (941±85).

Further investigations showed that lesion size was maintained along the length of the collar, although there was a tendency to decrease towards the distal end. The collar significantly increased the lumen area of the artery (1.548±0.069 cf. 0.863± 0.071 mm^2 in sham, $P<0.05$).

These results illustrate that enclosing a carotid artery within a silastic collar induces intimal growth throughout the length of the enclosed segment, mainly as a result of cell migration. The size of this lesion is influenced by the length and fit of the collar around the artery wall.

Booth RFG, Martin JF, Honey AC et al. (1989) Atherosclerosis 76, 257-268.

235P COLLAR-INDUCED NEO-INTIMA FORMATION IS INFLUENCED BY THE GEOMETRY OF THE COLLAR

G.R.Y. De Meyer, D.J.M. Van Put, H. Bult, M.M. Kockx[1] & A.G. Herman, Divisions of Pharmacology, University of Antwerp (UIA), B-2610 Wilrijk, and [1]Pathology, General Hospital Middelheim, B-2020 Antwerp, Belgium.

The positioning of a non-occlusive silicone collar around the rabbit carotid artery results in the formation of a neo-intima (Booth *et al.*, 1989; De Meyer *et al.*, 1994; Soma *et al.*, 1993). The mechanisms responsible for intimal thickening in this model are still debated. We investigated whether the diameter of in- and outlet of the collar or the impediment of the transmural fluid transport due to the collar wall contribute to neo-intima formation.

The following collar types (Figure 1) were positioned around the carotid artery of male rabbits for 14 days: collars with an internal volume of 278 µl and an inlet/outlet diameter of 3.1 mm (type 1A; very loose), 2.3 mm (type 1B; loose) or 1.8 mm (type 1C; fitting) and a collar with an internal volume of 134 mm^3 and an inlet/outlet diameter of 1.8 mm (type 2; fitting). In a second series of experiments the side wall of collar type 1A was largely removed, leaving two strips (one on top, one on the bottom) to connect the edges of the collar (window collar). Cross-sections (mid collar region) were used to measure the area of the neo-intima with a digitizing tablet.

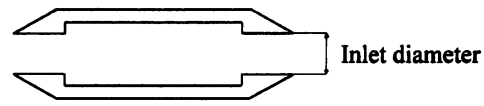
The area of the neo-intima (mm^2 10^{-3}) increased as the inlet diameter decreased and amounted to 24±4 (n=22), 38±8 (n=10), 53±9 (n=15) and 72±14 (n=15) with collar type 1A, 1B, 1C and 2 respectively (type 2 versus type 1A: $P<0.05$, analysis of variance, Student-Newman-Keuls test). Removal of the side wall of collar type 1A did not significantly alter the area of the neo-intima (33±8 in normal collar versus 20±4 mm^2 10^{-3} in window collar, n=13, Student's paired t-test).

In conclusion, the geometry of the collar influences the extent of neo-intima formation, whereby the diameter of the aperture appears to be an important parameter. More neo-intima formation is obtained with collars whose edges fit the carotid artery, rather than with loosely fitting collars. Impediment of transmural fluid transport does not seem to contribute to collar-induced neo-intima formation in loosely fitting collars.

G.R.Y. De Meyer is a Senior Research Assistant and D.J.M. Van Put is a Research Assistant of the Belgian National Fund for Scientific Research (N.F.W.O.).

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Figure 1.
Longitudinal section of the collar.



236P THE INVOLVEMENT OF ATP-SENSITIVE POTASSIUM CHANNELS IN β-ADRENOCEPTOR-MEDIATED VASORELAXATION OF THE ISOLATED PERFUSED RAT SUPERIOR MESENTERIC ARTERIAL BED

M.D. Randall, Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH.

Recent evidence has indicated that potassium channels may contribute towards β-adrenoceptor-mediated relaxations of pulmonary (Cook *et al.*, 1993) and vascular smooth muscle (Jackson, 1993). The involvement of K_{ATP}-channels in the mediation of vasodilator responses to a β-agonist has now assessed by the ability of glibenclamide to influence responses to isoprenaline in the isolated buffer-perfused rat mesenteric arterial bed. As a comparison the relaxant effects of verapamil have also been assessed in the absence and presence of glibenclamide.

Male Wistar rats (180-220g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1} i.p.) and the mesenteric arterial bed cannulated and perfused, as described previously (Randall & Hiley, 1988), with oxygenated Krebs-Henseleit solution at 5 ml min^{-1}. Perfusion pressure was continuously monitored by a pressure transducer placed close to the inflow cannula. In each case, following a 30 min equilibration, vascular perfusion pressure was raised by addition of methoxamine (10-30µM) and dose-response curves were constructed for the vasodilators in the absence or presence of 10µM glibenclamide.

Basal perfusion pressure was 19.9±0.7mmHg (mean±s.e.mean, n=12) and was increased by 105±8mmHg following the addition of methoxamine. Isoprenaline gave rise to dose-related relaxations of established tone (ED_{50} = 0.26±0.03nmol, R_{max} = 73.7±4.1%, n=8). Following the addition of glibenclamide there was a significant ($P<0.05$) rightward shift in the dose-response curve to isoprenaline (ED_{50} = 1.05±0.4nmol, R_{max} = 64.8±6.1%, n=8). Verapamil also gave rise to dose-related relaxations of tone (ED_{50} = 1.29±0.35nmol, R_{max} = 81.7±2.9%, n=4) which were unaffected by the subsequent addition of glibenclamide (ED_{50} = 1.24±0.27nmol, R_{max} = 86.3±5.5%, n=4).

The results of this investigation demonstrate that vasorelaxation to the β-agonist isoprenaline, but not that to verapamil, is partially sensitive to the selective K_{ATP}-channel blocker glibenclamide. This may suggest that β-adrenoceptor-mediated vasodilatation occurs partially via the activation of K_{ATP}-channels.

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237P EFFECTS OF CROMAKALIM AND GLIBENCLAMIDE ON RABBIT ISOLATED TENUISSIMUS MUSCLE DURING HYPOXIA OR NORMOXIA

J.M. Gurden & V.J. Hart, Pharmacology 2, Glaxo Research and Development Ltd., Park Road, Ware, Herts SG12 ODP.

ATP-dependent potassium (K_{ATP}) channels exist in skeletal muscle vasculature and myocytes (Quasthoff *et al.*, 1990). Cromakalim, a K_{ATP} channel activator, improves skeletal muscle function in a model of hindlimb ischaemia (Hatton *et al.*, 1991) but its site of action is uncertain. Using the rabbit isolated tenuissimus muscle, the effects of cromakalim were studied on skeletal muscle function during hypoxia or normoxia.

Tenuissimus muscles from heparinised (1000iu/kg i.v.), sacrificed New Zealand White rabbits (male, 2.93±0.03 kg, n=20) were sectioned transversely to give two tissues. Each was suspended (7g resting tension) in Krebs-Henseleit solution (Ca^{2+} 1.3mM) at 38°C and field stimulated (90% maximum voltage, 2Hz, 7ms) for 4 min every 45 min, during alternate periods of normoxia ($95\%O_2/5\%CO_2$) and hypoxia ($95\%N_2/5\%CO_2$). Residual muscle function (RMF) was calculated from tension developed at the end of each stimulation expressed as a percentage of the maximum achieved in that period (61.1±1.6% during normoxia and 37.5±1.5% during hypoxia, n=20). The effects of cromakalim (10nM-300μM) or vehicle on RMF were studied during hypoxia (H2) and normoxia (N2) and were compared to values obtained prior to addition of cromakalim (H1, N1). The ratios H2/H1 and N2/N1 were compared by Student's paired t-test to those for the paired vehicle-treated tissue (Table 1).

Cromakalim (10-300μM) caused a concentration-related exacerbation of muscle fatigue, which was more marked during hypoxia. Responses to a maximally-effective concentration of cromakalim (100μM) were abolished by the K_{ATP} channel blocker glibenclamide (10μM), which had no effect alone. These results demonstrate that this tissue is suitable for examining skeletal muscle function *in vitro* and that cromakalim causes hypoxia-selective exacerbation of muscle fatigue through glibenclamide-sensitive K_{ATP} channels.

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Table 1: Effect of vehicle or cromakalim on RMF during hypoxia or normoxia (n=4-8). Significance claimed (*) when P<0.05.

		Vehicle (V) or concentration (μM) of cromakalim (C) ± glibenclamide (G; 10μM)					
		1	10	30	100	300	G + 100
Hypoxia	V	100.8	105.5	87.9	107.3	99.1	104.1
(H2/H1, %)	C	98.4	85.7*	59.3*	54.9*	76.9*	117.0
Normoxia	V	107.8	108.2	109.9	111.5	109.7	119.5
(N2/N1, %)	C	113.3	92.5	80.8	83.3*	78.2*	105.5

238P INHIBITORY EFFECTS OF RUBIDIUM ON THE RESPONSES TO LEVCROMAKALIM AND P1060 IN THE HUMAN ISOLATED MYOMETRIUM

D.N. Criddle & R. Soares de Moura. Departamento de Farmacologia, Universidade do Estado do Rio de Janeiro, Brasil

The smooth muscle relaxant effects of K-channel openers (KCOs) are inhibited by rubidium in animal (Foster et al., 1989; Greenwood & Weston, 1993) and human isolated tissues (Criddle & Soares de Moura, 1994). Under conditions in which the bathing solution contains Rb^+ ions instead of K^+, the K-channel openers can no longer elicit hyperpolarisation or stimulate transmembrane ^{42}K movement, yet can fully relax blood vessels suggesting that they possess actions that are not mediated via the opening of plasmalemmal K-channels (Greenwood & Weston, 1993). Since KCOs exert inhibitory effects on the human isolated uterus (Cheuk et al., 1993; Morrison et al., 1993), we decided to assess whether inhibitory effects of rubidium on the actions of KCOs also occur in the human non-pregnant uterus, and if differences exist between structurally-different drugs by comparing the actions of the benzopyran levcromakalim (LK) with the cyanoguanidine pinacidil-derivative P1060.

Segments of uterus were obtained from patients undergoing hysterectomy (Institutional approval obtained). Strips were suspended in organ chambers filled with Krebs-Henseleit solution bubbled with 95% O_2 5% CO_2 at 37°C and isometric tension recorded using conventional techniques. Following stabilisation, tissues were exposed to either normal Krebs (K-PSS) or a modified solution in which the K^+ salts had been replaced by their Rb^+ equivalents (Rb-PSS) for 30 minutes. The strips were allowed to equilibrate for a further 30mins before cumulative concentration-response curves were constructed to LK, P1060 or verapamil.

Basal amplitudes and frequencies of contraction did not differ significantly between strips bathed in K-PSS and Rb-PSS (1.78±0.27g and 0.18±0.02min^{-1} (n=12) and 2.01±0.27g & 0.16±0.02min^{-1} (n=12), respectively). LK induced a concentration-dependent reduction of amplitude and frequency of contraction in K-PSS (IC_{50}s of 0.041 & 0.038μM, respectively) that was inhibited in Rb-PSS approximately 26- and 20-fold (IC_{50}s of 1.07 & 0.75μM, respectively, n=6). P1060 inhibited contractions of the uterus with a slightly greater potency than levcromakalim (IC_{50}s of 0.018 & 0.026μM, respectively, n=6) and these effects were inhibited approximately 138- and 160-fold in Rb-PSS (IC_{50}s of 2.48 & 4.15μM, respectively). In contrast, the inhibitory effects of verapamil were not significantly different between the K-PSS and Rb-PSS groups (n=4).

Our results show that LK and P1060 possess Rb-sensitive and Rb-insensitive mechanoinhibitory actions on the human uterus, the former being more important in the effects of P1060. Rb-PSS appears able to differentiate between the actions of structurally different KCOs and may thus indicate a future basis for develpoment of selectivity of drug action in the uterus.

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239P LOCAL ADMINISTRATION OF POTASSIUM CHANNEL OPENERS AND ISCHAEMIA-INDUCED VENTRICULAR ARRHYTHMIAS

K. Kaszala, A. Vegh, J. Gy Papp, K. Gyorgy & J.R. Parratt[1], Department of Pharmacology, A. Szent-Gyorgyi Medical University, Szeged, Hungary and [1]Department of Physiology and Pharmacology, University of Strathclyde, Glasgow Gl lXW.

There is still confusion regarding the effect of drugs that open K^+ channels on arrhythmias induced by myocardial ischaemia and of the role of these channels in mediating the antiarrhythmic effects of ischaemic preconditioning. In order to avoid peripheral vasodilatation, and resultant effects on cardiac sympathetic drive, we gave laevo cromakalim, the active enantiomer of cromakalim, by intracoronary infusion in a total dose of 3 $\mu g\ kg^{-1}$ given over a 30 min period into a side branch of the left anterior descending (LAD) coronary artery, immediately before a 25 min occlusion of that artery in mongrel dogs anaesthetised with chloralose and urethane (Vegh *et al.*, 1992). Under these conditions there were minimal effects of cromakalim on mean blood pressure (from 87 ± 5 to 76 ± 5 mmHg) and heart rate (from 134 ± 11 to 137 ± 8 beats min^{-1}) and increases in coronary blood flow (from 19 ± 2 to 25 ± 2 cm sec^{-1}; $P < 0.05$). Cromakalim failed to modify significantly the ventricular arrhythmias that resulted from LAD occlusion (incidence of ventricular fibrillation (VF) 56%, ventricular tachycardia (VT) 67%, VT episodes/dog 15.4 ± 7.3, ventricular premature beats (VPBs) 543 ± 257, survival after reperfusion 22%) compared with those dogs subjected to LAD occlusion in the absence of cromakalim (VF 47%, VT 80%, VT episodes 5.9 ± 1.7, VPBs 497 ± 109, survival 0%). These results demonstrate that opening myocardial K^+ channels with cromakalim prior to ischaemia is not protective but neither does it increase the severity of occlusion-induced arrhythmias.

In another seven dogs we compared these results with those obtained with nicorandil, a "mixed" K^+ opener and nitric oxide (NO) donor, given in a dose of 2.5 $\mu g\ kg^{-1}\ min^{-1}$ i.c. for 20 min prior to occlusion and then throughout the occlusion period (total dose 112.5 $\mu g\ kg^{-1}$). This reduced the incidence of VF during occlusion (0/7) and during reperfusion (2/7); the survival from combined occlusion-reperfusion insult was thus 71% (cp 0% in the controls). One explanation for this difference between the two K^+ channel openers might be the additional property of nicorandil as an NO donor.

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240P UPREGULATION OF SODIUM/POTASSIUM PUMPS BY THE LOOP DIURETIC ETHACRYNIC ACID: POSSIBLE ROLE OF PHOSPHOINOSITIDE TURNOVER

R. A. Murton & J. K. Aronson, University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE

The loop diuretic ethacrynic acid increases the number of Na/K pumps in the cell membranes of human lymphocytes (Rapeport *et al.*, 1986). The mechanism underlying this upregulation is unclear, but has been attributed to a raised intracellular sodium concentration. However, in rat cochlea ethacrynic acid alters phosphoinositide turnover, an effect that may underlie its ototoxic action (Bartolami *et al.*, 1993). Furthermore, the upregulation of Na/K pumps by lithium has also been attributed to an action on phosphoinositide turnover (Jenkins *et al.*, 1991). We have therefore tested the hypothesis that ethacrynic acid increases Na/K pump numbers in human lymphocytes by altering phosphoinositide turnover.

We exposed Epstein-Barr virus-transformed lymphocytes in culture for 72 h to ethacrynic acid alone (10–30 μM), myoinositol alone (10 mM), or ethacrynic acid (30 μM) with myoinositol (10 mM). We measured the numbers of Na/K pumps in the cell membranes by measuring the B_{max} of binding of [^{3}H]-ouabain (2.5–40 nM for 2 h; Kennedy *et al.*, 1990).

Ethacrynic acid increased Na/K pump numbers dose-dependently by 16–29% compared with control, without changing the affinity of ouabain binding; myoinositol prevented this increase without itself having any effect (Table 1).

These data support the view that the upregulation of Na/K pumps by ethacrynic acid in lymphocytes could be due, at least in part, to modulation of phosphoinositide signalling, since it is prevented by myoinositol.

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Table 1. Percentage increases in pump numbers due to ethacrynic acid and myoinositol separately and together

	Ethacrynic acid 10 μM	Ethacrynic acid 20 μM	Ethacrynic acid 30 μM	Myoinositol 10 mM	Ethacrynic acid (30 μM) + myoinositol (10 mM)
Control no. of pumps	305 000	282 000	242 000	265 000	279 000
Mean increase (%)	16	24	29	0.1	2.4
sem	3.7	5.1	5.8	2.6	2.9
n	15	13	9	7	8
P (versus control)*	<0.002	<0.002	<0.01	>0.5	>0.5
P (versus control)†	<0.007	<0.006	<0.004	1.0	>0.96

* Unpaired t-tests of percentage increases after arcsin transformation, with Bonferroni correction
† Sign tests comparing the absolute numbers of pumps with control values

241P THE ANTAGONISM OF PINACIDIL AND SDZ PCO400 BY GLIBENCLAMIDE AND GLIPIZIDE ON THE MOUSE INTESTINE CONTRACTED BY ELECTRICAL FIELD STIMULATION: A COMPARATIVE STUDY

C.K. Yeung, J.R. McCurrie, D. Wood, Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford, West Yorkshire, BD7 1DP, UK

The type of antagonism between pinacidil (PIN) and ATP-sensitive potassium channel (K_{ATP}) blockers is a matter of controversy. Using vascular smooth muscle McPherson & Angus (1990) have reported competitive antagonism whereas Steinberg *et al.* (1990) have found non-competitive antagonism. The present study, using intestine contracted by electrical field stimulation (EFS), aimed to characterise the nature of the antagonism of the relaxant effects of the potassium channel openers SDZ PCO400 (SDZ) and PIN by glibenclamide (GBC) and by a structurally related compound, glipizide (GPZ).

Mucosa-free preparations derived from the distal ileum of male BKW mice were placed in Krebs' solution under 0.5g tension (37°C, 95%O_2/5%CO_2). Following equilibration, isometric contractions were elicited by EFS (0.5Hz, supramaximal voltage). When consistent twitches were generated, cumulative concentration-response curves to PIN (0.3-100μM) or SDZ (0.3-60μM) or vehicle were performed. This procedure was repeated following 20 min incubation with a single dose of GBC (0.1-1μM) or GPZ (3-30μM) or vehicle. The effects of relaxants were expressed as mean ± s.e. mean of the EC_{50} (the concentration required to reduce twitch height by 50%). Statistical analysis was performed using the Student's paired t-test. Schild analysis of antagonism was performed (Arunlakshana & Schild, 1959) and slopes of the regression lines with 95% confidence limits were calculated ($n \geq 12$).

SDZ was a slightly more potent relaxant than PIN; EC_{50} values were 2.1±0.2μM (n = 27) and 3.3±0.3μM (n = 12) respectively. Maximal relaxation was similar (approx. 85%) for both compounds. GBC and GPZ caused concentration-dependent antagonism of the relaxant effects of both PIN and SDZ ($P<0.05$). The slopes of the Schild plots were close to unity using SDZ as the relaxant (GBC, 0.92; 0.53-1.32: GPZ 1.19; 1.00-1.37) but not with PIN (GBC 0.64; 0.30-0.93: GPZ 0.68; 0.41-0.95).

The pA_2 values (negative logarithm of the molar concentration of antagonist required to produce a two-fold shift in the EC_{50} for SDZ) using GBC and GPZ were 7.3 and 5.7 respectively. Thus GBC was approximately 40 times more potent than GPZ. Although it would not be appropriate to calculate pA_2 values for antagonism of PIN, the apparent effectiveness of the antagonists against PIN was similar to that observed for SDZ.

Thus in the mouse ileum there were differences in the ways in which GBC and GPZ antagonised PIN and SDZ. The results are consistent with the possibility that PIN has more than one site of action, possibly acting on calcium channels (Steinberg *et al.*, 1988) in addition to the K_{ATP} channel. SDZ, which appeared to be competitively antagonised by GBC and GPC, may act solely on K_{ATP} channels. In view of many previous studies which have shown competitive antagonism between PIN and GBC in a variety of vascular preparations, we suggest that the mechanism(s) underlying potassium channel modulation may be tissue and/or species dependent.

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242P EFFECTS OF DEXAMETHASONE (DEX) PRETREATMENT ON REGIONAL HAEMODYNAMIC RESPONSES TO LIPOPOLYSACCHARIDE (LPS) IN CONSCIOUS RATS

S.M. Gardiner, P.A. Kemp, J.E. March & T. Bennett. Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham NG7 2UH.

Although DEX is known to suppress some of the adverse cardiovascular effects of endotoxaemia (e.g., Wright *et al.*, 1992; Paya *et al.*, 1993), the haemodynamic details of this action are unknown. We have therefore assessed the effects of i.v. DEX (3 mg kg^{-1}; 3 mg kg^{-1} 24 h^{-1}, n = 9) or saline (SAL, 0.4 ml h^{-1}, n = 8) for the 24 h preceding, and the 24 h during, i.v. infusion of LPS (E coli serotype 0127 B8, 150 μg kg^{-1} h^{-1}) in conscious Long Evans rats (350-450g) chronically instrumented for recording renal (R), mesenteric (M) and hindquarters (H) haemodynamics, with pulsed Doppler probes and intravascular catheters (all surgery under sodium methohexitone anaesthesia, 40-60 mg kg^{-1} i.p.). One day after the onset of DEX infusion, there was an increase in mean BP and a bradycardia, together with falls in vascular conductance (VC) in all 3 vascular beds (Table 1).

Table 1. Resting cardiovascular variables before (C) and 24 h after onset of SAL (n = 8) or DEX (n = 9). * $P < 0.05$ (Friedman's test). Units for VC are [kHz mm Hg^{-1}]10^3; values are mean ± s.e. mean.

	SAL		DEX	
	C	24 h	C	24 h
HR (beats min^{-1})	331 ± 8	309 ± 5	326 ± 6	296 ± 5*
MAP (mm Hg)	100 ± 2	100 ± 1	100 ± 1	112 ± 2*
RVC (units)	76 ± 8	69 ± 7	63 ± 5	49 ± 5*
MVC (units)	70 ± 7	75 ± 9	82 ± 5	67 ± 4*
HVC (units)	43 ± 3	38 ± 4	37 ± 3	28 ± 2*

The early hypotensive and renal vasodilator effects of LPS (Gardiner *et al.*, 1992), which were maximal at 2 h in SAL-treated rats, were delayed in DEX-treated rats (maximal at 4 h, Table 2); in the latter, the maximal renal vasodilatation was attenuated also ($P < 0.05$, Mann-Whitney U-test). By 24 h after the onset of LPS infusion, the DEX-treated rats showed no fall in mean arterial pressure (MAP), no increase in heart rate (HR), an attenuated renal vasodilatation and no hindquarters vasodilatation (Table 2).

Table 2. Changes in cardiovascular variables during infusion of LPS in animals pretreated with SAL (n = 8) or DEX (n = 9).
* $P < 0.05$ (Friedman's test). Values are mean ± s.e. mean.

Time after start of LPS		2 h	4 h	24 h
Δ HR (beats min^{-1})	SAL	51 ± 11*	23 ± 12	92 ± 13*
	DEX	22 ± 9*	41 ± 8*	-2 ± 11
Δ MAP (mm Hg)	SAL	-7 ± 1*	5 ± 2	-9 ± 4*
	DEX	2 ± 2	-7 ± 2*	0 ± 2
Δ RVC (units)	SAL	51 ± 8*	41 ± 7*	71 ± 13*
	DEX	11 ± 4*	31 ± 5*	13 ± 4*
Δ MVC (units)	SAL	-28 ± 3*	-29 ± 6*	-9 ± 7
	DEX	-28 ± 2*	-9 ± 3*	-15 ± 3*
Δ HVC (units)	SAL	10 ± 5*	-9 ± 3*	29 ± 5*
	DEX	-4 ± 1*	-1 ± 2	4 ± 2

The ability of DEX to inhibit substantially the LPS-induced cardiovascular changes is consistent with its action to suppress induction of nitric oxide synthase and cyclo-oxygenase-2.

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243P INVOLVEMENT OF ANGIOTENSIN II AND VASOPRESSIN IN THE HAEMODYNAMIC RESPONSES TO LIPOPOLYSACCHARIDE (LPS) IN CONSCIOUS RATS

S.M. Gardiner, P.A. Kemp, J.E. March & T. Bennett. Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham NG7 2UH.

Although recent interest has focused on vasodilator mechanisms in endotoxaemia, LPS is a potent stimulus for angiotensin II (AII) generation and vasopressin (AVP) release (Schaller *et al.*, 1985), and both hormones can have marked haemodynamic effects. We have shown that pre-treatment with dexamethasone (DEX) can prevent the hypotension and markedly attenuate the peripheral vasodilatation seen during infusion of LPS (Gardiner *et al.*, 1994). In this study we assessed the effects of the AII (AT-1) receptor antagonist, losartan (10 mg kg^{-1}) and, 15 min later, the AVP (V_1)-receptor antagonist, [$d(CH_2)_5$0-Me-Tyr, Arg]-vasopressin (AVPX, 100 µg kg^{-1}; 100 µg kg^{-1} h^{-1}) in rats that had been infused with LPS (E coli serotype 0127 B8, 150 µg kg^{-1} h^{-1}) for 24h together with DEX (3 mg kg^{-1}; 3 mg kg^{-1} 24 h^{-1}) or saline (0.4 ml h^{-1}) starting 24h before, and continuing throughout the LPS infusion; all substances were administered i.v.

Animals were instrumented with pulsed Doppler probes and intravascular catheters to monitor renal, mesenteric and hindquarters haemodynamics (all surgery was carried out under sodium methohexitone anaesthesia 40-60 mg kg^{-1} i.p.). Prior to administration of losartan, resting values in the animals (n = 8) that had received LPS and saline (SAL) were:- heart rate (HR), 403 ± 14 beats min^{-1} (mean ± s.e. mean); mean arterial blood pressure (MAP), 91 ± 4 mm Hg; renal, mesenteric and hindquarters vascular conductance (RVC, MVC, HVC), 133 ± 14, 66 ± 5 and 67 ± 7 (kHz mm Hg^{-1})10^3, respectively. In animals (n = 9) that had received LPS + DEX, the corresponding values were:- HR, 298 ± 7 beats min^{-1}; MAP, 112 ± 3 mm Hg; RVC, 62 ± 8, MVC, 52 ± 4 and HVC, 33 ± 3 (kHz mm Hg^{-1})10^3. Table 1 summarises the responses to losartan and AVPX, expressed as changes relative to the resting values given above, and measured 15 min after administration of losartan and 5 min after AVPX.

Table 1. Cardiovascular changes after sequential administration of losartan and a vasopressin antagonist (AVPX) in rats receiving LPS + SAL or LPS + DEX. Values are mean ± s.e. mean; * $P < 0.05$ versus pre-losartan value; † $P < 0.05$ versus value after losartan (Friedman's test). Units for vascular conductance are [kHz mm Hg^{-1}]10^3.

	Losartan		AVPX	
	LPS + SAL	LPS + DEX	LPS + SAL	DEX + LPS
Δ HR (beats min^{-1})	34 ± 7*	22 ± 12	35 ± 5*	26 ± 14
Δ MAP (mm Hg)	-13 ± 3*	-4 ± 1*	-19 ± 4*†	-4 ± 1
Δ RVC (units)	43 ± 8*	6 ± 2	59 ± 12*†	2 ± 2
Δ MVC (units)	19 ± 6*	6 ± 2	29 ± 8*†	3 ± 2
Δ HVC (units)	9 ± 3*	3 ± 2	20 ± 5*†	3 ± 2

In the animals that had received LPS with saline, losartan caused tachycardia, hypotension, and increases in RVC, MVC and HVC. Subsequent administration of AVPX caused slight, additional hypotension and increases in RVC, MVC and HVC. In animals treated with DEX + LPS, losartan had a minimal effect on MAP, but no other action, and AVPX was without influence. The results indicate that AII and AVP exert vasoconstrictor influences after LPS, but not after DEX + LPS. It is possible this is because DEX prevents activation of the renin-AII system and AVP release, directly and/or indirectly, due to the relative elevation in MAP.

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244P THE EFFECTS OF AMRINONE AND GLUCAGON ON VERAPAMIL-INDUCED MYOCARDIAL DEPRESSION IN ISOLATED RAT HEART

Tuncok Y^1, Apaydin S^1, Gidener S^1, Guven H^1, Oto O^2, Ates M^1, Gure A^3. [1]Department of Pharmacology,[2] Chest and Cardiac Surgery and [3]Physiology. Dokuz Eylul University Medical Faculty 35340 Inciralti, IZMIR, TURKEY

Calcium channel blockers (CCBs) may produce profound myocardial depression, and an agent capable of reversing CCB-induced myocardial toxicity would be important. There is a little literature on treatment of CCBs with glucagon or amrinone alone (Zaritsky et al.1988 and Dagher et al.1989) and there are no studies using the combination of these inotropic agents or comparing their efficacy. The goal of this study is to compare the ability of glucagon and amrinone, used alone and in combination to improve the myocardial function in an rat isolated heart model of CCBs overdose. Unanesthetized male rats (200 to 300g) were killed by cervical dislocation. The heart was rapidly removed and placed in iced Ringer solution. The aorta was cannulated and the coronary circulation perfused by the Langendorff method with Krebs-Henseleit buffer solution at the flow rates that varied from 6-8 ml/min. Cardiac contractile force was estimated by monitoring isometric tension development generated through a hook attached to the apex of the heart by means of a force diplacement transducer. Changes in contractile force and heart rate derived from the transducer were displayed continuously on a strip chart recorder. After 15 min equilibration, all hearts were given 100µl bolus containing 10^{-4} mole verapamil. After producing 30-50 % depression in cardiac contractile force, hearts were injected 100µl bolus containing 10^{-5} to 10^{-3} mole amrinone, glucagon or amrinone-glucagon. Control experiments with saline or other solvents showed no effects on the heart rate or contractile force. Glucagon increased the heart rate from that observed after verapamil injection (from 225±3.9 to 292±9.7 beat/min with $3x10^{-6}$ mole glucagon, $p<0.01$). Amrinone increased the heart rate only at 10^{-3} mole concentration (from 225±3.9 to 272.1±11.2 beats/min, n=9, $p<0.05$). A positive linear correlation (r=0.86) was found between the log glucagon concentration vs percent recovery of baseline contractile force. While complete reversal of verapamil-induced myocardial depression was at glucagon concentrations $>3x10^{-6}$ mole (n=5), amrinone produced only 23.8±3.6 % recovery of baseline even in the highest concentration administered ($4x10^{-3}$ mole, n=5). When g lucagon ($3x10^{-6}$ mole) was administered with amrinone (10^{-3} mole), this combination did not result in higher increase in % recovery of contractile force than glucagon alone. We concluded that glucagon alone restored baseline contractility and heart rate on verapamil-induced myocardial depression in rat isolated heart. Amrinone has slight effect on contractility and heart rate.

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245P INFLUENCE OF DIHYDROPYRIDINE ON CALCIUM-INDUCED FACILITATION OF CALCIUM CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES

R Davies, SE Bates[1] & AM Gurney, Department of Pharmacology, UMDS, St. Thomas's Hospital, London SE1 7EH and [1]School of Applied Physiology, NESCOT, Ewell, Epsom, Surrey KT17 3DS

The rate at which cardiac muscle is stimulated influences the contractile force developed. This is partly due to frequency-dependent changes in the activity of L-type Ca channels, mediated by Ca^{2+} as it enters through the channels. Channel activity is also modified when the intracellular [Ca^{2+}] is rapidly elevated by flash-photolysis of the "caged" Ca^{2+} compound nitr-5. The Ca^{2+} current (I_{Ca}) recorded from guinea-pig ventricular myocytes responds to such a [Ca^{2+}] jump with a transient reduction followed by a pronounced facilitation. It was recently suggested that Ca^{2+}-dependent facilitation reflects a switch in channel behaviour from a fast to a slow gating mode and that facilitation occurs only when channels pre-exist in the fast mode (Tiaho *et al*, 1994). This hypothesis was based on the observation that the slower component of the biphasic decay of I_{Ca}, recorded from rat ventricular myocytes, was selectively enhanced following an increase in stimulation frequency. To examine the influence of channel gating in guinea-pig ventricular cells, we investigated the effects of photo-induced, intracellular [Ca^{2+}] jumps on I_{Ca} recorded in the presence of the dihydropyridines, nitrendipine or BayK 8644. These agents were selected because they differentially alter channel gating, nitrendipine promoting channel inactivation and BayK 8644 prolonging open time. Also, unlike other dihydropyridines, the effects of nitrendipine and BayK 8644 on I_{Ca} were not sensitive to light in the absence of nitr-5.

I_{Ca} was recorded using the whole-cell, patch-clamp technique. To block K^+ currents, pipettes were filled with a CsCl-based solution and cells were superfused with a K^+-free saline. Nitr-5 (2 mM) was introduced into cells by dialysis from the pipette solution. Cells were voltage clamped at -80 mV and I_{Ca} activated by a 300 ms step to 0 mV at 20s intervals, following a 100 ms pre-pulse to -40 mV to inactivate the Na^+ current. Nitrendipine, at 1 μM, reduced I_{Ca} amplitude by 33 ± 9 % (mean ± sem; n=4). The amplitude of the slower component of I_{Ca} decay (a_s) was affected more than the amplitude of the fast component (a_f), the ratio a_s/a_f being reduced from 1.6 ± 0.2 to 1.0 ± 0.2 (n=4). Nitrendipine had little effect on the time constant of the slow component, which was 103 ± 4 ms (n=19), although it lengthened the time constant of the fast component from 12 ± 2 ms to 23 ± 8 ms (n=4). In the presence of 1 μM nitrendipine, the facilitation of I_{Ca} induced by nitr-5 photolysis was significantly ($p<0.01$) suppressed, I_{Ca} increasing by only 56 ± 9 % (n=4) compared with 117 ± 10 % (n=5) under control conditions. In contrast, BayK 8644 (100 nM) increased I_{Ca} amplitude by 144 ± 22 % (n=5). This primarily reflected enhancement of the fast component of I_{Ca} decay, indicated by a reduction in a_s/a_f from 1.2 ± 0.2 to 0.58 ± 0.04 (n=5; $p<0.05$), without a change in either time constant. At the same time, BayK 8644 decreased the photolysis-induced facilitation of I_{Ca} to only 23 ± 5 % (n=3). Thus, dihydropyridine agonists and antagonists both suppress the facilitatory response to Ca^{2+}, despite their opposing effects on I_{Ca} amplitude and their different actions on I_{Ca} decay. While consistent with the hypothesis that channel gating influences the reponse to Ca^{2+}, these results are not easily explained by a simple Ca^{2+}-dependent switch from fast to slow gating modes.

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246P CYCLIC ADP-RIBOSE ACTIVATION OF THE SHEEP SKELETAL RYANODINE RECEPTOR Ca^{2+}-RELEASE CHANNEL IS DEPENDENT UPON LUMINAL Ca^{2+}

Rebecca Sitsapesan & Alan J. Williams (introduced by Sian Harding). Department of Cardiac Medicine, The National Heart & Lung Institute, London SW3 6LY.

Cyclic ADP-ribose (cADPR) has been shown to increase the open probability (Po) of cardiac ryanodine receptor Ca^{2+}-release channels (Mészáros et al, 1993; Sitsapesan et al, 1994) but it has been reported to have no effect on the skeletal isoform of the ryanodine receptor (Morrissette et al, 1993). We have demonstrated that cADPR may increase the Po of the cardiac channel by binding to the adenine nucleotide binding site (Sitsapesan et al, 1994). We also find that activation of skeletal channels by ATP is highly dependent on the luminal [Ca^{2+}] (Sitsapesan & Williams; unpublished observations). The experiments of Morrissette et al (1993)were performed at very low luminal [Ca^{2+}] which could explain why cADPR was without effect. Therefore, in the present study we have investigated if cADPR can activate the skeletal SR Ca^{2+}-release channel and if activation is dependent upon the luminal [Ca^{2+}]. Heavy sarcoplasmic reticulum vesicles containing Ca^{2+}-release channels were isolated from sheep skeletal muscle and incorporated into phosphatidylethanolamine lipid bilayers under voltage clamp conditions. The *cis* (cytosolic) chamber contained 250 mM HEPES, 125 mM Tris, 10 μM free Ca^{2+}, pH 7.4 and the *trans* (luminal) chamber contained 250 mM glutamic acid, 10 mM HEPES, pH brought to 7.4 with $Ca(OH)_2$ or $Ba(OH)_2$ (free divalent approximately 50 mM). In the presence of 50 mM luminal [Ca^{2+}] and 10 μM cytosolic [Ca^{2+}], 1 μM and 10 μM cADPR increased Po from 0.008 ± 0.005 (n=6; s.e. mean) to 0.068 ± 0.036 (n=4) and 0.188 ± 0.087 (n=4) respectively. No change in single channel conductance was observed. With 50 mM luminal Ba (contaminant luminal $Ca^{2+}<40$ μM), cADPR (1 & 10 μM) was unable to increase Po (normalised $nPo=0.147\pm0.05$ (n=8) before and 0.197 ± 0.065 (n=4) after 10 μM cADPR). However, after addition of 1 and 10 mM luminal Ca^{2+}, nPo was increased to 1 (n=8) and 6.96 ± 3.8 (n=5) respectively. As for the cardiac channel (Sitsapesan et al, 1994), the precursor to cADPR, βNAD$^+$, and the breakdown product ADP-ribose were also able to increase the Po of the channels in the presence of 10 μM cytosolic Ca^{2+} and millimolar luminal Ca^{2+}. 10 μM βNAD$^+$ and 10 μM ADP-ribose increased Po from 0.001 ± 0.001 to 0.120 ± 0.082 (n=4) and 0.043 ± 0.015 (n=4) respectively. Our results indicate that activation of the skeletal Ca^{2+}-release channel by cADPR is dependent upon the luminal [Ca^{2+}] and that cADPR is at least as effective at activating the skeletal channel as it is at activating the cardiac channel.

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247P ARE ADRENERGIC MECHANISMS INVOLVED WITH THE INOTROPIC RESPONSE OF THE HEART TO BENZODIAZEPINES?

A. Zeegers, R. S. Leeuwin & H. van Wilgenburg, Department of Pharmacology, University of Amsterdam, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

Benzodiazepines evoke positive and negative inotropic responses in the isolated rat heart, which are reversed significantly in the presence of the non-selective ß-adrenergic blocker propranolol (Leeuwin et al., 1993). This study describes inotropic responses elicited by diazepam or midazolam in the presence or absence of 1. the α-adrenergic blocker phenoxybenzamine, possessing relatively greater affinity for α_1 receptors; 2. the adrenergic ß-blocker atenolol, possessing almost exclusively $ß_1$-activity; and 3. metoprolol, in relatively low doses possessing exclusively $ß_1$-blocking properties and in relatively high doses $ß_2$-blocking properties as well.

Female rats (160-180g) were heparinized, and anaesthetized with pentobarbitone, 50mg/kg i.p. Hearts with cannulated aortae were excised and perfused with Tyrode's solution (37°C; pH 7.0; perfusion rate 8ml/min), gassed with CO_2+O_2. Contractile force was recorded isometrically, using a latex balloon filled with water, inserted in the left ventricle. After equilibration the heart was perfused with $2x10^{-5}$ to $6x10^{-4}$M diazepam or midazolam with or without an adrenolytic in the perfusate (n=7 for each concentration). Inotropic response was expressed as percentage change of contraction force.

Phenoxybenzamine, 10^{-7}M perfusate concentrations, did not affect the positive inotropic response to diazepam, whereas 10^{-6} and 10^{-5}M strongly (P<0.05) suppressed (maximum effects 137±8% vs 52±10%) it. The negative inotropic response to diazepam was significantly (P<0.05) antagonized by 10^{-7} to 10^{-5}M phenoxybenzamine, especially at the higher concentrations of diazepam. Phenoxybenzamine 10^{-7} to 10^{-5}M suppressed the positive inotropic response to midazolam significantly (P<0.05%): maximum effects 88 ± 7% vs 29 ± 4%. Concentrations as high as 10^{-4}M atenolol did not affect the positive inotropic response to diazepam, at the same time antagonizing the negative response significantly (P<0.05). Neither did atenolol, 10^{-4}M, interfere with the positive inotropic response evoked by midazolam. Metoprolol in a concentration of 10^{-6}M did not affect the positive or negative inotropic response to diazepam significantly. Both 10^{-5} and 10^{-4}M however, reversed these responses (P<0.05). Metoprolol 10^{-6} and 10^{-5}M did not affect the inotropic response to midazolam, whereas 10^{-4}M was highly effective in antagonizing it (P<0.05).

In conclusion: Actions of different adrenolytics on inotropic responses evoked by benzodiazepines seem complex. Either direct or indirect adrenergic mechanisms or both may be involved with inotropic actions of benzodiazepines.

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248P MECHANISM INVOLVED IN THE POSITIVE INOTROPIC EFFECT OF DIAZEPAM

E. Martinez, M.C Collado, J. Marin[1] & J . Hernández, Department of Pharmacology, Facultad de Medicina , Espinardo-Murcia and (1)Department of Pharmacology, Universidad Autónoma de Madrid. (Spain)

Diazepam produces some positive inotropic effects (Castillo-Ferrando, 1986; Gonzalez et al. 1990), but the mechanism responsible has not been established yet. Endogenous catecholamines could be involved in this effect since diazepam increases noradrenaline-induced cyclic AMP production in the rat heart (Elgoyhen & Adler- Graschinsky, 1989). We studied whether diazepam modified the inotropic response, elicited by a typical catecholamine releasing agent such as Tyramine on the electrically driven rat right ventricle.

Sprague-Dawley rats of either sex (250-350g) were used. All procedures were performed in Tyrode solution at 34 °C and saturated with 95% O_2- 5% CO_2. After dissection the heart was quickly removed and the free wall of the right ventricle was excised. Right ventricular strips were mounted longitudinally between two platinum electrodes under 1 g tension. The preparations were electrically stimulated (GRASS SD-9 stimulator), at frequency of 1.5 Hz; duration 4 ms and supramaximal voltage. Contractions were measured using a force-displacement transducer (GRASS FT-03) and recorded on a Dynograph Beckman Polygraph. Equilibration time was of 30 min after which an experiment was performed with subsequent increasing concentrations of drug. After washing, a second concentration-response curve was achieved for Tyramine in the presence of Diazepam (10 µM). Noradrenaline release was determined as previously described (Arribas et al. 1991) by using (±)- ^{3}H noradrenaline ClH 0.2 µM (Amersham, U.K). The results are summarized as follow:

Tyramine (5-100 µM) increased the contraction force of rat isolated right ventricular strips in a concentration-dependent manner, producing a maximal effect of 35.3 ± 7% increase of contractility (n = 4) at 100 µM. This effect was rapidly reversed after wash-out. In reserpinized rats, Tyramine showed no effect on contraction force. Diazepam (10 µM) which was devoid of effect in this preparation, produced a leftward shift in the Tyramine concentration-response curve and increased its maximal effect to 67.3 ± 7.2 % ($p< 0.01$) . Diazepam did not enhance the Tyramine-induced catecholamine release in this preparation. Control experiments with the diazepam solvent (DMSO) did not significantly modify the concentration-response curve to Tyramine.

In conclusion, Diazepam potentiates the inotropic effect of Tyramine in the isolated right ventricle of the rat. This effect is not consequence of an increase in catecholamine release but it might be related to the previously described enhancement of catecholamine-induced cAMP production (Elgoyhen & Adler-Graschinsky, 1989).

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249P HYPOXIC PRECONDITIONING OF HUMAN RIGHT ATRIAL TRABECULA INVOLES A PROTEIN KINASE C-DEPENDENT MECHANISM

M.E. Speechly-Dick, C.W. Pattison, W.B. Pugsley & D.M. Yellon. The Hatter Institute for Cardiovascular Studies, Department of Academic and Clinical Cardiology, University College Hospital, Grafton Way, London WC1E 6DB.

The term ischaemic preconditioning was first used by Murry *et al* (1986) who showed that brief periods of ischaemia with intermittent reperfusion make the heart more resistant to infarction when subjected to a subsequent prolonged ischaemic insult. The original definition of ischaemic preconditioning has expanded to include preconditioning with other stimuli, such as hypoxia and rapid pacing, and endpoints other than infarct size, eg recovery of contractile function. Ytrehus *et al* (1994) have recently shown that protein kinase C (PKC), an essential component in many intracellular transduction pathways, plays a central role in the mechanism of preconditioning in a rabbit model. They suggest that endogenous ligands such as adenosine initiate an intracellular pathway by acting on G-protein-linked receptors and via phospholipase C activate PKC, which phosphorylates a secondary effector to induce the protective effects of preconditioning. We have recently produced evidence for the central role of PKC in preconditioning in a rat model (Speechly-Dick *et al* 1994). The aim of the present study was to investigate the role of PKC in preconditioning in human myocardium.

Right atrial trabecula, obtained from patients during coronary artery bypass surgery, were suspended in an organ bath, superfused with Tyrode's solution and paced by field stimulation at 1Hz. After stabilisation for at least 45min each trabeculum underwent an ischaemic insult consisting of 90min of substrate-free hypoxic superfusion with rapid pacing at 3Hz followed by 2hr of reoxygenation at 1Hz with normal Tyrode's. In the two preconditioned groups this was preceded by 3min substrate-free hypoxia with rapid pacing and 7min reperfusion. We have previously demonstrated preconditioning in this model (Walker et al 1993).

To investigate whether preconditioning could be triggered or blocked by pharmacological manipulation of PKC, 1,2-dioctanoyl-*sn*-glycerol (DOG, 30μM), a diacylglycerol analogue and specific PKC activator, was given to one of the control groups prior to the long hypoxia to trigger preconditioning and chelerythrine (Che, 10μM), a potent selective PKC antagonist was given to one of the preconditioned groups to block preconditioning. Functional parameters recorded included time to peak contracture (TTPC) and developed tension (DT), presented here as percentage recovery of initial DT at 2hr reperfusion:

Group	DT recovery *(percentage)*	TTPC *(min)*
Control	29 ± 4.2	60 ± 6
Preconditioned	61 ± 4.8*	50 ± 4
Control + DOG	48 ± 3.6*	53 ± 2
Preconditioned + Che	24 ± 5.0	67 ± 6

Results are mean ± SEM.
**=p < 0.01 v Control. n=6 for each group.*

The results show that protection against post-ischaemic contractile dysfunction can be induced with a PKC activator and prevented with a PKC inhibitor. This study suggests a central role for protein kinase C in ischaemic preconditioning in human myocardium.

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250P SUPPRESSION OF ISCHAEMIA-INDUCED VENTRICULAR ARRHYTHMIAS BY THE LOCAL INTRACORONARY ADMINISTRATION OF NORADRENALINE

A. Vegh., J. Gy Papp & J.R. Parratt[1]. Department of Pharmacology, A. Szent-Gyorgyi Medical University, Szeged, Hungary and of [1]Physiology and Pharmacology, University of Strathclyde, Glasgow Gl lXW.

Although noradrenaline (NA) released under conditions of myocardial ischaemia is a major factor in determining the severity of the resultant ventricular arrhythmias, there is some evidence that the systemic administration of NA actually reduces arrhythmia severity under these conditions (Parratt *et al.*, 1981). Some have even suggested a role for NA in ischaemic preconditioning (Banerjee *et al.*, 1993). We examined the effects of the local intracoronary administration of NA (100 ng kg-1 min^{-1} for 10 min before and during the whole period of occlusion, into a side branch of the LAD) on the arrhythmias resulting from a 25 min coronary artery (LAD) occlusion in chloralose-urethane anaesthetised dogs (Vegh *et al.*, 1992). The haemodynamic effects of NA were an increase in mean arterial blood pressure (from 113 ± 5 to 124 ± 5 mmHg; $P < 0.05$), LVdP/dt max (from 3351 ± 200 to 4316 ± 315 mmHg sec^{-1}; $P < 0.05$) and in left circumflex diastolic blood flow (from 113 ± 5 to 126 ± 6 ml min^{-1}; $P < .05$). Ventricular arrhythmias during occlusion were reduced by NA (ventricular premature beats (VPBs) 142 ± 66 v 497 ± 109 in the controls; $P < 0.05$; ventricular fibrillation (VF) 23% v 47%; ventricular tachycardia (VT) 38% v 80%; episodes of VT 2.0 ± 1.2 v 5.9 ± 1.7). Those dogs that survived reperfusion (38% v 0%) were reoccluded in the absence of NA 2 h later. The arrhythmias in these dogs were more marked than in the presence of NA (VBPs 198 ± 116 v 17 ± 15; VF 25% v 0%; VT 50% v 0%; VT episodes 1.3 ± 0.9 v 0). We conclude that NA can reduce arrhythmia severity, perhaps by 'preconditioning' the myocardium through alpha-adrenoceptor stimulation. Possible explanations include an increase in action potential duration, enhanced conduction velocity, reduced potassium efflux and protein kinase C translocation.

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251P CHARACTERIZATION OF A NOVEL BINDING SITE FOR ANGIOTENSIN IV IN CULTURED PORCINE AORTIC ENDOTHELIAL CELLS

L. Riva & A.M. Galzin, Department of Preclinical Research, Synthelabo Recherche, 31 Avenue P.V. Couturier, 92225 Bagneux, France

Recently, a novel specific binding site for the hexapeptide fragment angiotensin II(3-8), (angiotensin IV, AIV), has been described in brain and cardiovascular tissues from various species (Swanson *et al.*, 1992). This site could be involved in the regulation of memory acquisition and retrieval, blood flow control and cell growth (Sardinia *et al.*, 1993). A specific AIV binding site has been found in endothelial cell lines from bovine coronary and aortic origin (Hall *et al.*, 1992), and a vasodilating effect has been suggested (Coleman *et al.*, 1992). The aim of the present experiments was to demonstrate the presence of a specific binding site for AIV on porcine endothelial cells obtained from primary culture.

Endothelial cells from fresh porcine aortae were isolated and cultured in 75 cm^2 flasks. Binding experiments were performed on membranes obtained from confluent cells between passages 3 and 8. Various concentrations of [^{125}I]-AIV were incubated with cell membrane preparation (100µg/ml) in assay buffer (NaCl 150 mM, Tris-HCl 50 mM, pH 7.4) at 37°C. Non-specific binding was defined with 1 µM AIV.

Association kinetic experiments indicate that [^{125}I]-AIV binding equilibrium was reached at 60 min (association rate constant k_1 = 4.10^6 $M^{-1}.sec^{-1}$, n=4), and was fully reversible (dissociation rate constant k_{-1}= $3.3.10^{-3}sec^{-1}$, n=6). The apparent kinetic equilibrium dissociation constant K_d was calculated as k_{-1}/k_1= 0.825 nM. Saturation equilibrium binding studies indicate the presence of a single high affinity site (K_D = 3.80 ± 0.56 nM, B_{max} = 10.44 ± 2.12 pmol/mg prot, n=3). Competition binding studies reveal a unique pharmacological profile, with high affinity for AIV and AIII, and no affinity for Losartan or PD123177 (Table 1).

Table 1.

Inhibitors	K_i (µM)
AIV	0.011 ± 0.003
AIII	0.079 ± 0.033
AII	1.46 ± 0.60
AI	3.14 ± 0.10
Sar^1Ile^8(AII)	14.4 ± 11.7
AII(1-7)	10.8 ± 4.1
Saralasin	> 100
Losartan	> 100
CGP 42112	> 100
PD 123177	> 100

Taken together, these data indicate that porcine aortic endothelial cells exhibit specific binding for AIV to a site different from the AT_1 or AT_2 receptor and support the view that AIV may play a role in the modulation of cardiovascular function.

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252P DO NEONATAL RAT CARDIAC MYOCYTES AND FIBROBLASTS SYNTHESIZE ANGIOTENSIN II?

Catharina A.M. van Kesteren*, A.H. Jan Danser, [1]Jos M.J. Lamers, [1]Han A.A. van Heugten, [2]Maarten A.D.H. Schalekamp, Pramod R. Saxena. Depts. of Pharmacology, [1]Biochemistry and [2]Internal Medicine I, Erasmus University, Rotterdam, The Netherlands.

Neonatal rat cardiomyocytes, grown for 1 day in the presence of serum and subsequently cultured for 5 days under serum-free conditions, have been reported to synthesize angiotensin II (AII) (Dostal *et al.*, 1992). Mechanical stretch of myocytes causes release of AII in the medium and this AII may act as an initial mediator of the stretch-induced hypertrophic response (Sadoshima *et al.*, 1993). These data suggest that myocytes can synthesise AII independently from the circulating renin-angiotensin system (RAS). However, it is possible that myocytes have sequestered RAS components from serum during plating. In addition, myocyte cell cultures are always contaminated with some fibroblasts. To investigate AII formation by cardiomyocytes in more detail we measured renin (R), prorenin (PR), angiotensinogen (Ao) in the medium and angiotensin I (AI) and AII in the medium and cell lysate of neonatal rat cardiac myocytes and fibroblasts cultured in the presence or absence of serum. Cells were isolated by enzymatic dissociation of cardiac tissue from 1-3 day old Wistar strain pups and grown for 1 day in Dulbecco's modified Eagle's medium : medium 199 (4:1) containing 5 % horse serum and 5 % fetal calf serum. After 1 day the cells (1.5-3x10^6 cells per dish) were either serum-deprived or maintained in serum-containing medium for 5 days. At the end of the 5-day period medium and cells were collected. PR (after activation to R by plasmin) and R were measured by enzyme-kinetic assay in the presence of excess rat Ao. Ao was measured as the maximum amount of AI generated in the presence of excess R. AI and AII were measured by radioimmunoassay after SepPak extraction and HPLC separation (detection limit for AI and AII in medium 0.2 and 0.1 fmol/ml and in cells 0.3 and 0.2 fmol/10^6 cells, respectively). In a separate series of experiments AI-to-AII conversion by ACE was studied, in the absence or presence of 0.5 mM captopril, over a 2-hour period after the addition of AI (1 pmol/ml) to intact myocytes that had been cultured for 5 days.

The levels of RAS components in the medium of myocytes and fibroblasts cultured in the presence of serum were less than or equal to the levels in unconditioned serum-containing medium (R and PR 0.18 ± 0.04 and 1.28 ± 0.02 fmol AI/min per ml, Ao 31 ± 2 pmol/ml, AI and AII 1.3 ± 0.3 and 0.6 ± 0.2 fmol/ml; n=5, mean ± SD). All RAS components were undetectable in unconditioned serum-deprived medium. In the medium of fibroblasts and myocytes cultured in the absence of serum, PR, Ao and AI were < 10 % of the levels in serum-containing medium. These low levels were most likely caused by contamination of the cells with the serum-containing medium of the first 24-hour plating period. R and AII were undetectable. Cellular AI and AII were below the detection limit under all conditions. AI incubated with myocytes was rapidly metabolised ($t_{1/2}$ with and without serum 15 ± 3 and 68 ± 40 min respectively, n=4) with AII being a major metabolite. Captopril significantly ($p < 0.05$) prolonged the half life of AI ($t_{1/2}$ with and without serum 87 ± 33 and 222 ± 237 min respectively, n=4). The results suggest that myocytes or fibroblasts do not synthesize R, PR or Ao in concentrations that are detectable or high enough to result in AI or AII concentrations of physiological relevance. Serum-deprived myocytes are capable of converting AI into AII via ACE, suggesting that ACE might be synthesised by these cells.

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253P ELECTROPHYSIOLOGICAL EFFECTS OF ZATEBRADINE IN GUINEA-PIG VENTRICULAR MUSCLES

O. Pérez, P. Gay, L. Franqueza, C. Valenzuela, E. Delpón & J. Tamargo. Dept. of Pharmacology. School of Medicine. Universidad Complutense. 28040 Madrid. Spain.

Zatebradine (UL-FS 49) is a specific bradycardic agent which has been found effective in the treatment of effort-induced angina pectoris, particularly in patients with depressed ventricular function (Kobinger, 1985; Kobinger & Ellie, 1987). In the present study we studied the electrophysiological effects of zatebradine.

The experiments were performed in guinea pig papillary muscles perfused with Tyrode solution (34°C). Transmembrane action potentials were recorded with standard microelectrode techniques. The maximum upstroke velocity (V_{max}) of the action potential was used as an indirect index of the sodium current.

In papillary muscles driven at 1 Hz, zatebradine $\geq 5 \times 10^{-6}$M decreased the V_{max} without altering the resting membrane potential and exerted biphasic effects on the action potential duration (APD). At concentrations up to 10^{-5}M it significantly lengthened, while at higher concentrations it shortened the APD.

In muscles driven by trains of stimuli at rates between 0.5 and 3 Hz zatebradine, 5×10^{-5}M, reduced the V_{max} of the first action potential of the train (i.e. tonic block) by 8 ± 1%. However, in the presence of zatebradine repetitive stimulation led to an exponential decline of the V_{max} (onset rate 0.32 ± 0.02 per action potential at 2 Hz) to a new plateau level. This frequency-dependent block was more marked at fast (2 and 3 Hz) than at slow (0.5 and 1 Hz) driving rates ($P < 0.01$). Zatebradine also increased the time constant for the recovery of V_{max} from the frequency-dependent block (offset) from 20 ± 3 ms to 2.9 ± 0.1 s (n = 7).

Zatebradine, 5×10^{-5}M, shifted the relationship between V_{max} and the resting membrane potential from which the action potential arises in a hyperpolarizing direction by 4 ± 1 mV ($P < 0.05$). This indicated that the depressant effects on V_{max} were more pronounced at depolarized compared to normally polarized membrane potentials.

Zatebradine only at 10^{-4}M significantly reduced the amplitude and V_{max} of the slow action potentials elicited by isoprenaline (10^{-6}M) in K-depolarized (27 mM) muscles driven at 0.1 Hz.

These results demonstrated that in guinea pig papillary muscles zatebradine produced a frequency- and voltage-dependent block of sodium channels. From the onset and offset kinetics of the frequent-dependent V_{max} block, zatebradine can be considered as an intermediate kinetics (class IA) sodium channel blocker.

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254P MECHANISMS OF REDUCTION OF ISCHAEMIA-INDUCED ARRHYTHMIAS BY ENDOTOXIN IN THE *EX VIVO* PERFUSED RAT HEART

Wu Song, Brian L Furman, James R Parratt, Department of Physiology and Pharmacology, University of Strathclyde, George Street, Glasgow Gl lXW, Scotland.

Low dose endotoxin reduced ischaemia-induced arrhythmias *ex vivo* in the perfused rat heart (Wu Song et al., 1994). The effect required at least 4 h to be evident and was partially reversed by dexamethasone pretreatment. The present work was undertaken to determine the ability of cycloheximide (a protein synthesis inhibitor), L-NAME (an inhibitor of nitric oxide synthase) and indomethacin (a cyclooxygenase inhibitor) to modify this action of endotoxin.

Rats were treated with endotoxin (*E. coli* Boivin preparation, Difco 2.5 mg kg^{-1} i.p.) or saline. Eight hours later the hearts were isolated under pentobarbitone (60 mg kg^{-1} i.p.) anaesthesia and perfused via the aorta (Krebs Henseleit 37° C, 95% O_2/5% CO_2; 10 ml min^{-1}). An epicardial electrogram was recorded using fine platinum electrodes and the main left coronary artery ligated for 30 minutes. During this period control hearts showed a large number of ventricular premature beats (VPBs) (1165 ± 144) ventricular tachycardia (VT, 100%) and ventricular fibrillation (VF, 50%). Endotoxin treatment markedly reduced the number of VPBs (53 ± 13 $P < 0.01$) and the incidences of VT (0% $P < 0.05$) and VF (0% $P < 0.05$). The effect of endotoxin was significantly reversed by cycloheximide, 0.1 mg kg^{-1} 40 h before endotoxin injection (cycloheximide alone, VPBs 1084 ± 156; cycloheximide plus endotoxin 566 ± 79; $P < 0.01$ vs endotoxin alone). Perfusion of hearts with L-NAME (10^{-3} M) before and throughout the occlusion period did not modify the number of VPBs (1062 ± 278) but significantly reduced the effect of endotoxin (251 ± 56 VPBs; $P < 0.05$ vs endotoxin). Similarly, indomethacin (10^{-5} M) reduced the effects of endotoxin (202 ± 44 VPBs, $P < 0.05$ vs endotoxin) without itself modifying the number of VPBs in control hearts (1262 ± 189). Perfusion with a combination of L-NAME (10^{-3} M) and indomethacin (10^{-5} M) appeared to produce an additive effect in reversing the effects of endotoxin (592 ± 76 VPBs, $P < 0.01$ vs endotoxin). All treatments reversed the protective effect of endotoxin against VT. Hearts obtained from endotoxin-treated rats and perfused with indomethacin still failed to show VF although cycloheximide, L-NAME and the combination of L-NAME and indomethacin reversed the effects of endotoxin in preventing VF. Among the treatments, only a combination of indomethacin and L-NAME significantly increased pre-occlusion perfusion pressure (control mmHg 31 ± 19; indomethacin + L-NAME 43 ± 1.5 $P < 0.01$).

These data suggest that the effects of endotoxin require *de novo* protein synthesis, involve the L-arginine/nitric oxide pathway, possibly via inducible NO synthase and involve a metabolite generated via the cyclooxygenase pathway, possibly via an inducible form of the enzyme. The inability of a combination of L-NAME and indomethacin to reverse completely the effects of endotoxin may implicate an additional mechanism.

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255P THE EFFECTS OF DIFFERENT TYPES OF INOTROPIC AGENTS IN ISOLATED HEARTS FROM HYPERTENSIVE-DIABETIC RATS

O.H.M. Beenen, M. Pfaffendorf & P.A. van Zwieten, Dept of Pharmacotherapy, Academic Medical Centre, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Hypertension is often prevalent in the diabetic individual and is known to aggravate the cardiovascular complications associated with diabetes. Several abnormalities in cellular handling of calcium have been suggested to occur in hearts of these individuals which may lead to altered responses to drugs which increase cardiac contractile force. In the present study we investigated the profile of the contractile effects of α_1-adrenoceptor agonists, calcium ions and the Ca^{2+} entry promotor Bay K 8644 in isolated perfused hearts taken from spontaneously hypertensive rats (SHR) with simultaneous diabetes.

SHR and normotensive Wistar Kyoto rats (WKY) of 12 weeks of age were made diabetic by an injection of streptozotocin (55 mg/kg i.v). 8 Weeks later the inotropic effects of cirazoline, ST587 (2-chloro-5-trifluoro-methylphenylimino-2-imidazolidine), extracellular calcium and Bay K 8644 were assessed in paced (6Hz) Langendorff hearts (37°C) and expressed as left ventricular pressure (LVP) and +dP/dt max.

Mean arterial pressures (MAP) of both non-diabetic (mean values ± SEM, $n \geq 26$, 170 ± 4 mmHg) and diabetic (123 ± 8 mmHg) SHR were significantly ($p < 0.05$) higher compared to both non-diabetic (105 ± 4 mmHg) and diabetic (95 ± 5 mmHg) WKY. The presence of diabetes in the hypertensive rats significantly lowered the MAP.

The developed left ventricular pressure of control WKY ($n \geq 22$, 86 ± 3 mmHg) and control SHR hearts (84 ± 3 mmHg) were significantly higher compared to both diabetic WKY (51 ± 3 mmHg) and diabetic SHR hearts (51 ± 3 mmHg). The inotropic responses to both cirazoline and ST587 were increased ($p < 0.05$) in hearts from diabetic WKY ($n = 5$-8, max. increase in LVP 52 ± 1 mmHg vs 46 ± 2 mmHg) when compared with those from control WKY (35 ± 1 mmHg vs 29 ± 2 mmHg). Additional hypertension of the donor animals however, impaired the inotropic response to ST587 (SHR: 18 ± 1 mmHg vs diabetic SHR: 28 ± 1 mmHg) but not that to cirazoline (SHR: 33 ± 2 mmHg vs diabetic SHR: 52 ± 1 mmHg when compared to (diabetic) WKY hearts.

The combination of hypertension and diabetes appeared to decrease ($p < 0.05$) the inotropic response to extracellular calcium ions ($n = 5$-8, Ca^{2+}: 3.15 mM, max LVP: 56 ± 6 mmHg) when compared to WKY hearts (80 ± 4 mmHg). The Ca^{2+} influx promotor Bay K 8644 did not influence the maximally developed LVP in (diabetic) SHR and WKY preparations.

The inotropic effect of ST587 is known to require the influx of extracellular calcium ions, whereas the response to cirazoline is mediated by an intracellular Ca^{2+} release only. Accordingly, the influence of hypertension and diabetes on the contractile responses to α_1-adrenoceptor stimulants and to calcium ions is reflected by substantial changes in calcium handling in such hearts.

256P BTS 67 582, A NOVEL GLUCOSE LOWERING AGENT

C.L. Kaul[1], A.R. Marita[1], K. Dickinson & R.B. Jones, Boots Pharmaceuticals Research Department, Nottingham, NG2 3AA and [1]Boots Pharmaceuticals, Bombay, India.

BTS 67 582, 1,1-dimethyl-2-[2-(4-morpholinophenyl)]guanidine monofumarate, is currently under clinical trial for the treatment of type II diabetes. The glucose lowering properties of BTS 67 582 have been evaluated in normal fasted and in glucose primed rats. Its mechanism of action has been investigated using ligand binding techniques and isolated tissue preparations.

Normal male Wistar rats were starved overnight and orally dosed with either BTS 67 582, glibenclamide, tolbutamide or vehicle (0.25% cellosize). Heparinised blood samples were taken from the tail vein by venepuncture after prewarming rats for 15 minutes in a heated cabinet at 37°C, prior to and at 1, 2 and 4 hours after dosing and plasma assayed for glucose and insulin content. Glucose primed rats (Sprague Dawley) were administered glucose (800 mg/kg in saline) subcutaneously immediately after oral dosing. Binding of BTS 67 582 to the 'sulphonylurea receptor' was evaluated using the ligand [^{3}H]-glibenclamide in crude membrane preparations from rat brain and the pancreatic beta insulinoma cell line HIT-T15 (Schmid-Antomarchi et al 1987). The ability of BTS 67 582 to modulate potassium channel activity was assessed in isolated rat aortic strips precontracted with phenylephrine (10^{-8} M) and relaxed with cromakalim.

In fasted rats BTS 67 582 caused glucose lowering with an ED_{50} 2 hours after dosing of 18.5 mg/kg po, slightly less potent than tolbutamide (ED_{50} 13.7 mg/kg) and glibenclamide (ED_{50} 0.4 mg/kg). In glucose primed rats BTS 67 582 attenuated the rise in plasma glucose (ED_{50} 16.5 mg/kg 2 hours after dosing) and accelerated its disappearance. The glucose lowering activity of BTS 67 582 was accompanied by stimulation of insulin release. Glibenclamide and tolbutamide displaced [^{3}H]-glibenclamide with IC_{50} values of approximately 1.5 nm and 37 µM respectively in both tissues. BTS 67 582 at concentrations up to 1 mM had no effect on this binding. Both BTS 67 582 and glibenclamide antagonised the cromakalim induced relaxation of phenylephrine contracted rat aortic strips with a pA2 of 5.88 and 7.47 respectively indicating functional potassium channel blockade.

In conclusion BTS 67 582 is a novel agent which causes glucose lowering and stimulates insulin secretion in fasted and glucose primed normal rats. It does not displace [^{3}H]-glibenclamide from its binding site but possesses functional potassium channel blockade in rat aortic strips. BTS 67 582 could therefore represent a new class of potassium channel blocker with potential utility in the treatment of type II diabetes.

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257P OBESE AND LEAN ZUCKER RATS: GENERAL CHARACTERISTICS AND DRUG-INDUCED VASOCONSTRICTOR AND-DILATOR EFFECTS IN ISOLATED RESISTANCE VESSELS

K.L. Kam, M. Pfaffendorf & P.A. van Zwieten, Department of Pharmacotherapy, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

In several pathologic conditions such as atherosclerosis, diabetes mellitus and hypertension changes at the level of the arterial smooth muscle have been suggested to be involved in the pathogenesis of each of these diseases. The recently introduced obese (OZR) and lean (LZR) Zucker rat have been proposed as a potentially valuable animal model to study the syndrome of obesity-hypertension-insulin resistance-hyperlipoproteinaemia. We used these animals at an age of 22 weeks. Basal parameters (n≥7 ± s.e.mean) like the systolic (179.8±3.2 versus 152.1±2.7), diastolic (113.3±2.3 versus 106.5±1.7) and mean arterial (135.5±2.3 versus 121.7±2.0) blood pressures (mmHg) of the OZR were moderately but significantly ($p<0.05$) higher than in the LZR. However, heart rate values (beats/min) were significantly lower in the OZR (329±5) compared to the LZR (346±7). Other significantly different (OZR versus LZR) general parameters were: body weight (587.0±6.8 versus 407.2±5.2 g), plasma glucose (11.0±0.8 versus 6.2±0.2 mM), plasma insulin (59.7±4.0 versus 15.6±2.5 mIU/l), cholesterol (4.28±0.41 versus 1.78±0.06 mM), HDL (2.83±0.39 versus 1.12±0.06 mM), LDL (0.93±0.17 versus 0.41±0.08 mM), triglycerides (1.18±0.17 versus 0.54±0.09 mM), urinary albumin (3.0±0.1 versus 2.1±0.1 scale 0-4) and urinary glucose (1.6±0.6 mM versus normal).
The morphological characteristics of the small arteries, the tunica media thickness and tunica-media-thickness-to-lumen-diameter-ratio were the same (14.47±1.67 versus 14.58±1.83 µm and 5.71±0.58 versus 5.64±0.65 %, n=7) when comparing the OZR and LZR.
Mesenteric small arteries were prepared and normalised to their individual optimal lumen diameter (± 260 µm for both groups) in an isometric wire-myograph. The sensitivities (-log EC_{50} M) of the cumulative concentration-response curves (CRC) for methoxamine (5.67±0.17 versus 5.75±0.04), noradrenaline (5.59±0.06 versus 5.50±0.08), calcium chloride (3.47±0.03 versus 3.45±0.08) and potassium chloride (1.22±0.03) showed no differences when constructed for the small arteries (n=7) isolated from OZR and LZR, respectively. Neither the relaxation to sodium nitroprusside (6.11±0.12 versus 6.18±0.16) nor the relaxation to methacholine (6.64± 0.10 versus 6.78±0.10) showed differences (-log EC_{50} M) when assessed on the preparations (n=7) from OZR and LZR, respectively. The sensitivities of the CRC (-log EC_{50} M) of cromakalim (5.76±0.20 versus 5.57±0.18) and the antagonistic effect on this relaxation (-log IC_{50}) by 3 µM glibenclamide (4.83±0.09 versus 4.93±0.08) were not affected when comparing the OZR and LZR, respectively. Also the sensitivities of the CRC (-log IC_{50} M) for nifedipine (on K^+-induced contractions, 9.28±0.18 versus 9.50±0.16) were the same in preparations (n=7) isolated from OZR and LZR.
In conclusion, functional pharmacological responses to both vasoconstrictor and -dilator agents in isolated resistance vessels appear not to be influenced by the syndrome charac-'teristic for OZR. The degree of hypertension and the corresponding vascular hypertrophy are too mild to cause similar changes as found in the vascular system of spontaneously hypertensive rats and of hypertensive patients.

258P TERFENADINE INCREASES THE FORCE OF CONTRACTION OF GUINEA-PIG VENTRICULAR STRIPS BY A HISTAMINE H_2-DEPENDENT MECHANISM

I. Cardelus, J. Llenas, R.W. Gristwood and J.M. Palacios, Research Dept, Almirall, Cardener 68-74, 08024 Barcelona, Spain.

The cardiac effects of several histamine H_1-receptor antagonists, diphenhydramine, d-chlorpheniramine, loratadine, ebastine, cetirizine and terfenadine were studied using ventricular strips isolated from reserpinized guinea-pigs.

Segments of right ventricles were removed from male guinea-pigs (450-550 g) and placed in organ baths containing Krebs-Henseleit solution (mM): NaCl (118), $NaHCO_3$ (25), KCl (4.7), $MgSO_4$(1.6), NaH_2PO_4 (1.2), D-glucose (11), $CaCl_2$ (2.5), at 37° C and gassed with 95% O_2/5% CO_2. Following 30 minutes stabilization, the preparations were electrically stimulated to contract at 1 Hz. Changes in isometric tension were recorded from a resting tension

Terfenadine, but not the other antihistamines, increased the force of contraction (by 179 + 45 % and 441 + 52 % at 10 and 100 µM, respectively). Pretreatment with cimetidine (1-10 µM) inhibited this effect of terfenadine, indicating the involvement of histamine H_2-receptors.

It is concluded that terfenadine increases the force of cardiac contraction in the guinea-pig by a histamine H_2-receptor dependent mechanism. This effect was not observed with diphenhydramine, ebastine, loratadine, cetirizine or d-chlorpheniramine and probably occurs as a consequence of histamine release from cardiac mast cells.

259P DESENSITISATION OF HISTAMINE H_1-RECEPTOR-MEDIATED CALCIUM RESPONSES IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

G. McCreath, Dickenson, J.M.*, I.P.Hall & S.J.Hill*, Division of Therapeutics and *Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH.

Histamine H_1-receptor activation can increase intracellular Ca^{2+} (Hallam et al., 1988) and stimulate inositol phosphate formation in human umbilical vein endothelial cells (HUVEC; McCreath et al., 1992). We have previously demonstrated desensitisation of H_1-receptor mediated inositol phosphate formation in HUVEC (McCreath et al., 1992). In the present study we have examined the regulation of histamine-mediated intracellular Ca^{2+} responses in these cells.

HUVEC were obtained as previously described (McCreath et al., 1992) and cultured at 37°C in humidified air/CO_2 (95:5). HUVEC were grown to confluence on glass coverslips and loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 AM for Ca^{2+} determination (Dickenson & Hill 1993).

Histamine (10μM), in Ca^{2+}-free buffer, induced a transient 260 ± 15% increase in the level of $[Ca^{2+}]_i$ (Ca^{2+} release) in HUVEC (n=13). 100 Seconds after the return of Ca^{2+} to basal unstimulated levels, re-introduction of 2mM extracellular Ca^{2+} induced a 100 ± 12% increase in $[Ca^{2+}]_i$ over basal levels (Ca^{2+} influx). The Ca^{2+} release in response to histamine (10μM; in Ca^{2+}-free medium) was abolished by mepyramine (1μM). Exposure of HUVEC to mepyramine (1μM) immediately following the initial response to histamine (10 μM) in Ca^{2+}-free medium also prevented the response to subsequent Ca^{2+} addition (2mM). Pre-exposure to 10μM histamine (500s) produced a significant reduction in both the Ca^{2+} release (by 95 ± 3%; $p<0.05$; n=10) and Ca^{2+} influx (34 ± 2%, $p<0.05$; n=10) produced by a second addition of histamine (10μM; 100s after removal of the initial stimulus).

Prior exposure to phorbol dibutyrate (PdBu;1μM,15 min) produced a significant reduction in Ca^{2+} release (by 45 ± 3%, $p<0.05$, n=13) and Ca^{2+} influx (by 88 ± 1%, $p<0.05$; n=13) in response to 10μM histamine stimulation. The PKC inhibitor, RO 31-8220 (10μM), completely inhibited the PdBu-induced reduction of both responses to 10μM histamine (n=9). However, RO 31-8220 was without effect on the histamine-induced desensitisation of intracellular Ca^{2+} release (n=6).

These data show that histamine-induced intracellular Ca^{2+} release and subsequent Ca^{2+} influx in HUVEC is dependent on H_1-receptor occupation and can be modified by PKC activation. PKC does not, however, appear to play a major role in histamine-induced densensitization of H_1-receptor-mediated Ca^{2+} responses in HUVEC.

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I.P.H. is a National Asthma Campaign Senior Research Fellow.

260P ANALYSIS OF TISSUE-DEPENDENT INSURMOUNTABLE ANTAGONISM OF HISTAMINE H_1-RECEPTORS ON GUINEA-PIG ISOLATED TISSUE BIOASSAYS

G.F. Watt, N.P. Shankley & J.W. Black, Analytical Pharmacology, King's College School of Medicine & Dentistry, 123 Coldharbour Lane, London SE5 9NU.

Tissue-dependent insurmountable antagonism has been observed on both $5HT_2$- and angiotensin receptor systems (Robertson *et al.*, 1994). Although similar behaviour has been reported for histamine (HA) H_1-receptor antagonism (Labrid *et al.*,1977), the reduction in the agonist concentration-effect curve maxima only occurred with high concentrations of the antagonists (>100-fold pA_2). We have studied the interaction between HA and the selective antagonists, mepyramine and triprolidine on the guinea-pig aorta, gallbladder and gastric muscle assays using established methods and protocols (Bishop *et al.*, 1995). With the exception of the HA-triprolidine interaction on the gallbladder, the results were consistent with expectations for competition at a single site and the affinity values estimated for both antagonists (Table 1) were similar to published values (Ash & Schild, 1966; Ison & Casy, 1971). Triprolidine produced concentration-dependent insurmountable antagonism on the gallbladder assay (3nM gave a 71±4% depression of maximum response), although the pA_2 estimated from the lowest dose ratio, obtained in the presence of 1nM triprolidine, was similar to the pK_B estimates on the other two assays. On the gallbladder assay, triprolidine (3nM) had no significant effect on responses to either CCK8-S or 5-methylfurmethide. Additional pharmacological tests (washout and receptor protection using 30nM mepyramine) indicated that the action of triprolidine was readily reversible and its site of action could be occluded by mepyramine. This behaviour appeared to be independent of tissue differences in HA efficacy because the HA $p[A]_{50}$ values between assays were indistinguishable and the antagonism remained surmountable on the guinea-pig gastric muscle assay following partial receptor inactivation using phenoxybenzamine (0.3μM for 5min). As yet, we have been unable to quantitatively account for these data using existing pharmacological models of insurmountable antagonism as recently reviewed by Robertson *et al.* (1994).

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Table 1	aorta (tension)		gallbladder (tension)		gastric muscle (length)	
	pK_B±s.e	b±s.e. (d.f.)	pK_B±s.e.	b±s.e. (d.f.)	pK_B±s.e.	b±s.e. (d.f.)
mepyramine	9.27±0.07	1.13±0.11 (26)	9.38±0.14	1.13±0.11 (24)	9.13±0.09	1.01±0.09 (26)
triprolidine	9.38±0.08	1.07±0.07 (52)	9.71±0.11	-* (22)	9.68±0.10	1.00±0.09 (29)

b - Schild slope parameter, *- insurmountable antagonism, therefore $pK_B=pA_2$ estimated with 1nM triprolidine.

261P KINETICS OF DOPAMINE, 5-HT AND THEIR AMINO ACID PRECURSORS UPTAKE IN OK CELLS IN CULTURE

M. A. Vieira-Coelho & P. Soares-da-Silva, Institute of Pharmacology & Therapeutics, Faculty of Medicine, 4200 Porto, Portugal.

The cellular uptake of L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan (L-5-HTP) has been suggested to rate limit the synthesis of dopamine (an endogenous natriuretic agent) and 5-hydroxytryptamine (an endogenous antinatriuretic agent) in tubular epithelial cells, but information concerning the mechanism(s) involved and the nature of the process(es) of cell membrane transport of the two amino acid precursors in epithelial cells of renal tubules is lacking (Soares-da-Silva, 1994). The present work reports on the kinetics and characteristics of cellular uptake of L-DOPA, L-5-HTP, DA and 5-HT in opossum kidney (OK) cells in culture. OK cells (ATCC 1840-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524). After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 40 μg of cell protein. In uptake studies, OK cells were preincubated (30 min) with Hanks medium with added pargyline (100 μM), tolcapone (1 μM) and benserazide (50 μM) in order to inhibit the enzymes monoamine oxidase, catechol-O-methyltransferase and aromatic L-amino acid decarboxylase, respectively. Determination of initial rate of uptake was performed in experiments in which OK cells were incubated with L-DOPA, L-5-HTP, DA or 5-HT (100, 250 and 500 nM) during 1, 3, 6, 12, 30, 60 and 120 min; using this procedure the compounds were applied from the apical cell border. L-DOPA and L-5-HTP, but not DA and 5-HT, rapidly accumulated in OK cells. The parameters of an exponential saturation equation ($C_i/C_o=k_{in}/k_{out}.(1-e^{-Kout.t})$) were fitted to the experimental data; C_i and C_o represent the intracellular and extracellular concentration of the substrate, and t the incubation time. The analysis revealed for L-DOPA and L-5-HTP, respectively, a rate constant of total inward transport (k_{in}) of 68±5 and 164±13 μl mg protein^{-1} min^{-1}, a rate constant of total outward transport (k_{out}) of 7.9±2.8 and 13.2±2.0 μl mg protein^{-1} min^{-1} and an equilibrium factor of accumulation (C_i/C_o) of 158±20 and 511±8 (n=5). Saturation experiments were performed in OK cells incubated for 6 min with concentrations of L-DOPA and L-5-HTP which ranged from 0.1 to 250 μM; non-linear analysis of the saturation curve revealed for L-DOPA and for L-5-HTP, respectively, a K_m of 33 (29, 39; 95% confidence limits) and 13 (8, 24) μM and a V_{max} of 3.6±0.1 and 2.8±0.2 nmol mg protein^{-1} min^{-1}. Accumulation of D-DOPA and D-5-HTP (5 to 250 μM) in OK cells was found to be non-saturable, the amount accumulated corresponding to less than 10% obtained with the L forms. In another series of experiments, OK cells were cultured on permeable membranes (Costar Transwell 3413) and the substrates were applied to the cells only from the basal border. The kinetics (V_{max} in nmol mg protein^{-1} min^{-1} and K_m in μM) of L-DOPA, L-5-HTP, DA and 5-HT uptake through the basal border were as follows: L-DOPA, V_{max}=3.6±0.5 and K_m=18 (13, 28); L-5-HTP, V_{max}=0.6±0.1 and K_m=21 (10, 48); DA, V_{max}=1.2±0.1 and K_m=7 (4, 12); 5-HT, V_{max}=0.4±0.1 and K_m=11 (6, 20) (n=5). It is concluded that L-DOPA and L-5-HTP are taken up into OK cells through both the apical and basal borders, whereas DA and 5-HT are taken up through only the basal border. When applied from the apical cell border, L-5-HTP is cleared from the incubation medium more efficiently than L-DOPA, whereas at the basal border L-DOPA is transported more efficiently than L-5-HTP, DA or 5-HT.

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262P UPTAKE AND ACCUMULATION OF N-METHYL-4-PHENYLPYRIDINIUM (MPP+) BY RAT HEPATOCYTES

F. Martel, H. Russ*, I. Azevedo & E. Schömig*, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal and *Department of Pharmacology and Toxicology, University of Würzburg, 97078 Würzburg, Germany

1-Methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was previously shown to be taken up and accumulated by hepatocytes, where it induces toxicity (Di Monte *et al.*, 1987; Singh *et al.*, 1988), but the mechanism responsible for the uptake of MPP^+ is unknown. The aim of this study was to characterize the hepatic uptake of [^{3}H]-MPP^+ in primarily cultured rat hepatocytes.

Initial rates of specific [^{3}H]-MPP^+ transport into rat hepatocytes were found to be saturable, the K_m being 12.6 (9.3, 17.0) μM and the V_{max} being 40.1±2.6 pmol mg protein^{-1} min^{-1} (n=4). The rate constant of inward transport of [^{3}H]-MPP^+ into hepatocytes (k_{in}) was 16.1±3.2 μl mg protein^{-1} min^{-1}, the rate constant of outward transport of [^{3}H]-MPP^+ from hepatocytes (k_{out}) was 0.113±0.027 min^{-1} and the equilibrium factor of accumulation (A_{max}) was 56.4±3.8 (n=24). [^{3}H]-MPP^+ accumulation was sensitive to cyanine863, decynium22, quinine, clonidine, quinidine and O-methylisoprenaline. The most potent compounds were cyanine863, decynium22 and quinine, with IC_{50}'s of 97 (71, 140), 230 (180, 290) and 790 (490, 1300) nM, respectively (n=4). The monovalent organic cation tetraethylammonium (100 μM) and the bile salt taurocholate (100 μM) both reduced the transport of [^{3}H]-MPP^+ by about 50%.

We conclude that [^{3}H]-MPP^+ is efficiently taken up into hepatocytes by a carrier-mediated mechanism. [^{3}H]-MPP^+ uptake into rat liver cells is brought about by a system with kinetic and pharmacological characteristics that distinguish it from the known hepatic and renal organic cations carriers, and that also distinguish it from the noradrenaline carrier systems ($uptake_1$ and $uptake_2$). The carrier responsible for [^{3}H]-MPP^+ uptake into hepatocytes seems to be a new member of a family of membrane-bound transporters which is sensitive to cyanine-related compounds.

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263P INOSITOL POLYPHOSPHATE CONCENTRATIONS ARE INCREASED IN SKELETAL MUSCLE DURING PORCINE MALIGNANT HYPERTHERMIA

M. Steinfath, P.H. Tonner, J. Scholz, A. Richter, W. Loescher, N. Roewer & J. Schulte am Esch (introduced by J. Neumann), Department of Anaesthesiology, University of Hamburg, University Hospital Eppendorf, D-20246 Hamburg, Germany

Malignant hyperthermia (MH) is a potentially lethal, inherited pharmacogenetic disorder characterized by an increased calcium level in the myoplasm of skeletal muscle (MacLennan & Phillips, 1992). Intracellular calcium is released from the sarcoplasmic reticulum by ryanodine and inositol 1,4,5-trisphosphate (1,4,5-IP_3) receptors. A defect of the ryanodine receptor has been proposed to be responsible for the MH syndrome. In swine and humans increased levels of 1,4,5-IP_3 have been measured in MH susceptible (MHS) subjects at rest as compared to non-susceptible (MHN) individuals (Foster et al., 1989; Scholz et al., 1991; 1993). This study was aimed to determine inositol polyphosphates before and during MH crisis triggered by halothane and after dantrolene treatment.

With approval of the local animal care committee 7 MHS and 7 MHN pigs were studied. Anaesthesia was induced with metomidate. After 15 min 3 vol% halothane were added for a period of 15 min. When halothane was discontinued dantrolene was administered at a dose of 3.5 mg/kg i.v.. Heart rate, blood pressure, endexpiratory CO_2 ($P_{et}CO_2$), and body temperature were monitored throughout. Two muscle biopsies were taken in randomized order after each period (basal, halothane, dantrolene). Measurement of inositol polyphosphates was accomplished by HPLC-metal-dye-detection method (Mayr, 1988; Scholz et al., 1993). Data are expressed as mean±SEM. Statistical significance was determined by Student's t-test for paired and unpaired observations ($p<0.05$).

During halothane challenge all MHS pigs developed clinical signs of a MH crisis as determined by an increase in heart rate, $P_{et}CO_2$, and body temperature as well as a decrease in blood pressure ($p<0.05$). In MHS- and MHN-pigs 1,3,4-IP_3, 1,4,5-IP_3, 1,3,4,5-IP_4, 1,3,4,6-IP_4, IP_5, IP_6 were detected. In MHN-pigs the concentrations of inositol polyphosphates remained close to basal levels after administration of halothane and dantrolene. In MHS-pigs basal concentrations of 1,3,4-IP_3, 1,4,5-IP_3, 1,3,4,5-IP_4, 1,3,4,6-IP_4 were significantly ($p<0.05$) higher than in MHN. After halothane challenge inositol polyphosphate levels increased significantly ($p<0.05$) in MHS swine (*1,3,4-IP_3:* 1.03±0.07; *1,4,5-IP_3:* 0.87±0.05; *1,3,4,5-IP_4:* 0.39±0.05; *1,3,4,6-IP_4:* 0.16±0.01 pmol/mg wet weight) compared to basal values (*1,3,4-IP_3:* 0.89±0.07; *1,4,5-IP_3:* 0.74±0.04; *1,3,4,5-IP_4:* 0.22±0.03; *1,3,4,6-IP_4:* 0.10±0.01 pmol/mg wet weight). Treatment with dantrolene reduced these inositol polyphosphate concentrations close to baseline.

The results demonstrate that the inositol polyphosphates are markedly increased during MH crisis. Although changes in the ryanodine receptor complex have been reported it is conceivable that the enhanced calcium concentration in the myoplasm during MH crisis could additionally be due to an altered inositol polyphosphate system.

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264P EFFECT OF GENTAMICIN ON ENZYME ACTIVITIES IN A HUMAN RENAL CELL LINE

A. Kumar, J. McAulay & J.M. Sneddon, School of Health Sciences, University of Sunderland, Sunderland SR1 3SD

We have previously reported (Charlton & Simmons, 1993) our findings relating to an immortal human renal cell line, G401 which retains the specific characteristics of the proximal tubule where most damage occurs by xenobiotics. To be of any value in predicting nephrotoxicity *in vivo*, the cell line should be susceptible to the same toxic insult *in vitro*; our preliminary studies suggest that this may be the case. Gentamicin, an aminogylcoside antibiotic, is nephrotoxic in about 10% of patients. We have investigated the effect of gentamicin on the G401 cell line by measuring the activity of three enzyme markers to the drug; lactate dehydrogenase (LDH), a marker of non-specific cellular injury, alkaline phosphatase (AP), an apical membrane-bound enzyme and leucine aminopeptidase (LAP), a marker of the basolateral membrane.

G401 cells were grown to confluence in McCoy's 5α medium supplemented with 10% foetal calf serum. Once confluent, cells were incubated with varying concentrations of gentamicin (0-5mM) for 24, 48, or 72 hours. At the end of the incubation period cells were washed, homogenised and enzyme activities and protein content determined. Results are shown in Table 1.

Table 1 The effect of varying concentrations of gentamicin (0-5mM) on the specific activity of lactate dehydrogenase (LDH), (IU/mg protein); alkaline phosphatase, AP (mU/mg protein); leucine aminopeptidase, LAP, (mU/mg protein) at three different incubation times, 24, 48, & 72h. Results are expressed as mean ± s.e. mean, n=3.

*p<0.05, **p<0.01, compared with 0mM gentamicin for each time, one tailed paired t-test.

	Gentamicin Concentration				
Incubation time:	0μM	10μM	100μM	1mM	5mM
LDH					
24h	0.08 ± 0	0..06 ± 0.006	0.07 ± 0.003	0.05* ± 0.003	0.04** ± 0.003
48h	0.09 ± 0.003	0.07* ± 0.0	0.05** ± 0.0	0.06* ± 0.003	0.04* ± 0.003
72h	0.07 ± 0.006	0.06 ± 0.007	0.05* ± 0.003	0.04* ± 0.006	0.02* ± 0.003
AP					
24h	0.45 ± 0.009	0.39 ± 0.10	0.33 ± 0.05	0.30* ± 0.09	0.25* ± 0.05
48h	0.64 ± 0.14	0.41 ± 0.06	0.43 ± 0.09	0.26* ± 0.03	0.20* ± 0.04
72h	0.32 ± 0.04	0.33 ± 0.06	0.29 ± 0.06	0.21* ± 0.03	0.13** ± 0.03
LAP					
24h	11.32 ± 0.20	10.36 ± 0.28	9.83 ± 0.56	9.11* ± 0.46	7.95* ± 0.36
48h	10.49 ± 0.69	9.71 ± 0.45	9.06 ± 0.57	8.19* ± 0.41	7.34* ± 0.40
72h	10.25 ± 0.87	7.50 ± 0.57	7.54 ± 0.63	7.11* ± 0.86	4.93* ± 0.96

Our results suggest that the G401 cell line is susceptible to toxic insult by gentamicin and that the G401 may be of value in predictive nephrotoxicology.

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R. Whelpton, A.T. Michael-Titus & Z. Yaqub, Department of Pharmacology, Queen Mary & Westfield College, Mile End Road, London E1 4NS.

Temoporfin, (tetra(m-hydroxyphenyl)chlorin, m-THPC) a new photosensitizer for the photodynamic therapy of cancer, is undergoing clinical trials (Ris *el al.*, 1991). Binding by lipoproteins may affect the biodistribution of compounds of this type and it has been suggested that they may enter tumour cells via LDL receptors (Mazière *et al.*, 1991).

Serum was separated from the blood of 5 healthy volunteers (23-46 y, 59-83 kg; 1 female) and incubated with [^{14}C]-temoporfin (10, 1 or 0.1 μg/ml) for 30 min at room temperature (23-25°C) or for 20 h at 4°C. Lipoprotein fractions, corresponding to very low density (VLDL), low density (LDL), lipoprotein(a) (Lp(a)) and high density lipoprotein (HDL), were obtained by differential flotation (Naito, 1986). KBr solutions were used to give final densities of 1.006, 1.063, 1.087 and 1.21 g/ml prior to centrifugation. The purity of the fractions was assessed by agarose gel electrophoresis. Protein content was determined by Lowry's method and total cholesterol and albumin using commercially available kits (Sigma). Association of drug and lipoprotein was confirmed by autoradiography of electrophoresis gels and size exclusion chromatography (Vercaemst & Rosseneu, 1983). Data are expressed as mean (s.e.mean) and were tested by one-way ANOVA.

The distribution of temoporfin between serum fractions after incubation for 30 min is shown in Table 1. The proportions of drug in each fraction were unaffected by the drug concentration or incubation conditions ($P > 0.05$). Although the HDL layer contained measurable amounts of albumin (HDL/Residue = 0.22 ± 0.3, n = 5) it was clear that binding to albumin was minimal in the presence of lipoproproteins.

Table 1. Temoporfin distribution as a function of concentration

Fraction	Percent of initial radioactivity (s.e.mean) 10 μg/ml	1 μg/ml	0.1 μg/ml
VLDL	5.1 (1.3)	6.6 (1.4)	7.5 (1.0)
LDL	20.2 (2.1)	23.4 (3.0)	24.0 (2.6)
Lp(a)	15.8 (1.6)	17.8 (1.8)	16.5 (1.8)
HDL	38.3 (1.1)	38.9 (1.7)	38.7 (1.7)
Residue	18.5 (1.3)	15.3 (1.6)	15.0 (1.4)

In common with other photosensitizers, temoporfin is extensively bound to human lipoproteins, the proportion bound being unaffected by concentration over a 100 fold range. If tumour uptake is dependent on LDL binding, the fact that <25% of the drug is found in this fraction need not be clinically important as equilibrium between the classes is rapid. Moreover, if the drug did not bind to serum lipoproteins it is to be expected that the blood concentrations *in vivo* would be considerably lower, as such a hydrophobic drug would be taken up by tissues. This may or may not result in higher tumour concentrations.

[^{14}C]-Temoporfin was a gift from Scotia Pharmaceuticals.

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266P EFFECT OF HYPERBARIC OXYGEN AND 5-FU ON THE GROWTH OF HUMAN COLONIC CANCER CELLS

L.C. McMillan, J.A.S. Ross[1] & H.M. Wallace, Departments of Medicine & Therapeutics, Biomedical Sciences and [1]Environmental & Occupational Medicine, University of Aberdeen, Aberdeen AB9 2ZD

Hyperbaric oxygen (HBO) therapy involves intermittent exposure to 100% oxygen at between 2-3 atmospheres and is used in the treatment of divers suffering from decompression sickness. HBO also has a role in the treatment of cancer where the necrotic centres of malignant tumours are often hypoxic and resistant to radiotherapy. Here HBO is the best method for enhancing the effect of radio-therapy on cancer tissue (Henk, 1986). Colorectal cancer is a leading cause of death in the Western World and in Grampian 260 new cases are diagnosed annually. Treatment is limited, and 5-fluorouracil (5-FU) is the primary agent used with metabolism being essential for its action (Pinedo & Peters, 1988). Our ultimate aim is to combine the two treatments in an attempt to produce an enhanced anti-tumour effect. In this study, we examined the effects of exposure to 5-FU and to HBO on the growth of the human colonic cancer cell line, HT115.

Cells were grown as monolayer cultures in DMEM with Hepes and 10% (v/v) horse serum at a seeding density of 2.1 x 10^4 cells/cm^2. Cultures were grown for 48-72h at 37°C in a humidified atmosphere of 95% air:5% CO_2 and exposed to either HBO for 2 or 4h or 5-FU (0-10μM) for 24/48h. The effects on cell growth were determined.

Table 1: Effect of exposures to 5-FU and to HBO on Protein Content of HT115 cells. Results are mean values +/- SD (n=4) *p<0.05/ **p<0.01

Time after exposure (h)	Protein Content (mg/culture) Control	5FU(10μM)	Control	HBO (4h)
24	0.831 ± 0.047	0.669 ± 0.06**	0.520 ± 0.061	0.497 ± 0.036*
48	0.926 ± 0.066	0.558 ± 0.04**	0.709 ± 0.02	0.640 ± 0.008*

Both exposures to 5-FU decreased significantly protein content with the longer exposure time having the greater effect (Table 1). A single 4h exposure to HBO affected protein content at 24 & 48h (Table 1). The effect of the combination of HBO and 5-FU exposure is currently under investigation.

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267P OESTROGEN RECEPTOR FUNCTION IN RAT URERUS IS DIFFERENTLY INHIBITED BY TAMOXIFEN AND ICI 182,780

A. Ferreira Mendes, M. M. Caramona, M. Celeste Lopes
Laboratório de Farmacologia, Faculdade de Farmácia, Universidade de Coimbra
3000 Coimbra, Portugal

Tamoxifen (TAM), a triphenylethylenic antioestrogen, is widely used in the clinical management of breast cancer and some endocrine disorders. However, it displays some oestrogen-like effects (Van den Koedijk *et al.*, 1994), what brings about the need for a new, "pure" antioestrogen to be developed. It is now being postulated that ICI 182,780 (ICI)*, a steroid drug, behaves as such, fully antagonising the oestrogen receptor (ER) function (Wakeling & Bowler, 1992).

The aim of the work was to compare the antiuterotrophic potency of those two drugs in rat uterus. To evaluate their interaction with the ER, we assessed the receptor level in both the cytosolic (ER_C) and nuclear (ER_n) fractions of uteri homogenates from treated rats.

Ovariectomized Wistar rats, 10-12 weeks old, have been administered with either vehicle alone (0.5 ml saline with 2% ethanol), oestradiol (E_2, 220 µg/kg), TAM (440 µg/kg), ICI (720 µg/kg), or E_2 plus each of the drugs in study (n=7-8 per group). Uteri were removed 18 hours after administration and used to assess the uterotrophic/antiuterotrophic effect and the ER levels in the cytosolic and nuclear fractions. An immunoenzymatic method, the "ER-EIA" kit from the Abbott Laboratories, has been used for those determinations.

In the cytosolic fraction, the highest value (ER_C=389 ± 36 fmol/mg prot.) was obtained with ICI. This concentration was about twice as high as the one obtained with E_2 (ER_C=195 ± 50 fmol/mg prot.). On the other hand, ICI gave the lowest result in the nuclear fraction (ER_n=110 ± 16 fmol/mg DNA), whereas E_2 gave the highest (ER_n=346 ± 85 fmol/mg DNA). With TAM, the concentrations were: ER_C=110 ± 35 fmol/mg prot. and ER_n=338 ± 58 fmol/mg DNA.

Our results suggest that ICI 182,780 inhibits ER translocation to the nucleus, probably by inhibiting ER dimerization, thus preventing DNA binding and transcription activation. TAM doesn't inhibit ER translocation to the nucleus, suggesting that it doesn't interfere with receptor dimerization. Its mechanism of action must lie on some subsequent step, namely modifying the ER interaction with oestrogen responsive elements (ERE) in DNA.

* Kindly supplied by Zéneca Farmacêutica, Portugal.

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268P PARACETAMOL INHIBITS DNA SYNTHESIS IN THE RAT TESTIS, THYMUS AND OTHER ORGANS AFTER NON-CYTOTOXIC ORAL DOSES

Catherine Lister & André E M McLean, Clinical Pharmacology, UCL Medical School, 5 University St London WC1E 6JJ.

Paracetamol is known to quench the tyrosyl free radical in ribonucleotide reductase (RR) in V79 cells in culture, thereby bringing DNA synthesis to a halt. (Hongslo et al 1990). We have measured inhibition of DNA synthesis in various tissues in the rat following a single oral dose of paracetamol in the range 1g/kg to 0.125g/kg (Chang and Looney, 1965).

Table 1 shows marked inhibition of thymidine incorporation into DNA especially in testis and thymus, 30-60 minutes after paracetamol. Liver, duodenum and stomach are less affected, particularly at the 0.125g/kg dose.

These differences between tissues in inhibition of DNA synthesis are unrelated to the cytotoxic effects of paracetamol, which in rats are found mainly in the liver, and only at higher oral doses than those used in the present experiments.

The most likely explanation for the inhibition of DNA synthesis in vivo is that paracetamol acts as a phenolic antioxidant-radical trapping agent that penetrates cells and prevents precursor synthesis by inhibiting RR at doses about ten times those in therapeutic use.

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Table 1. Effect of paracetamol on DNA synthesis in different tissues in the rat.

	Thymidine incorporation DPM/mg tissue.			
DOSE	control	0.125g/kg	0.5g/kg	1g/kg
n	6	3	3	3
TISSUE				
Testis	16.1 ± 5.0	8.5 ± 1.4†	1.7 ± 0.9†	0.73 ± 0.15†
Thymus	97.0 ± 15.0	62.9±16.8†	12.8± 2.0†	4.5 ± 1.4†
Liver	21.4 ± 5.3	25.6± 4.6	11.6 ± 2.9†	10.0 ± 2.0†
Duodenum	37.9 ± 13.2	34.0± 16.4	9.8 --	9.7 --
Stomach	26.7 ± 8.5	17.9± 4.1	14.2 ± 1.1	7.3 ± 3.0†

MEANS±SD for 3 to 6 separate animals. (*only one result available † denotes $p<0.05$ as compared with control group.). ^{3}H Thymidine, 10uCi per 100g body weight was given intravenously 30 minutes after oral paracetamol dosing.The animals were kept in a warm cage and killed 30 minutes later. Tissues were taken and homogenised, macromolecules precipitated with trichloracetic acid, washed, dissolved in NaOH, and counted.Acid soluble radioactivity was not significantly affected by paracetamol.

269P INFLUENCE OF PRENATAL CARBON MONOXIDE EXPOSURE IN PERIPHERAL MYELINOGENESIS IN THE RAT

V. Cuomo, MR. Carratù, Institute of Pharmacology, School of Medicine, University of Bari, Italy

Studies dealing with the consequences of prenatal exposure to carbon monoxide (CO), an air pollutant as well as one of hundreds of constituents of cigarette smoke, have shown that such exposure can affect nervous system function and behaviour of the offspring in both animals and humans (Annau & Fechter, 1994). Based on our recent findings showing that prenatal exposure to relatively mild CO concentrations markedly affects both central and peripheral nervous system functions (Di Giovanni *et al*, 1993; Carratu *et al*, 1993), experiments have been designed in order to investigate possible influences of gestational exposure to CO on peripheral myelinogenesis in the rat.

Rat pups born to dams exposed to CO (0, 75 and 150 ppm) throughout gestation were sacrificed at timed intervals after birth (3, 8, 13, 18, 28 and 40 days of age); sciatic nerves were quickly removed and processed for computer-assisted planimetric measurements on Toluidine blue-stained sections and immunohistochemistry with polyclonal antibody to myelin basic protein (MBP).

In rat pups born to dams exposed to CO (75 and 150 ppm) throughout gestation, a linear relationship was found between growth in myelin sheath thickness and age as in control rats, thus indicating that in both nonexposed and CO-exposed rats, myelin develop occurs *pari passu* with age. However, in 40-day old rats exposed to 150 ppm of CO throughout gestation myelin sheath thickness was found to be signficantly decreased in comparison with control rats (control: 0.76±0.08 μm, n=29; 150 ppm: 0.34±0.02 μm, n=32, p<0.01). A linear relationship was also found between myelin sheath thickness and axon diameter in both nonexposed and CO-exposed rats; however, the slope of the line relating these two physiological variables in CO-exposed rats was found to be significantly different in comparison with control rats (control: r 0.99, slope 0.23±0.001; 75 ppm: r 0.97, slope 0.16±0.0018*; 150 ppm: r 0.98, slope 0.15±0.0014**; *p<0.02 vs control; **p<0.01 vs control), thus indicating that growth in axon diameter and myelin sheath thickness was asynchronous in the offspring of dams exposed to this gas throughout gestation. Immunohistochemistry with polyclonal antibody to IBP has shown that in 150 ppm CO-exposed rats immunostain intensity of sciatic nerve sections was notably reduced in comparison with control rats at each tested age. In 75 ppm CO-exposed rats immunostain of sciatic nerve sections was less intense at 8 and 13 days of age in comparison with control rats; in 18-, 28- and 40-day old rats no difference was found between non-exposed and CO-exposed rats.

In sum, the results of the present study provide the first straightforward evidence that prenatal exposure to relatively mild concentrations of carbon monoxide impairs peripheral myelinogenesis in the rat and hint at CO influence on the broad array of signals between Schwann cells and axons which promote mutual growth and differentiation.

This work was supported by the CNR-ENEL Project Interactions of Energy Systems with Human Health and Environment and by MURST.

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270P THE ROLE PLAYED BY THE Na^{2+}/Ca^{2+} EXCHANGER DURING GLUCOPENIC AND ANOXIC CONDITIONS

L. Annunziato, S. Amoroso, *S. Montagnani, A. Bassi, R. Caporaso, E. Iannotti, N. Caso, G.M. Russo and G. F. Di Renzo. Unit of Pharmacology, Department of Neurosciences, *Institute of Anatomy, School of Medicine, University "Federico 11" Naples, Via S. Pansini 5, 80131, Naples, Italy.

Homeostasis of intracellular Ca^{2+} and Na^+ ions, which is altered under glucopenic and anoxic conditions, is mainly regulated by the Na^+/Ca^{2+} exchanger system. This exchanger couples the uphill extrusion of Ca^{2+} to entrance of Na^+ ions into the cell. However, this mechanism can also operate in a reversible fashion by coupling Na^+ ion efflux to Ca^{2+} ion influx, depending not only on the intracellular and extracellular concentrations of Na^+ and Ca^{2+} ions but also on membrane potential. The aim of the present study was to characterize the mode of operation of the Na^+/Ca^{2+} exchanger under glucopenic and anoxic conditions and the role played by this exchanger in the cellular survival in primary culture of fetal hippocampal neurons and C6 glioma cells, a line which exhibits several characteristics of astrocytes and has been extensively used as a model for cerebral glia.

Glucopenic and anoxic conditions were obtained by adding to the incubation medium oligomycin (5μg/ml) plus 2-deoxy-glucose (2mM), in the absence of extracellular glucose. This treatment induced a time-dependent release of LDH, an intracellular enzyme used as an index of cytotoxicity. The activation of the Na^+/Ca^{2+} exchanger as a Na^+ extrusion pathway coupled to Ca^{2+} ion entry, obtained by the removal of Na^+ ions from extracellular medium, reduced the release of LDH induced by anoxia + glucopenia (Table 1).

Table 1

Treatment	Extracellular LDH (mlU/ml)
Control	50 ± 2
Anoxia + glucopenia	800 ± 56
Na^+-free + anoxia + glucopenia	200 ± 19*

*P <0.05 vs all groups

This effect was reverted by the specific Na^+/Ca^{2+} exchanger inhibitor, 5-(N-H-chlorobenzyl)2'-4'-dimethylbenzamil amiloride (DMB), in a dose dependent manner. Ouabain (1 mM), which causes an increase in intracellular Na^+ ion concentra-tions and thus activates the Na^+/Ca^{2+} exchanger as Na^+ ion efflux pathway, reduced LDH increase induced by glucopenic and anoxic treatment. This ouabain effect was also counteracted by CBDMB (3 uM). (Table 2).

Table 2

Treatment	Extracellular LDH (mlU/ml)
Control (145 mM Na^+	20 ± 2
Anoxia + glucopenia	609 ± 41
Ouabain (1 mM)	10 ± 2
CBDMB (3 μM)	12 ± 2
Ouabain + anoxia + glucopenia	420 ± 37*
Ouabain+anoxia+glucopenia+CBDMB	850 ± 34

* P < 0.05 versus all groups

By contrast the 5-(N,N-hexamethylene) amiloride (HMA), another pyrazine analogue which specifically inhibits the Na^+/Ca^{2+} antiporter and has no inhibitory effect on the Na^+/Ca^{2+} exchanger, did not affect LDH release induced by glucopenic and anoxic conditions. Finally, gadolinium, used at the concentration which specifically blocks extracellular Ca^{2+} entry through voltage-sensitive calcium channels, did not modify LDH release induced by glucopenic and chemical anoxia treatment.

Collectively, these results suggest that Na^+/Ca^{2+} exchanger, under glucopenic and anoxic conditions, is operating as Na^+ efflux coupled to Ca^{2+} entry. This mode of operation seems to have a beneficial effect upon anoxic insult.

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271P THE ROLE OF NO PRODUCTION IN THE ANTI-ADRENERGIC EFFECT OF ADENOSINE ON CALCIUM CURRENT IN ISOLATED RABBIT ATRIO-VENTRICULAR CELLS

A.E. Martynyuk, K.A. Kane[1], S.M. Cobbe & A.C. Rankin, Department of Medical Cardiology, Royal Infirmary, Glasgow G31 2ER and [1]Department of Physiology and Pharmacology, University of Strathclyde, Glasgow Gl lXW.

Adenosine, like acetylcholine, has been shown to attenuate isoprenaline stimulated calcium current (I_{Ca}) in myocytes from various regions of the heart (Pelleg and Belardinelli, 1993) but its effect on atrio-ventricular (A-V) nodal cells has not been studied. Recently, it has been suggested that this effect of acetylcholine involves the production of nitric oxide (NO) (Han *et al.*, 1994). The aim of the present study was to study the anti-adrenergic effect of adenosine in isolated A-V nodal cells and its dependence on NO production.

Spontaneously active A-V nodal cells, enzymatically isolated from rabbit hearts, were studied at 35 - 37° C. I_{Ca} was measured using the whole cell patch clamp technique with step pulses from a holding potential of -50 mV (to inactivate sodium current). Outward potassium currents were blocked by using potassium free pipette solution by replacing the potassium by caesium.

Extracellular application of isoprenaline (0.1 μM) increased peak I_{Ca} from 611 ± 71 to 1035 ± 114 pA (mean ± S.E., n = 18). This stimulatory effect of isoprenaline on I_{Ca} was antagonised by the extracellular application of adenosine. The isoprenaline-induced increase in I_{Ca} (186 ± 20% of control, n = 18) was abolished in the presence of 10 μM adenosine (I_{Ca} 100 ± 4% of control, n = 4, $P<0.05$). The adenosine A_1 receptor antagonist CPDPX (8-cyclopentyl-1,3-dipropylxanthine), in a concentration of 0.1 μM, blocked the effects of adenosine, 10 μM, on I_{Ca} activated by isoprenaline. The effect of adenosine on the isoprenaline stimulated I_{Ca} was prevented by pre-incubation of the cells with the NO synthase (NOS) inhibitor L-nitro-arginine methyl ester (L-NAME, 0.5 - 1 mM) for 3 - 4 h. In L-NAME pre-treated cells, the isoprenaline-induced increase in I_{Ca} (231 + 68% of control, n = 4) was not reduced by the addition of 10 μM adenosine (209 ± 55% of control, n = 4). Co-incubation of cells in L-NAME with L-arginine, (1 mM, the endogenous substrate of NOS) restored the adenosine-induced attenuation of I_{Ca}. In these cells, isoprenaline resulted in an increase in I_{Ca} (157 ± 7% of control, n = 6), and, following addition of adenosine, 10 μM, I_{Ca} was reduced to 107 ± 8% ($P<0.05$).

In conclusion, the anti-adrenergic effect of adenosine on the AV-node may, in part, be due to its attenuation of the catecholamine stimulated increase in I_{Ca}. This effect of adenosine is mediated by the A_1 receptor and it is dependent upon the production of NO.

This work is supported by the British Heart Foundation.

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272P PHOTO-INDUCED ADEQUATE NITRIC OXIDE (PIANO) GENERATING SYSTEM AS A USEFUL TOOL FOR THE INVESTIGATION OF NO-MEDIATED RESPONSES

K.C.Chang Department of Pharmacology, College of Medicine, Cardiovascular Research Institute, Gyeongsang National University, Chinju, Korea (introduced by W. C. Bowman)

Chang et al (1993) and Chung & Chang (1994) reported that a so-called photo-induced adequate nitric oxide (PIANO) system, in which a NO- or NO_2-carrying molecule is photoactivated to release NO, can be exploited to investigate the role of NO in various smooth muscle functions. In the present study, comparisons were made between PIANO-mediated and NANC nerve stimulation-mediated relaxations of the isolated rat gastric fundus, the fundus having been pre-contracted either by 5-hydroxytryptamine (5-HT, 10 μM) or by high KCl (65.4 mM). The fundus strips were pretreated with guanethidine (10 μM) and atropine (1 μM) to inactivate noradrenergic and cholinergic mechanisms. Each observation was made on 4 or more fundus preparations. NANC nerve stimulation was evoked by field stimulation (20 V, 1 ms, 16 Hz for 10 s). PIANO was evoked by exposure to UV (Chung and Chang, 1994) for 15 s in the presence of the NO-carrying molecule streptozotocin (100 μM). In 5-HT-contracted strips, both NANC nerve stimulation and PIANO produced transient and reproducible relaxations. Relaxations evoked by NANC nerve stimulation were abolished by the NO synthase inhibitor L-NAME (5×10^{-4}) or by tetrodotoxin (TTX, 10^{-6}M). D-NAME (5×10^{-4} M) was without effect. PIANO-induced relaxations were unaffected by the same concentrations of L-NAME, D-NAME or TTX.

In high K^+-contracted preparations, NANC-nerve stimulations failed to produce relaxations, possibly because the axon membranes were depolarized and nerve conduction was thereby abolished. However, an alternative explanation, requiring further study, is that the response to NO, rather than its release, is impaired by depolarization of the smooth muscle cell membranes. PIANO-induced relaxations, though significantly reduced in amplitude ($P < 0.01$) compared to those in 5-HT-contracted tissue, were still clearly produced in K^+-contracted tissues. The reduced amplitude in high K^+ may have been a consequence of postjunctional depolarization, since the amount of NO released by PIANO was the same during both 5-HT-induced and K^+-induced contractions. The amount of NO released was measured indirectly as nitrite by Griess reagents (Stuehr & Griffith, 1992). M&B 22948, a selective inhibitor of cGMP phosphodiesterase, potentiated the PIANO-induced relaxations during both 5-HT- and K^+-induced contractions, indicating, as expected, that c-GMP is responsible for the relaxations. The results provide further evidence that the PIANO method provides an additional tool for investigation of NO-mediated responses.

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273P COMPARISON OF N-NITRO-L-ARGININE METHYL ESTER AND N-NITRO-L-ARGININE ON BASAL AND CARBACHOL-STIMULATED NITRIC OXIDE SYNTHASE

W.Elbishti & D.Pollock, Department of Pharmacology, University of Glasgow, Glasgow, G12 8QQ, Scotland.

Recently, Buxton *et al.* (1993) have reported that the nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L-NAME) may act as a muscarinic antagonist, whereas N-nitro-L-arginine (L-NOARG) does not block muscarinic receptors. In contrast to the effect of L-NAME on NOS, muscarinic blockade can not be reversed by L-arginine. This has cast doubt on the validity of the proposal by Randall and Griffith (1991) that there might be isoenzymes of NOS involved in basal and agonist-stimulated NO synthesis, since it was based on the ability of L-arginine to reverse L-NAME-induced inhibition of basal but not acetylcholine-stimulated NO release. This study was undertaken to compare the effects of L-NAME and L-NOARG on basal and carbachol-stimulated NO release in rat pulmonary artery, and their prevention by L-arginine.

Male Wistar rats were killed by stunning and exsanguination. Rings from the 2nd order branches were isolated, mounted under 1g tension in organ baths containing Krebs' buffer (37°C), gassed with 95 % O_2 / 5 % CO_2. Basal NO release was assessed indirectly by measuring the potentiation by L-NAME or L-NOARG of contractions induced by phenylephrine (PE), which does not release NO in this preparation. Concentration-response curves to PE (10^{-9}-10^{-4} M) were obtained in the absence and presence of L-NAME ($2x10^{-4}$M) or L-NOARG (10^{-4}M) alone or with L-arginine (10^{-2} M). Cumulative concentration- response curves to carbachol (CARB, 10^{-9}-10^{-4} M) were obtained in PE-precontracted rings ($9.5x10^{-8}$ or $9.5x10^{-9}$ M) in the absence and presence of L-NAME or L-NOARG alone or with L-arginine. Contractile effects were expressed as mg tension. mg^{-1} tissue. Relaxant effects were expressed as % relaxation of PE-induced tone.

PE induced concentration-dependent contractions with an EC_{50} value of $2.3x10^{-9}$ M and a maximum response of 323.6± 23 $mg.mg^{-1}$ tissue (mean ± s.e.mean, n=8). Pretreatment with L-NAME or L-NOARG potentiated PE-induced contractions yielding EC_{50} values of $2.8x10^{-10}$ and $4.1x10^{-10}$ M, respectively (n=8, $0.01>P>0.001$). There were, however, no significant differences in the maximum responses (370.7 ± 22.8 and 356.6 ± 12.5 $mg.mg^{-1}$ tissue, respectively). The potentiating effects of L-NAME and L-NOARG were completely prevented by L-arginine (n=8). In PE-precontracted rings, CARB induced concentration-dependent relaxations reaching a maximum response of 102.1 ± 4% (n=9). This response was greatly reduced by L-NAME to 6.6 ±1.8% (n=9, $P< 0.001$), and abolished by L-NOARG (n=9). Treatment with L-arginine only partially prevented the effects of L-NAME and L-NOARG on CARB-induced relaxation. In this case, the maximum relaxations induced by CARB were 44.±1.8 and 37.6±1.3%, respectively (n=9, $P< 0.001$).

These results confirm the findings of Randall and Griffith (1991), and support the view that there might be isoenzymes of NOS involved in basal and agonist-stimulated NO release. The similarity in the inhibitory effects of L-NAME and L-NOARG on CARB-induced relaxation and their partial prevention by L-arginine suggests that L-NAME has no significant antagonistic activity on muscarinic receptors mediating relaxant responses to CARB in rat pulmonary artery, and therefore can be used for studying such responses in this preparation.

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274P EFFECT OF NITRIC OXIDE ON THE MIGRATION OF LEUKOCYTES THROUGH HUMAN ENDOTHELIAL CELL MONOLAYERS

H. Parry[1,2], G. Whitley[2], & P. Vallance[1], [1]Departments of Pharmacology and Clinical Pharmacology and [2]Cellular Molecular Sciences, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE.

The accumulation of leukocytes in tissues is a central event involved in the inflammatory response. The passage of leukocytes across the endothelium is stimulated by cytokines and mediated by adhesion molecules. Recent work has suggested a role for nitric oxide (NO) in the regulation of this process. Adhesion is the first step in transendothelial migration and a variety of studies have reported the inhibition of leukocyte binding to endothelium by NO (Kubes *et al.*, 1991; Radomski *et al.*, 1987; Bath *et al.*, 1991). We investigated the effect of nitric oxide on the migration of leukocytes through an endothelial cell monolayer.

The human umbilical vein endothelial cell line, SGHEC-7 (Fickling *et al.*, 1992) was grown to confluency on the gelatin coated porous membrane (8μm pore size) of Costar Transwell inserts and cultured in complete tissue culture medium. Lipopolysaccharide (LPS) at 10μg/ml and recombinant Human interferon-γ (rHuIFNγ) at 150U/ml or N$^{\omega}$-mono-methyl-L-arginine (L-NMMA) at 10^{-3}M, S-nitrosoglutathione (GSNO) 10^{-3}, 10^{-4} or 10^{-5}M or L-arginine at $6x10^{-3}$M were added 30mins prior to the addition of ^{111}Indium labelled leukocytes. The leukocytes were allowed to migrate through the endothelial cell monolayers for 24h whereupon the cells were harvested and the radioactivity determined. The results are expressed as a percentage of the control (mean ± S.E.M.).

A combination of LPS and rHuIFNγ increased the migration of leukocytes through the endothelial cell monolayers by 186 ±40% (after 30min preincubation, n=21) and by 198 ±11% $p<0.001$ (after 24h preincubation, n=41). Addition of blocking antibodies for ICAM-1 decreased migration through stimulated endothelial cell monolayers by 68 ±2% $p<0.05$ (n=9). The addition of GSNO at 10^{-3} 10^{-4} or 10^{-5}M and no significant effect on leukocyte transendothelial migration over 24h (125 ±10%, n=15, 117 ±2.9%, n=6 and 90 ±15%, n=12 respectively). Similarly the addition of either L-NMMA or L-arginine had no significant effect on leukocyte transendothelial migration (144 ±28%, n=27 and 141 ±38%, n=9 respectively).

In this experimental system neither endogenous nor exogenous NO had any effect on the migration of leukocytes through human endothelial cell monolayers. It remains to be determined whether NO has a role in modifying the migration of leukocytes through chronically activated endothelial monolayers.

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275P MICROINFUSION OF Nω-NITRO-L-ARGININE METHYL ESTER (L-NAME) INTO THE INFERIOR COLLICULUS DEPRESSES AUDITORY MIDDLE LATENCY RESPONSES IN THE RAT

G. Bagetta[1,2], C. Grassi[3], R. Santarelli[3], S. Nisticò[3] & G.B. Azzena[3], [1]Faculty of Pharmacy, Calabria University, Cosenza (Italy), [2]"Mondino-Tor Vergata" Neurobiol. Center, University of Rome "Tor Vergata", 00133 Rome & [3]Institute of Human Physiology, Faculty of Medicine, Catholic University, 00168 Rome (Italy)

Nitric oxide (NO), a highly reactive short-lived radical species, is thought to function as neuronal messenger in the central nervous system. Receptor agonists which increase intraneuronal Ca^{2+} activate NO synthase (NOS), a highly regulated enzyme which generates NO via the oxidation of the guanidino group of l-arginine with stoichiometric production of l-citrulline (see Knowles & Moncada, 1994). In the rat brain, immunocytochemical and *in situ* hybridization studies have shown that the inferior colliculus (IC), an important relay of the acoustic pathway, is among the most rich nuclei in NOS-containing neurones (Bredt & Snyder, 1992). These are abundant in the cortical region of the IC which receives nerve terminals mainly from the auditory cortex; by contrast, the central nucleus, which projects to the auditory cortex, contains a low density of NOS positive neurones with the exception of the ventrolateral region. Here we report evidence indicating that tonic NO production in the IC may be involved in the processing and transmission of acoustic signals to the auditory cortex of rat.
Under anaesthesia (ketamine 66 mg kg^{-1} and xylazine 13 mg kg^{-1} i.p.; 1 ml kg^{-1} volume) and stereotaxic guidance (Paxinos & Watson, 1982) adult male Wistar rats (200-300 g) were implanted with a stainless steel guide cannula to allow drug injection (1 µl volume of infusate, 1 µl min^{-1} rate) into one IC. Auditory potentials were evoked by monoaural click stimuli of supraliminal intensity and with 250 ms interstimulus interval; the evoked potentials were recorded via a ball Ag/AgCl electrode placed onto the previously exposed auditory cortex. Signals were filtered (0.003-3 KHz) and 250-1000 traces were averaged with the aid a computer program for studying middle latency (MLR) and brain stem (BSER) responses, respectively.
In agreement with previously reported data in rats (see Di & Barth, 1993), MLRs began with a biphasic positive (P1)-negative (N1) sharp wave with a latency of (mean±s.e.mean) 17.7+1.8 ms and 23.3+0.9 ms, respectively (control P1-N1 amplitude= 118±14 µV; n=5). Microinfusion of L-NAME (1 mM) into the IC contralateral to the stimulated ear did not affect the latency of MLR components (P1=18.4±2.1 ms; N2=22.9±0.9 ms; n=5) nor did it alter BSER. By contrast, this treatment reduced the amplitude of P1-N1 by 40% to 70% as compared to control; the less active enantiomer, D-NAME (1 mM; n=4), was ineffective (reduction ranging from 5% to 15% of control amplitude). The inhibitory effect of L-NAME started 3.5±1.4 min after the beginning of injection and lasted 24-30 min. A more dramatic MLR depression (>90%) was recorded in rats receiving 5 mM L-NAME (n=2), whereas a dose of 0.5 mM (n=2) was ineffective. Microinfusion into the IC of MK801 (1 µM; n=3), a use-dependent N-methyl-D-aspartate (NMDA) receptor antagonist, reduced the amplitude of the MLR components by 60% to 85%, without affecting their latencies.

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276P S-NITROSO-N-ACETYL-PENICILLAMINE (SNAP)-INDUCED RELEASE OF ENDOGENOUS AMINO ACIDS FROM RAT STRIATAL SLICES

Nicola J. Knott and J.A. Davies, Department of Pharmacology & Therapeutics, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN.

Nitric oxide (NO) is considered a retrograde messenger in the CNS and its synthetic enzyme, NO-synthase, has been identified in the striatum in a discrete, small (2%) population of medium-sized aspiny interneurons which receive a prominent cortical glutamatergic innervation. GABAergic, medium-sized spiny neurons predominate in the striatum and contain guanylate cyclase (Ariano, 1983), a major target for NO and subsequent cGMP production. In this study we investigated the effects of the NO-releasing agent, SNAP, on the release of amino acids from striatal slices.

Coronal slices (250µm) were prepared from striata of female Wistar rats (200-250g) using a McIlwain tissue chopper and perfused with gassed (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) at 37°C. Two-minute aliquots were collected and assayed for amino acids by HPLC with fluorometric detection. Transmitter release was elicited with a four-minute pulse of SNAP (100µM, 250µM, 500µM) in normal (NaCSF), magnesium-free (MgF) or calcium-free (CaF) aCSF. Haemoglobin (Hb, 10µM) was used as a scavenger for NO. The results are presented as the mean stimulated release per two-minute sample as a percentage of basal release.

SNAP (250µM and 500µM) produced a significant increase in glutamate release of 125±10% (n=7, P<0.05) and 327±48% (n=8, P<0.01) respectively. SNAP-induced release (500µM) was potentiated in MgF when compared to NaCSF (327±48% and 144±21% respectively, n=8, P<0.01). Penicillamine (500µM, MgF) had no effect on glutamate release but was shown to account for the release of glycine (136±13%, n=8, P<0.05) and GABA (134±8%, n=8, P<0.05). Hb (10µM) abolished the effect of SNAP (67±10%, n=8, P<0.001)). The SNAP-induced release of glutamate was also reduced to basal values upon removal of calcium from MgF (107±9%, n=8, P<0.001).

These results demonstrate that the increased release in glutamate was due to SNAP donating NO, as this effect was abolished by the NO-scavenger, haemoglobin. The release was also calcium-dependent suggesting a presynaptic mechanism for the action of NO. The potentiated effect seen in MgF implies that a portion of the release is NMDA receptor mediated as a result of increased endogenous release of glutamate. As our results have demonstrated, it is necessary when using SNAP to perform a control using penicillamine as this compound was found to be responsible for the increased release of certain amino acids. These results provide further evidence for the role of NO as an intercellular messenger in the CNS.

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277P LACK OF EVIDENCE FOR CYCLIC AMP-DEPENDENT REGULATION OF NMDA RECEPTOR-STIMULATED NITRIC OXIDE SYNTHESIS IN CULTURED RAT CEREBELLAR GRANULE CELLS

N.J. Toms and P. J. Roberts, Department of Pharmacology, University of Bristol, Bristol BS8 1TD.

In the immature rat cerebellum, the Ca^{2+}/calmodulin-dependent enzyme nitric oxide synthase (NOS) catalyses the formation of L-citrulline from L-arginine. NOS has been shown to be concentrated in granule cells where it can be stimulated via NMDA receptor activation (Bredt & Snyder, 1989; Bredt *et al.* 1990; Garthwaite *et al.*, 1989; Kiedrowski *et al.*, 1992). We have previously described the potentiation of NMDA-stimulated nitric oxide synthesis by dibutyryl cAMP and forskolin in immature rat cerebellar slices and its abolition by the protein kinase A inhibitor H-89 (Toms & Roberts, 1994). In this study we have investigated the effects of dibutyryl cAMP and forskolin on both NMDA-stimulated [^{3}H]-citrulline formation and $^{45}Ca^{2+}$ entry into cultured cerebellar granule cells.

Granule cells were prepared from 7-day-old rat pups and cultured using a serum-free method modified from that of Kingsbury *et al.* (1985). Briefly, cerebella were incubated in versene and triturated through a series of fire-polished glass pipettes (1-0.3mm). The resulting cell suspension was centrifuged and the pellet re-suspended in chemically-defined medium. Cells ($3x10^6$cells/35mm well) were then seeded onto a diluted (1/40 v/v) Matrigel matrix and incubated. Both NOS activity, determined by measurement of [^{3}H]-citrulline synthesis, and $^{45}Ca^{2+}$ influx were based on the methods as described by Kiedrowski *et al.* (1992) in the presence of 10μM glycine.

Initial experiments were performed to assess the optimum cellular age for the experiments. 100μM NMDA-stimulated $^{45}Ca^{2+}$ influx, relative to basal, increased with number of days *in vitro* (DIV) to peak at 6 DIV (age used in subsequent assays). NMDA produced concentration-dependent elevations in both $^{45}Ca^{2+}$ influx and [^{3}H]-citrulline synthesis; maximal stimulation occurred at 100μM NMDA (2.7pmol $^{45}Ca^{2+}$/5min and 180% above basal NOS activity, respectively). NMDA (100μM) stimulation of both $^{45}Ca^{2+}$ influx and [^{3}H]-citrulline synthesis was selectively inhibited by the NMDA antagonist D-AP5 (50μM) and the NOS inhibitor L-NAME (50μM), respectively. Pre-incubation with dibutyryl cAMP (1mM) or forskolin (60μM) had no significant effect on submaximal (10μM) NMDA-stimulated [^{3}H]-citrulline synthesis or $^{45}Ca^{2+}$ influx.

These data demonstrate that NMDA stimulates both NOS activity and $^{45}Ca^{2+}$ influx in cultured cerebellar granule cells as previously reported by Kiedrowski *et al.* (1992). However, under the present culture conditions, elevations of intracellular cAMP do not appear to modulate either NOS activity or $^{45}Ca^{2+}$ influx, in contrast to our findings in immature rat cerebellar slices. The locus and mechanism of cAMP/PKA-mediated potentiation of NMDA-stimulated NOS activity, observed in slices, remains to be determined.

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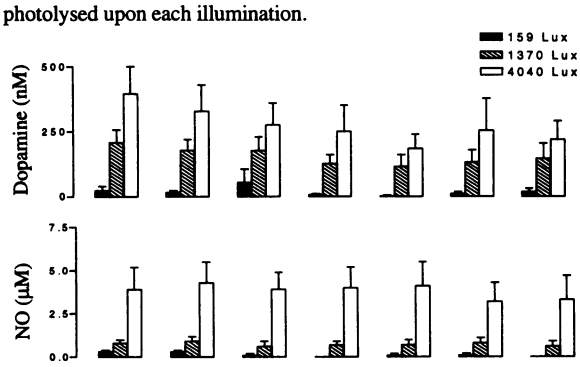
278P SIMULTANEOUS MEASUREMENT OF ENDOGENOUS DOPAMINE AND EXOGENOUS NITRIC OXIDE FROM THE RAT STRIATUM *IN VITRO*

M.D. Black & P. P. A. Humphrey. Glaxo Institute of Applied Pharmacology, University of Cambridge, Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1QJ.

Nitric oxide (NO), produced by photoactivation of Roussin's Black salt (RBS), causes dopamine (DA) release from a rat striatal slice *in vitro* (Black *et al.*, 1994). However, the amount of NO released from RBS by light has not been measured. Using a voltammetric electrode sensitive to NO (Linton Instruments), we have now quantified the amounts of NO liberated from RBS by light in concert with measurement of endogenous striatal DA efflux.

Brain slices (350μm) containing the striatum were prepared from male AHA Sprague-Dawley rats (150-200g). Slices were placed in a perspex bath superfused with oxygenated artificial CSF at 32°C (flow rate = 1.2ml min^{-1}). DA efflux was monitored using fast cyclic voltammetry as described by Palij and colleagues (1990). RBS (30μM) was added to the perfusion medium for 30min and then washed off for 30min before photoactivation was commenced. The experiments were performed under darkened conditions throughout. Each electrode was calibrated on each experimental day (the DA electrode with 300nM DA and the NO electrode with 1.6μM NO).

Illumination of brain slices before RBS treatment had no effect on dopamine or NO measurements. After 30min washout of the RBS, the brain slice was illuminated with three increasing intensities of white light (159, 1370 and 4040 Lux). This caused a graded and intensity dependent increase in the DA and the NO signal. Both the DA and NO signal took approx. 1-2min to reach a plateau at each light intensity; upon cessation of the illumination both signals decreased (DA invariably below the original baseline while NO returned to its pre-illumination levels). The reproducibility of the light intensity related increases in DA and NO were determined every 30min on seven occasions (see Figure). It can be seen that over this period the amounts of DA released by illumination decreased somewhat, this may be due to depletion of neuronal stores. Whereas the amounts of NO released stayed relatively constant; this suggests that only a small proportion of the total RBS within of the brain slice is photolysed upon each illumination.



Histograms showing the increases in DA and NO during illumination of brain slices, pre-treated with RBS, every 30min at three increasing light intensities (N=5).

We conclude that RBS treatment of brain slices and subsequent photoactivation is a reliable, quantitative method of administering NO to brain tissue *in vitro*. The levels of NO required to elicit DA release are in the high nanomolar/ low micromolar range.

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279P CHRONIC CLONIDINE ADMINISTRATION-INDUCED BEHAVIOURAL HYPERACTIVITY: EFFECTS OF IMIPRAMINE, PROPRANOLOL AND DIAZEPAM

N. Enginar, H. Koyuncuoglu, I. Hatipoglu, R. Disci# & L. Eroglu. Dept of Pharmacology & #Dept. of Public Health, Istanbul Faculty of Medicine, University of Istanbul, 34390, Capa, Istanbul, Turkey.

The addition of clonidine to drinking water for 13 days has been found to produce depression-like behaviour in rats (Hoffmann & Weiss, 1986; Rosenwasser, 1989). Being easier and less stressful than repeated injections (Enginar & Eroglu, 1990), this treatment seems more suitable for producing a clonidine-induced depression model in rats. However, in our preliminary studies, rats receiving clonidine in their drinking water for 13 days appeared agitated and with increased motor activity. Using the same dosing regimen, we investigated the effect of chronic clonidine on immobility in the forced swimming test and exploratory activity in the hole board, and evaluated the influence of imipramine, diazepam and propranolol on clonidine-induced behavioural changes in rats.

Rats were divided into 4 groups: control and clonidine for Experiment 1 and control and clonidine for Experiment 2. The rats separated for Experiment 1 were subjected to a pre-test session of the forced swimming test. Twenty-four hours later, clonidine administration was started. Clonidine groups received clonidine in their drinking water (5 μg/ml); control animals received tap water. On day 14, clonidine administration was stopped. Control and clonidine groups were divided into 5 subgroups: no administration; saline (1 ml/kg); imipramine (30 mg/kg); diazepam (1 mg/kg); and propranolol (5 mg/kg). Behavioural tests were carried out 1 h after the IP injections. In Experiment 1, animals (n = 7-10) were subjected to forced swimming test for the measurement of total duration of immobility for 5 min. In Experiment 2, animals (n = 5-8) were tested for exploratory activity. Over a period of 5 min, the number of head dips, areas crossed and defecation were counted.

The animals in the clonidine groups showed a significant reduction (245 ± 6.1 vs 289 ± 4.1 s; $p<0.020$ in the total duration of immobility and a significant increase in the number of areas crossed (37 ± 8.0 vs 13 ± 3.9; $p<0.05$) and head dips (15 ± 2.8 vs 4 ± 1.2; $p < 0.02$). The clonidine-induced anti-immobility effect was significantly intensified (205 ± 10.5 vs 120 ± 18.7 s; $p<0.001$) and attenuated (205 ± 10.5 vs 264 ± 9.2 s; $p<0.01$) by imipramine and propranolol treatment respectively. These drugs showed no significant difference in clonidine-induced exploratory activity changes. Diazepam was ineffective in changing the effects of clonidine in both tests.

The results appear not to confirm the putative depressogenic action of clonidine, and demonstrate that the chronic treatment schedule produces a hyperactivity syndrome in rats which could be partly reversed by propranolol.

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280P EFFECTS OF VARIOUS DOSES OF CLONIDINE ON PENTOBARBITAL SLEEP AND PENTYLENETETRAZOL CONVULSIONS IN MICE

N. Enginar & H. Koyuncuoglu. Department of Pharmacology, Istanbul Faculty of Medicine, University of Istanbul, Capa 34390, Istanbul, Turkey.

Behaviourally, clonidine displays sedative, anxiolytic, hypnotic and anaesthetic effects, all of which have been suggested to be mediated by α-adrenoceptors (Nichols & Ruffolo, 1991). However, in addition to our findings (Enginar *et al*, 1994), some reports (van der Poel *et al.*, 1991) imply that clonidine may induce anxiety and aggression. The discrepancy between these findings presumably depends upon the doses used, since clonidine's effects on pre- and post-synaptic α-adrenoceptors may show dose dependency (Anden *et al.*, 1976). Thus, for the evaluation of the relationship between the dose and the paradoxical central effects, we investigated the effects of various doses of clonidine on pentobarbital-induced sleep and pentylene-tetrazol-induced convulsions in mice.

Mice were pre-treated with clonidine HCl (0.01, 0.1, 1, 10, 15, 20 or 50 mg/kg i.p.) or saline 10 min before sodium pentobarbital (25 or 35 mg/kg i.p.; n = 7-13) or pentylene-tetrazol (40 or 60 mg/kg s.c.; n = 5-10), respectively. Following the pentobarbital injection, sleep latency and sleeping time were measured and significant differences determined using Student's t-test. After the pentylenetetrazol injection, mice were observed over the following h for the incidence and onset of clonic convulsions. Significant differences were determined using X^2 test.

Clonidine at a dose of 1 and 0.1 mg/kg increased sleeping time significantly (38 ± 6.5 to 73 ± 12.1 min; $p<0.05$ and 38 ± 6.5 to 95 ± 9.7 min; $p<0.001$, respectively) in 35 mg/kg pentobarbital-treated mice. The effects of the other doses of clonidine on both doses of pentobarbital-induced sleep were insignificant. Clonidine at doses of 1 (80%), 10 (75%), 15 (88%), 20 (83%) and 50 (100%) mg/kg increased the incidence of clonic convulsions induced by 40 mg/kg pentylenetetrazol (10%) significantly ($p<0.01$, $p<0.05$, $p<0.01$; $p<0.02$ and $p<0.01$, respectively. Although none of the doses of clonidine affected the incidence of clonic convulsions induced by 60 mg/kg pentylenetetrazol (36%), the number of mice which died during convulsion was significantly higher in the 15 (90%; $p<0.05$) and 50 (100%; $p<0.05$) mg/kg clonidine pre-treated mice. Neither sleep latency nor the time of onset of clonic convulsions was affected by clonidine pretreatment.

These results indicated that clonidine may, in low doses, prolong pentobarbital-induced sleep and, in high doses, potentiate pentylenetetrazol-induced convulsions.

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281P EFFECTS OF CAM1028 ON THE DEVELOPMENT OF TOLERANCE TO THE ATAXIC EFFECTS OF ETHANOL

Wilson, J. and Little, H.J., Pharmacology Department, The Medical School, University Walk, Bristol BS8 1TD.

The CCK_B subtype of receptors for the neuropeptide cholecystokinin (CCK) have been reported to be involved in the processes of drug dependence and adaptations to drugs. The CCK_B antagonist, CI988, antagonised the anxiogenesis and proconvulsant effects of benzodiazepine withdrawal (Singh *et al.*, 1992) and prevented the development of tolerance to morphine antinociception (Xu et al., 1992). The present study investigated the effects of another selective CCK_B antagonist, CAM1028 (PD135,158, see Hughes et al, 1990), on tolerance to the ataxic effects of ethanol.

Male TO mice (25-30g), n=8-10 per treatment group, were administered ethanol by liquid diet. All mice received control diet for 3 days, then 2 days of 3.5% v/v ethanol diet then 5 days of 7% v/v ethanol diet. Control groups were pair-fed a control diet. CAM1028 (0.03-3 mg/kg) was injected, i.p., twice daily (6 p.m. and 12 midnight) for the whole of the 10 day treatment. There were no significant differences ($P > 0.1$, Student's t test and ANOVA) between the groups in the amount of ethanol consumed (in g/kg/day)

Following this chronic treatment schedule, ethanol (2 g/kg) was injected, i.p., 3h after withdrawal (at 8 a.m.) from ethanol. Time (s) spent on the rotorod was recorded every 10 min for 120 min; a cut-off time of 180s was used. Tolerance to the effect of ethanol was observed (control vs. ethanol alone, $P<0.05$, 20-100 min). CAM1028 reduced the tolerance developed to ethanol (see Table) when it was given at doses of 0.03 mg/kg ($P<0.05$, 20-50 min) or 0.1 mg/kg ($P<0.05$, 20-30 min) and, to a very small extent, with the 0.3 mg/kg dose ($P<0.05$, 20 min). After 120 min, there were no differences between these treatment groups. At doses of 1 and 3 mg/kg, however, CAM1028, had no effect on the development of tolerance. We showed previously that chronic CAM1028 alone, with a control diet, did not alter the effects of ethanol.

Repeated administration of low doses of CAM1028 therefore decreased the tolerance developed to ethanol whilst higher doses had no effect. Further experiments are required to investigate the mechanism of this action.

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CAM1028 mg/kg:-	0	0	0.03	0.1	0.3	1	3
Diet:-	Control	Ethanol	Ethanol	Ethanol	Ethanol	Ethanol	Ethanol
10 inin	2.9 ± 0.7	17.3 ± 8.9†	4.9 ± 1.1	3.5 ± 0.7	3.6 ± 1.3	26.8 ± 21.9	27.9 ± 21.9
20 min	2.9 ± 0.9	101.0 ± 31.0††	3.6 ± 1.1*	3.6 ± 2.0*	12.3 ± 7.2*	74.3 ± 28.4	119.1 ± 30.1
30 min	7.0 ± 4.7	120.4 ± 28.1††	16.1 ± 18.8*	27.6 ± 21.8*	88.3 ± 27.6	95.3 ± 29.5	131.1 ± 28.3
40 min	25.4 ± 22.1	133.9 ± 22.8††	52.1 ± 23.9*	69.0 ± 26.4	134.4 ± 23.3	113.9 ± 30.7	116.9 ± 29.5
50 min	49.3 ± 28.6	147.8 ± 17.9†	48.6 ± 25.2**	103.9 ± 30.1	164.6 ± 15.4	140.8 ± 26.0	133.3 ± 26.8

Table 1: Data are mean ± s.e.m. of time (s) spent on rotorod. Time in min is that after acute ethanol injection (2 g/kg). †$P<0.05$, ††$P<0.01$ cf. controls; *$P<0.05$, **$P<0.01$ cf. ethanol alone. Comparisons by the Mann-Whitney U-test.

282P CARDIOVASCULAR EFFECTS OF ANXIOGENIC AND ANXIOLYTIC AGENTS IN CONSCIOUS RATS

J.K. Webb, S. Boyce, and N.M.J. Rupniak, Neuroscience Research Centre, Merck Sharp & Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

Intravenous (i.v.) injection of CCK-B receptor agonists CCK-4 or pentagastrin provokes panic-like attacks in man (Bradwejn *et al* 1990; Abelson *et al*, 1991). This suggests that CCK-B receptor antagonists may be useful in treating anxiety or panic disorders. However, such compounds fail to exhibit consistent anxiolytic profiles in most recognised behavioural screens in rodents. In man, CCK-4 induced panic attacks are associated with a transient elevation in blood pressure. We now examine whether this physiological response can be provoked by anxiogenic agents in the conscious rat, and whether this response can be inhibited by the CCK-B receptor antagonist CI-988 and the benzodiazepine receptor agonist chlordiazepoxide (CDP).

The caudal vein and artery of Sprague-Dawley rats were cannulated under isoflurane anaesthesia. Animals were placed in commercial restrainers and allowed to recover for 1h. Blood pressure was monitored continuously using a six channel Graph-Tech Linearcorder. Dose response curves to pentagastrin (0.1-100µg/kg) or to the β-carboline FG 7142 (0.001-3mg/kg) were constructed by cumulative i.v. dosing every 10 min in the same animal. The ability of CI-988 (0.5-3mg/kg) and CDP (3 or 10mg/kg), administered i.v. 15 min previously, to inhibit pentagastrin or FG 7142-induced pressor responses was examined using separate animals. Data were subjected to ANOVA and Dunnett's t-tests where appropriate.

Bolus i.v. injection of either pentagastrin (0.1-100µg/kg) or FG 7142 (0.001-3mg/kg) produced a dose dependent increase in both systolic and diastolic blood pressure. The maximum increases in systolic and diastolic blood pressure over baseline were 18.7 ± 3.72 and 22.0 ± 2.23 mmHg respectively for pentagastrin and 41.25 ± 1.77 and 20.0 ± 1.77 mmHg for FG7142. Administration of CDP (3 or 10 mg/kg i.v.) 15 min before pentagastrin (1 µg/kg) or FG 7142 (0.3 mg/kg) produced a dose-dependent decrease in the pressor response compared to baseline values. Similarly, CI-988 (0.5-3 mg/kg) caused a dose-dependent decrease in the pressor response to pentagastrin, but failed to inhibit the pressor response induced by FG 7142 (Table 1).

Table 1	% of Control Pressor Response	
	Pentagastrin (1 µg/kg)	FG7142 (0.3 mg/kg)
Vehicle	100 ± 8	100 ± 8
CDP (10mg/kg i.v.)	63 ± 7*	36 ± 10*
CI-988 (1mg/kg i.v.)	51 ± 4*	74 ± 15

*$p < 0.05$ compared with vehicle treated animals, Dunnett's t-test (n =3-9).

These findings suggest that cardiovascular measurements in conscious rats may be useful for preclinical evaluation of putative anxiolytic agents. This approach has the advantage of measuring a physiological variable that can be directly compared in man and may help to establish the active dose range for compounds such as CI-988 *in vivo*. The clinical efficacy of CCK-B antagonists in anxiety disorders has not yet been established.

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283P A COMPARISON OF THE EFFECT OF CHLORDIAZEPOXIDE ON SOCIAL COMPETITION IN TRIADS AND DIADS OF RATS

K.L. Woodall, A.M. Domeney[1] & M.E. Kelly, Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, West Yorkshire BD7 1DP and [1]Battelle Europe, 7 Route de Drize, CH 1227 Carouge, Geneve.

Previous research has demonstrated that dominant-subordinate relationships measured in small groups of rats competing for palatable substances can be altered by administration of a number of pharmacological agents e.g. buspirone, chlordiazepoxide (cdp) and p-chlorophenylalanine (Gentsch *et al.*, 1990; Joly & Sanger, 1991; Kostowski *et al.*, 1984). Recently it has been suggested that social competition in groups of rats is associated with different levels of fear and therefore could be a suitable animal model of anxiety (Joly & Sanger, 1991). The purpose of this study was to compare the development and stability of a social hierarchy in diads of rats with social hierarchies in triads of rats. In addition the effect of cdp on the dominant-subordinate relationship in both diads and triads of rats was determined to investigate whether the size of the social group has any effect on the outcome of the drug treatment.

Baseline levels of competition were established for a 6 week period during 5 min access to sweetened milk in diads (n=7) and triads (n=9) of male Lister Hooded rats weighing 380±12g at the start of the study. For each group the social hierarchy was established by observing which animal had access to the spout at 5s intervals throughout the testing period (e.g. 60 observations for one 5 min trial). All groups were tested twice a week. The effect of cdp (1.25, 2.5 and 5.0 mg/kg i.p.) on the access to sweetened milk was assessed 40 min after treatment of either the dominant or subordinate rat. Data were converted to % drinking for each rat in each group and analysed using one factor ANOVA followed by Dunnett's t-test.

In the diads the dominant and subordinate rat had access to the drinking spout for 56.3±2.3% and 43.7±1.8% of the 5 min testing period respectively. In the triads the dominant, intermediate and subordinate rat had access to the drinking spout for 44.5±2.2%, 34.9±1.4% and 20.6±1.4% of the 5 min testing period respectively. Administration of cdp to the subordinate animals in diads of rats significantly increased access to the sweetened milk at 2.5 ($P<0.01$) and 5.0 ($P<0.05$) mg/kg i.p. compared to saline injected controls, 43.7±1.8% increased to 52.8±3.7% and 55.5±2.6% respectively. Administration of cdp to subordinate animals in triads of rats significantly increased access to the sweetened milk at 2.5 ($P<0.001$) and 5.0 ($P<0.05$) mg/kg i.p. compared to saline injected controls, 20.2±2.3% increased to 35.9±2.1% and 29.7±4.6% respectively. Administration of cdp to dominant animals in either diads or triads had no significant effect at any of the doses tested.

These results show that stable hierarchies measured in terms of access to sweetened milk can be demonstrated in both diads and triads of male Lister Hooded rats. The size of the social group did not appear to influence the increase in social competition seen in subordinate rats or the lack of effect seen in dominant rats following treatment with cdp.

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284P THE DIOXAPHENALENE DERIVATIVE BARAKOL EX-TRACTED FROM *CASSIA SIAMEA*: A NATURAL ANXIOLYTIC?

W. Thongsaard[1,3], C. Deachapunya[1], S. Pongsakorn[1], E.A. Boyd[2], G.W. Bennett[3] & C.A. Marsden[3], [1]Dept of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand. [2]Dept of Pharmaceutical Sciences, Nottingham University, University Park, Nottingham, NG7 2RD. [3]Dept of Physiology & Pharmacology, Queen's Medical Centre, Nottingham University, Nottingham, NG7 2UH

Barakol, 3a,4-dihydro-3a,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene (Bycroft *et al.*, 1970), is an active ingredient in the alcoholic extract of *Cassia siamea*, a plant used in Thai traditional medicine. It was shown to have dopamine agonist effects, to increase nociceptive threshold and suppress serotonergic system activity by decreasing head shake behaviour induced by 5-hydroxytryptophane (Tongroach *et al.*, 1992). The present study examined the behavioural effects of an extract of barakol in rats in comparison with diazepam using the elevated plus maze; a behavioural test for anxiolytic drugs (File, 1987).

The study consisted of two experiments both performed using male Wistar rats (150-170 g). Firstly, a range of doses of barakol (10, 25, 50, 75 mg/kg i.p., 30 min before testing) were examined for their effects on behaviour on the plus maze. The second experiment compared the effects of barakol (10 mg/kg i.p.) with diazepam (1 mg/kg i.p. 30 min before testing) and a combination of these two when diazepam was given 30 min before barakol and behaviour on the plus maze tested 30 min after barakol administration. Both barakol and diazepam were dissolved in 0.9% saline.

Barakol (10 mg/kg) significantly increased total arm entries and the number of open arm entries and time spent in the open arms ($p<0.05$ n=10 Mann Whitney) on the plus maze. In contrast, barakol (25 and 50 mg/kg) had no effect on total arm entries but significantly increased ($p<0.05$ n=10) both the number of open arm entries and the time in the open arms.

Barakol (10 mg/kg) produced a marked increase in the number of rears (control = 2.0 ± 0.3, barakol 27 ± 1.2), while 25 (3.9 ± 0.4) and 50 mg (3.2 ± 0.7) produced much smaller increases. At 75 mg/kg barakol had no significant effect on any of the parameters measured in the plus maze. In the second experiment, diazepam (1 mg/kg) produced a similar behavioural profile to barakol except that it did not increase rearing (Fig 1). Diazepam was given 30 min before barakol, the effects on plus maze behaviour were similar to the two drugs given alone except that there was a significant decrease in the number of rears compared to barakol alone (Fig 1).

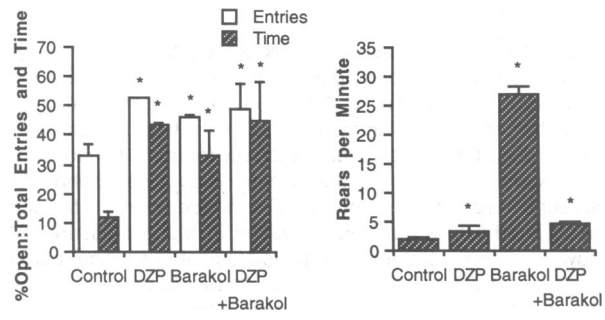


Fig 1. Effect of barakol (10 mg/kg) and diazepam (1 mg/kg) alone and in combination on open arm entries and rearing on the plus maze. *p<0.05 Mann Whitney U test, n=10 for each group.

The results indicate that barakol has an anxiolytic profile but the mechanisms involved are unknown. Barakol, however, differs from diazepam in that it increases arousal and exploration (increase rears and total arm entries) which may be due to agonist effects at dopamine receptors. At a high dose of barakol (75 mg/kg i.p.) the anxiolytic and arousal properties were lost, indicating other pharmacological actions come into play which need to be identified.

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285P INFLUENCE OF *TILIA EUROPAE* EXTRACTS ON $GABA_A$ RECEPTOR

C. Cavadas[1]; C. A. Fontes Ribeiro[2]; I. Araújo[1]; P. J. Isabel[3]; M. S. Santos[4]; A. Proença da Cunha[3]; T. R. A. Macedo[2]; M. M. Caramona & M. D. Cotrim[1,2] - [1]Laboratório de Farmacologia da Faculdade de Farmácia; [2]Departamento de Farmacologia (IBILI); [3]Laboratório de Farmacognosia da Faculdade de Farmácia; [4]Centro de Neurociências. Universidade de Coimbra, 3000 Coimbra - PORTUGAL

The extracts or infusions obtained from inflorescences of *Tilia europeae* have been used in popular medicine by reason of their sedative/anxiolytic properties, but its mechanism of action is not known. Since centrally-active $GABA_A$ receptor agonists and modulators can produce sedation we have examined the possible interaction of tilia with the $GABA_A$ receptor complex in rat brain.

In order to test tilia extract affinity for the GABA binding site in $GABA_A$ receptor-complex, postsynaptic membranes of rat cortices were isolated (Zunkin *et al.*, 1974) and competition curves with [^{3}H]muscimol were performed using a centrifugation technique (Bernasconi *et al.*, 1980) .

To study the influence of the extract on $^{36}Cl^-$ fluxes, synaptoneurosomes were isolated (DeLorey & Brown, 1992) and $^{36}Cl^-$ uptake determinated (Schwartz *et al.*, 1986). Aqueous extract from *Tilia europeae* inflorescences was prepared and padronized by determining the concentration of rutin and caffeic acid.

Tilia extracts displaced [^{3}H]muscimol bound to postsynaptic membranes of rat cortices (IC_{50}=2.7 ± 0.3 µg/ml) and stimulated the $^{36}Cl^-$ uptake by synaptoneurosomes (9 mg/ml increased 120 ± 25 nmol $^{36}Cl^-$ /mg protein; in the presence of 1 mM picrotoxin this value was 18 ± 6nmol $^{36}Cl^-$ /mg protein).

The amino acids content of tilia extract was determinated by HPLC in a Gilson-Asted system, being several amino acids detected, including GABA. The GABA content of tilia extract (about 100 µM), probably responsible for the displacement of the [^{3}H]muscimol in post-synaptic membranes, can not explain its anxiolytic/sedative effects since this amino acid does not significantly cross the brain blood barrier.

Therefore, the sedative/anxiolytic effect of tilia extract has a mechanism of action still unclear.

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286P THE POTENTIAL ANXIOLYTIC ACTIVITY OF *TILIA EUROPAE* AQUEOUS EXTRACT IN MICE

I V Figueiredo[1], M D Cotrim[1,2], C Cavadas[1], C A Fontes Ribeiro[2], P J Isabel[3], A Proença da Cunha[3], M M Caramona[1,2], T R A Macedo[2] [1]Laboratório de Farmacologia; [3]Laboratório de Farmacognosia - Faculdade de Farmácia. [2]Instituto Biomédico de Investigação da Luz e da Imagem. Universidade de Coimbra
3000 Coimbra, Portugal

Tilia europeae is popularly used for the treatment of symptoms of anxiety, migraine, nervousness and insomnia (J. Duke, Handbook of Medicinal Herbes, 1985). These uses resemble those of the hypnotic-sedative drugs.

This study was undertaken to determine the action of *Tilia europeae* on the central nervous system and the possible sedative, anxiolytic and sleep-inducing properties in male albino mice. With this aim, we have observed the behaviour of mice confronted with some experimental situations. The sedative properties have been checked by measuring curiosity (wood slabe containing 16 inspection holes, 3 cm diameter, spaced 5 cm between them; this wood slabe is placed 50 cm above the floor; we measured the number of head dips during 5 min.) and equilibrium (Rota-rod test) behaviour of mice. Sleep-induction has been studied in mice treated with a hypnotic dose of pentobarbital (30 mg/kg) (Jones *et al.*, 1987; Maluf *et al.*, 1991; Soulimani *et al.*, 1991).

The tilia aqueous extracts (3%) were prepared from powdered dessicated inflorescences gathered from Jardim Botânico (Coimbra) and administered by the oral route (10 ml/kg).

We obtained the following results: 1) Tilia decreased the spontaneous curiosity of mice (53.0 ± 3.8 % of reduction; $p< 0.05$, n=35) compared with controls (n=22), 45 min after oral administration. Tilia extracts did not modify the behaviour of mice (n=13) on the Rota-rod, 45 min after administration. 3) The pentobarbital sleeping time of mice treated with aqueous extract of tilia was significantly different ($p< 0.05$) from that of controls (46.6±2.6 min, n=24, and 36.9±2.4 min, n=31, respectively). Significant differences in pentobarbital sleeping time ($p< 0.05$) were detected between the control group (36.9±2.4 min) and the group receiving 0.25 mg/kg diazepam (72.7±3.8 min., n=24).

Tilia extracts showed possible sedative activity but more studies are needed to characterise the pharmacological actions on the CNS of this medicinal plant.

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287P EFFECTS OF *TILIA EUROPAE* ON GUINEA-PIG ILEUM AND AORTA

M D Cotrim[1,2], I V Figueiredo[1], C Cavadas[1],C A Fontes Ribeiro[2], P J Isabel[3], A Proença da Cunha[3], M M Caramona[1,2], T R A Macedo[2] [1]Laboratório de Farmacologia; [3]Laboratório de Farmacognosia - Faculdade de Farmácia. [2]Instituto Biomédico de Investigação da Luz e da Imagem. Universidade de Coimbra 3000 Coimbra, Portugal

Traditional medicine is an important source of new drugs with therapeutic applications. However, most of the effects of such drugs have not yet undergone analysis by experimentation. The use of medicinal plants as teas or decoctions for therapeutic purposes is a common practice in Portugal.

This work aimed to study the influence of *Tilia europeae* extracts on the guinea-pig ileal and aortic contractions induced by several agonists *in vitro* . Aqueous extract from *Tilia europeae* inflorescences was prepared according to C.Cavadas *et al.*, 1994, P118, in this Meeting. A segment of the ileum was removed from male guinea-pigs which were killed by cervical dislocation and cut into 2.5-2 cm long sections. The ileal portions were placed in organ baths containing Krebs solution, aerated with 95% O_2 / 5% CO_2 and maintained at 37 °C. After an equilibration period of 60 min, a concentration-response (c-r) curve to tilia extracts (0.2-1.8 mg/ml), acetylcholine, histamine or 5-hydroxytryptamine (5-HT) (6 nM-0.3 mM) was performed; then, after several washings tilia extract (0.2-1.8 mg/ml) or the solvent (controls) or atropine (0.07 μM) were added and a second c-r curve performed. Meanwhile, some of the experiments with 5-HT were performed in the presence of 0.1 μM ketanserin. From the same animal the aorta was removed and cut into rings; cumulative c-r curves were then constructed to noradrenaline (NA, 6 nM-130 mM), before and after tilia extracts.

The following results were obtained: 1) Tilia extracts induced contractions which were inhibited by atropine; 2) Acetylcholine or histamine-induced contractions were not significantly affected by tilia; 3) 5-HT was non-competitively antagonized, in a concentration-dependent way, by tilia extracts (related to controls, and in the absence of ketanserin, the maximal effect (E_{max}) obtained in the presence of 0.2, 0.6 or 1.8 mg/ml tilia was 80±10.5, 52.5±15.3 or 37.3±10.4 %, respectively; with ketanserin the E_{max} obtained in the presence of 0.7 mg/ml tilia was 51.1±12 %); 4) Noradrenaline-induced contractions in guinea-pig aorta rings were not affected by tilia.

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288P ANTICONVULSANT EFFECTS OF THE IMIDAZOQUINAZOLINES, NNC 14-0185 AND NNC 14-0189: COMPARISON WITH DIAZEPAM

H.C. Jackson, H.C. Hansen, M. Kristiansen, P.D. Suzdak, M.E. Judge & M.D.B. Swedberg, Novo Nordisk A/S, Novo Nordisk Park, Måløv, DK-2760, Denmark

NNC 14-0185 (3-(3-cyclopropyl-5-isoxazolyl)-6-fluoro-5-morpholino-imidazo[1,5-a]quinazoline) and NNC 14-0189 (3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-7-fluoro-5-(4-methyl-1-piperazinyl)-imidazo[1,5-a] quinazoline) potently displace [^{3}H]flunitrazepam from its binding sites in bovine cortex (IC50 5.6 and 7.9 nM respectively compared with 11 nM for diazepam). In this study we have assessed the anticonvulsant and muscle relaxant effects of NNC 14-0185 and NNC 14-0189 in mice and compared them with those of diazepam.

Subjects were male NMRI mice (20±2g). Treatment groups contained 8-10 animals. In the first experiment, mice were injected i.p. with vehicle or different doses of the test drug. Thirty min later animals were treated with pentylenetetrazol (PTZ) 160 mg/kg s.c. and observed for the presence of clonic seizures for 15 min. Rotarod performance (the number of animals in each group having more than 2 falls from a rotating rod in a 2 min period) was assessed immediately before seizure testing. ED50 values (the dose of drug preventing seizures or causing rotarod impairment in 50% of the animals) and 95% confidence levels were determined and used to calculate therapeutic indices (TI; rotarod ED50 / anticonvulsant ED50). In the second experiment, mice were treated with vehicle or the benzodiazepine receptor antagonist flumazenil (10 mg/kg i.p.) and injected concurrently with either NNC 14-0185 (10 mg/kg i.p.) or NNC 14-0189 (10 mg/kg i.p.). Clonic seizure thresholds (mg/kg i.v.; means ± s.e.mean) were determined 15 min later by infusion of PTZ (15 mg/ml; 12 ml/h) through the tail vein. In the third experiment, the development of tolerance to the anticonvulsant effects of NNC 14-0185, NNC 14-0189 and diazepam was investigated. Mice were treated twice a day for 3 days with vehicle or test drug (10 mg/kg i.p.). On day 4 mice were injected with drug (10 mg/kg i.p.) and 30 min later clonic seizure thresholds determined using the PTZ infusion test.

The ED50 values for NNC 14-0185, NNC 14-0189 and diazepam for protection against the clonic seizures induced by PTZ s.c. were 3.6 (2.0-6.4); 3.1 (1.4-6.7); and 0.8 (0.6-1.2) mg/kg i.p. respectively. The corresponding ED50 values for impairment of rotarod performance were 35 (11-1631); 56 (31-135) and 1.3 (0.5-4.5) mg/kg i.p. Hence NNC 14-0185 and NNC 14-0189 potently prevented PTZ-induced seizures. Both compounds showed a better separation between their anticonvulsant and ataxic effects than diazepam (the TI for NNC 14-0185 was 10; the TI for NNC 14-0189 was 18 and the TI for diazepam was 1.6). The anticonvulsant effects of both NNC 14-0185 (10 mg/kg i.p.) and NNC 14-0189 (10 mg/kg i.p.) were significantly (*P<0.05; Dunnetts test) blocked by flumazenil (10 mg/kg i.p.). Clonic seizure thresholds mg/kg PTZ i.v. were: NNC 14-0185 + vehicle 268 ± 21; NNC 14-0185 + flumazenil 152 ± 15; NNC 14-0189 + vehicle 305 ± 25; NNC 14-0189 + flumazenil 121 ± 26. Flumazenil alone did not alter PTZ seizure thresholds (flumazenil 77 ± 6; vehicle 63 ± 6). Tolerance did not develop to the anticonvulsant effects of NNC 14-0185 10 mg/kg i.p. (clonic seizure thresholds mg/kg PTZ i.v.: acute 200 ± 29; chronic 219 ± 17) or NNC 14-0189 10 mg/kg i.p. (seizure thresholds mg/kg PTZ i.v.: acute 228 ± 22; chronic 224 ± 22). In comparison, the anticonvulsant effects of diazepam 10 mg/kg i.p. were significantly reduced in mice treated twice a day with diazepam (10 mg/kg) for 3 days (seizure thresholds mg/kg PTZ i.v.: acute 219 ± 14, chronic 159 ± 8; *P<0.05, Dunnetts test).

In conclusion, NNC 14-0185 and NNC 14-0189 are potent anticonvulsant agents in mice. These effects appear to be mediated, at least in part, by benzodiazepine receptors. Both NNC 14-0185 and NNC 14-0189 have some advantages over diazepam in that they are less likely to produce muscle relaxation at anticonvulsant doses and that tolerance did not appear to their anticonvulsant effects in a 4-day test. Further studies are required to assess the potential use of these compounds in the treatment of epilepsy.

289P FACILITATION OF MUSCLE FUNCTION BY THE BENZODIAZEPINE ALPRAZOLAM

Z.Yu, R.S. Leeuwin and H. van Wilgenburg, Department of Pharmacology, University of Amsterdam, Academic Medical Centre, Amsterdam, The Netherlands.

Although neuromuscular actions (usually myorelaxation) of the benzodiazepines are well known, such responses have not been described for alprazolam, marketed in the 1980's for the treatment of anxiety associated with depression. It has long been assumed, by general consensus, that the site of these responses is located in the CNS (Harvey, 1985), although studies from our group have revealed direct peripheral effects of benzodiazepines on muscle function (Duval et al., 1991). This study is a first attempt to map out neuromuscular actions of alprazolam.

Isolated phrenic nerve-diaphragms from female rats (160-180g), killed by decapitation, were used. They were placed in organ vessels containing Krebs-Ringer solution (37°C) and gassed with 5% CO_2 in O_2. Preparations were stimulated with supramaximal rectangular pulses. Stimulation frequencies were: 0.1 Hz (twitch) during 5 min, and 10 to 50 Hz (tetanus) during 3 sec with intervals of 20 sec. Both indirect (0.2 msec width) or direct (2.0 msec width) pulses were applied. In the latter case transmission was blocked using pancuronium. Alprazolam, bath concentration $2.7x10^{-5}$ to $2.0x10^{-4}$M dissolved in 96 % ethanol, was added to the bath non-cumulatively according to a Latin Square. Contractions were measured isometrically. Each concentration was examined using 6 preparations. Effects were expressed as percentage area under the curve of control. Estimated Emax's were obtained using Scott plots and linear regression. Equilibration dissociation constant (K) and Hill coefficient (Hn) were calculated using Hill plot graphing.

Tetanic contractions were potentiated by alprazolam both after indirect and direct stimulation, which was related to the concentration at 10-30 Hz ($P<0.05$), but not at 40-50 Hz. Emax's after indirect stimulation were 435 ± 33.6; 748 ± 57.5; 541 ± 30.4; 239 ± 8.0; 136 ± 3.7 and after direct stimulation 302 ± 21.9; 825 ± 103; 466 ± 22.1; 175 ± 7.9; 107 ± 5.2 (average ± SEM; respectively, at 10, 20, 30, 40 and 50 Hz). At frequencies higher than 20 Hz the initial enhancement decreased and the dose-response relationship abated. The K-values at 10, 20 and 30 Hz were frequency-dependent as well. K and Emax after indirect stimulation differed significantly ($P<0.01$) from those after direct stimulation except at 20Hz. The pooled Hn was 0.96 ± 0.15.

In conclusion, the results suggest that the benzodiazepine derivative alprazolam affects muscle function, i.e. it produces potentiation of the tetanic contractions, in contrast to other benzodiazepines (e.g. diazepam), eliciting inhibitory responses as well. This potentiating response is frequency- as well as concentration dependent. The phrenic nerve may facilitate this potentiation.

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290P THE ROLE OF AMYGDALA AND HYPOTHALAMUS IN $GABA_A$ RECEPTOR-MEDIATED CARDIOVASCULAR RESPONSES

Z.Gören, K.Berkman, N.Aslan, Ş.Oktay, F.Onat,
University of Marmara, School of Medicine, Department of Pharmacology, Haydarpaşa, 81326, İstanbul, Turkey.

GABA is an inhibitory neurotransmitter in the central nervous system. Besides having a role in neurodegenerative diseases, GABA also plays an important role in central control of cardiovascular functions. GABA exerts its effects via specific receptors termed as $GABA_A$ and $GABA_B$. It has been previously shown that microinjection of drugs that impair GABA mediated synaptic transmission in the CNS generates cardiovascular and behavioral changes. I.c.v. administration of $GABA_A$ antagonist, BMI, elicits marked increases in blood pressure and heart rate due to an increase in sympathetic activity (Di Micco(1979);Gillis(1984). Previous evidence indicates that a forebrain periventricular GABAergic system exerts a tonic inhibitory influence on the sympathetic nervous system.The purpose of this study was to examine the role of unilateral lesion of the central nucleus of amygdala (CeA) and paraventricular (PVN) and dorsomedial (DMH) nuclei of the hypothalamus in BMI-induced cardiovascular responses. A monopolar isolated stainless steel wire electrod was placed into CeA, PVN and DMH, 3-5 days before the experiments. An anodal lesion was made with 2 mA and 1 mA for 20 and 10 sec for the lesioning of the nuclei of amygdala and hypothalamus, respectively. Sham-lesion groups underwent the identical surgical procedure with the exception that no current was passed. After removal of the electrode, the stainless steel guide cannulas were implanted stereotaxically into the lateral cerebral ventricle of sham-lesioned and lesioned rats. Only rats with proper CeA, PVN and DMH lesions and icv cannula placements were included in the study. In conscious unrestrained male Sprague Dawley rats, polyethylene catheters were inserted into the iliac artery to monitor blood pressure (MAP) and heart rate (HR). I.c.v. injection of BMI at the doses of 0.3-0.5 nmol led to dose-dependent increases in MAP and HR. The cardiovascular effects of administration of i.c.v. BMI were significantly reduced by electrolytic ablation of CeA, PVN and DMH. Table I shows the influence of BMI(0.5 nmol/rat) on MAP in sham-lesioned and CeA-, PVN- and DMH-lesioned animals. The responses to BMI in PVN- and DMH-lesioned rats were lower than those of CeA-lesioned animals. These results indicate that hypothalamus and to a lesser extent amygdala are both involved in GABAergic control of the cardiovascular function.

Table I. the influence of BMI(0.5 nmol/rat) on MAP

Time(min)	0	1	3	5	10	30
Sham	96±1.5	108±3.6	111±2.5	109±2.7	103±3	96±2
CeA	100±4.8	102±2.8	106±2.5	103±2.5	99±3	99±3
PVN	98±3.3	101±3	103±3.4	104±4.1	104±3	99±2
DMH	97±4.4	103±4.6	98±4.2	99±7	101±7	101±6

Responses in CeA-, PVN- or DMH-lessioned groups were statistically different than those of the sham-lesioned animals (two-way ANOVA).

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291P A FUNCTIONAL COMPARISON OF THE ANTAGONISTS BICUCULLINE AND PICROTOXIN AT RECOMBINANT MURINE GABA$_A$ RECEPTORS

B.J. Krishek & T.G. Smart, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

Mammalian γ-aminobutyric acid (GABA$_A$) receptors are characterised by the possession of a variety of allosteric sites from which a number of agents can modulate receptor function. Previous studies performed with recombinant GABA$_A$ receptors have established that some pharmacological properties are critically dependent on the presence of a γ subunit in the receptor complex, e.g., inhibition by Zn^{2+} or enhancement by benzodiazepines (Smart *et al.*, 1994). The aim of this study was to examine whether the inhibitory action of the classical GABA antagonists, bicuculline and picrotoxin was similarly dependent on the receptor subunit composition. *Xenopus laevis* oocytes were injected with cDNAs encoding for α1β1, α1β1γ2S or α1β1γ2L polypeptide subunits and studied using a two-electrode voltage clamp recording technique.

Equilibrium concentration response curves for GABA were constructed for each receptor construct. Increasing the bicuculline concentration (5 - 100μM) laterally shifted the curves in a parallel manner with an unaltered maximum response, compatible with a competitive mode of antagonism. The GABA EC_{50}s were correspondingly increased in the presence of bicuculline by: α1β1 (Control, 5.87 ± 0.7μM; +100μM bicuculline, 356 ± 21μM); α1β1γ2S (Control, 10 ± 0.2μM, +100μM bicuculline, 554 ± 16μM); α1β1γ2L (Control, 10.2 ± 0.5μM; +50μM bicuculline, 320 ± 38μM). The affinity of bicuculline was determined using the Schild equation. The slopes of the Schild plot were not significantly different from unity and thus pK_B values for bicuculline on each receptor construct were determined as (± 95% confidence limits, n=3): α1β1 (5.87 ± 0.24); α1β1γ2S (5.96 ± 0.21); α1β1γ2L (5.99 ± 0.23). The application of 0.01-100μM picrotoxin to recombinant GABA$_A$ receptors caused a lateral shift in the concentration response curve with a concomitant reduction in the maximum response to GABA. This type of antagonism is in accordance with a mixed inhibitory mechanism. The apparent dissociation constants (K_d) for the antagonism of a response to 10 μM GABA by picrotoxin were determined in the three GABA$_A$ receptor constructs (n=4): α1β1 (0.55 ± 0.03μM); α1β1γ2S (0.64 ± 0.1μM); α1β1γ2L (0.46 ± 0.1μM).

The apparent pK_B values for bicuculline and the K_ds for picrotoxin were relatively unaffected by the GABA$_A$ receptor isoforms used in this study. Unlike Zn^{2+} or benzodiazepines, both bicuculline and picrotoxin were particularly unaffected by the absence of the γ2 subunit in the α1β1 construct. In comparison, it is probably not surprising that pA_2 values for bicuculline of 5.93 (Simmonds, 1982) and 6.1 (Kemp *et al.*, 1986) have been reported using native neuronal GABA$_A$ receptors which are likely to be representative of heterogenous GABA$_A$ receptor populations.

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292P NEUROSTEROIDS ENHANCE MUSCIMOL AND ADENOSINE POTENTIATION IN THE PRESENCE OF BARIUM

S. Akhondzadeh & T.W. Stone, Department of Pharmacology, University of Glasgow, G12 8QQ, Scotland.

We have previously shown that the effect of muscimol on population spike size was enhanced by adenosine while the adenosine activated potassium channel was blocked by barium 1 mM (Akhondzadeh & Stone, 1994a). In addition to this, DIDS (4,4-diisothiocyanatostilbene-2,2'-disulfonic acid, disodium salt) a chloride channel blocker (Miller & White, 1984) and CPT (cyclopentyltheophylline), an adenosine A_1 receptor antagonist, decreased this potentiative interaction (Akhondzadeh & Stone, 1994b). We now report a study of the effect of some neurosteroids (alphaxalone, 5α-pregnan-3α-ol-20-one) that have positive modulatory effects on the GABA$_A$ receptor (Miller, 1991) on the adenosine inhibitory response and on the interaction between adenosine and muscimol on the spike size.

Hippocampal slices 450 μm thick were prepared from male Wistar rats (170-210 g) and were superfused at 30°C with ACSF (in mM : KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, Glucose 10, $MgCl_2$ 1.2 saturated with 95% O_2/ 5% CO_2). Extracellular recordings of orthodromic potentials were made in the CA1 pyramidal cell layer following stimulation of Schaffer collateral fibres in the stratum radiatum.

Adenosine 2 μM reduced spike amplitude by 12.58% ± 0.86 (mean ± s.e.m., P≤0.001, n=4, Student's t-test) and alphaxalone 1 and 5 μM decreased the population spike by 0.65% ± 0.27, (ns, n=4) and 8.93 ± 0.42 (P≤0.001, n=4) respectively. In the presence of barium 1 mM adenosine was ineffective while alphaxalone 5 μM reduced the spike size by 9% ± 0.37 (P≤0.001, n=4). The combination of adenosine 2 μM with alphaxalone 1 and 5 μM in the presence of barium reduced the spike size by 5.90% ± 1.16 (ns, n=4) and 20.13% ± 1.17 (P≤0.01, n=4) respectively. The same experiment was performed using 5α-pregnan-3α-ol-20-one 1 and 5 μM reduced the spike size by 2.5% ± 0.89(ns, n=4) and 11% ± 1.1 (P≤0.001, n=4).The combination of adenosine 2 μM and 5α-pregnan-3α-ol-20-one 1 and 5 μM in the presence of barium reduced the spike size by 6.10% ± 0.77 (ns, n=4) and 23.20% ± 1.11 (P≤0.01, n=4). In another set of experiments, in the presence of barium, muscimol 1 μM reduced the spike amplitude by 13.32% ± 0.71 (P≤0.001, n=4) while adenosine 2 μM was ineffective. The combination of muscimol 1 μM with adenosine 2 μM reduced the spike size by 29.73% ± 0.83 (P≤0.001, n=4). This reduction was increased in alphaxalone 1 and 5 μM to 39% ± 1.29(P≤0.01, n=4) and 49.50% ± 1.04 (P≤0.001, n=4). This interaction between adenosine and muscimol on the spike size was also enhanced by 5α-pregnan-3α-ol-20-one 1 and 5 μM to 40% ± 1.87(P≤0.01, n=4) and 50.17% ± 3.02 (P≤0.001, n=4).

The results indicate a potentiative interaction between adenosine, muscimol and neurosteroids which, because it occurs in the presence of the potassium channel blocker barium, may occur at the level of chloride channels.

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293P NEUROSTEROIDS POTENTIATE GABA AND ADENOSINE INHIBITION OF HIPPOCAMPAL EVOKED POTENTIALS

S. Akhondzadeh & T.W. Stone, Department of Pharmacology, University of Glasgow, G12 8QQ, Scotland.

GABA, a major inhibitory neurotransmitter in the vertebrate central nervous system, mediates fast synaptic inhibition through the activation of receptor-linked chloride channels. Certain synthetic and endogenous steroids like alphaxalone and 5α-pregnan-3α-ol-20-one have been reported as positive modulators of $GABA_A$ receptors (Harrison & Simmonds, 1984). They have been shown to have two distinct action on the $GABA_A$ receptor. At low concentrations, they potentiate $GABA_A$ action, while at higher concentrations they can directly open the chloride channel of the $GABA_A$ receptor (Puia et al, 1991). We have previously shown that the effect of muscimol on hippocampal population spike size was enhanced by adenosine while the adenosine activated potassium channel was blocked by barium 1 mM (Akhondzadeh & Stone, 1994). We now report a study of the effect of alphaxalone and 5α-pregnan-3α-ol-20-one as positive modulators of $GABA_A$ with adenosine and muscimol on the spike size.

Hippocampal slices 450 μm thick were prepared from male Wistar rats (170-210 g) and were superfused at 30°C with ACSF (in mM : KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, Glucose 10, $MgCl_2$ 1.2 saturated with 95% O_2/ 5% CO_2). Extracellular recordings of orthodromic potentials were made in the CA1 pyramidal cell layer following stimulation of Schaffer collateral fibres in the stratum radiatum.

Alphaxalone 5 and 10 μM reduced spike amplitude by 8.46% ±0.95 and 16.42% ± 1.06 (mean ± s.e.m., P≤0.001, n=4, Student's t-test; 5α-pregnan-3α-ol-20-on 5 and 10 μM reduced spike size by 10.82% ± 0.63 and 23.08 ± 0.7 respectively (P≤0.001, n=4). Lower concentrations did not reduce the spike size significantly. Muscimol 1 μM reduced the spike size by 13.97% ± 0.65 whereas, adenosine 2 μM reduced it by 12.85% ± 0.53 (P≤0.001, n=4). Alphaxalone 0.5 and 1 μM that had no significant effect on the spike size, potentiated the inhibitory effect of muscimol to 20.82% ± 0.84 (P≤0.001, n=4) and 32.50% ± 1.19 (P≤0.001, n=4); adenosine inhibition was increased to 13.10% ± 0.6(ns) and 19.63 ± 0.63 (P≤0.01, n=4) respectively. In another set of experiments 5α-pregnan-3α-ol-20-one 1 μM enhanced the effect of muscimol 1 μM and adenosine 2 μM to 37.18% ± 1.13(P≤0.001, n=4) and 21.30% ± 1.12 (P≤0.001, n=4) respectively.

The results indicate that at low concentrations these neurosteroids enhance the inhibitory effect of adenosine. This action may be the result of interaction between adenosine and endogenous GABA.

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294P CHOLESTEROL ENRICHMENT OF NEURONAL MEMBRANES ALTERS THE ENHANCEMENT OF [3H]-FLUNITRAZEPAM BINDING BY PREGNANOLONE

P. Bennett & M.A. Simmonds, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

The potentiation of receptor-mediated responses by neurosteroids shows clear structure-activity requirements (Simmonds, 1991) compatible with the idea that there are specific recognition sites for the neurosteroids on the $GABA_A$ channel protein. We are now investigating the possibility that membrane cholesterol might influence the potency of neurosteroids by competing for the same binding sites. This report concerns the enrichment of membranes with cholesterol and associated changes in the effect of pregnanolone (5β-pregnan-3α-ol-20-one) as an enhancer of [^{3}H]-flunitrazepam (FNZ) binding.

Membranes were prepared from the cerebral cortex, cerebellum and spinal cord of adult male Wistar rats. The "buffy coat" fraction (Zukin *et al.*, 1974) was obtained and washed 3 times with a 5 mM Tris-HCl buffer containing 1 mM EDTA at pH 7.4 prior to storage at -20°C. Liposomes containing equal proportions of cholesterol and phosphatidylcholine were prepared by sonication in SET buffer (0.25 mM sucrose, 1 mM EDTA, 50 mM Tris, 0.02% sodium azide, pH 6.9). Cholesterol enrichment of the membranes was achieved by incubation of membranes (0.4 mg protein.ml^{-1}) in SET buffer containing 1% BSA and approximately 0.25 mg total liposome lipids.ml^{-1} for 4 h at 37°C. The membranes were then washed with the binding assay buffer (50 mM Tris, 150 mM NaCl, pH 7.4) at 4°C and frozen until required. Unenriched membranes were subjected to the same procedures with the omission of the liposomes. Membrane cholesterol was assayed by means of a Sigma diagnostics kit and protein was assayed with the Biorad reagent.

Concentration - effect curves were determined for the enhancement of [^{3}H]-FNZ binding to the membranes by pregnanolone. Pregnanolone 30 nM - 10 μM was incubated with washed membranes (0.2 mg protein. ml^{-1}) at 37°C for 10 min. 1 nM [^{3}H]-FNZ was then added and the incubation continued at 4°C for 60 min. The membranes were harvested by filtration and the bound radioactivity determined by liquid scintillation counting. Non-specific binding of 5 - 10 % was determined by addition of 10 μM unlabelled FNZ. Pregnanolone was initially dissolved in DMSO which was present at a final concentration of 0.45% in all assay tubes.

The cholesterol enrichment procedure had no direct effect on the binding of FNZ as determined by the displacement of [^{3}H]-FNZ by unlabelled FNZ (10 pM - 1 μM). The effects of pregnanolone on [^{3}H]-FNZ binding were, however, changed. In unenriched membranes from all three areas of CNS, the threshold concentrations of pregnanolone for enhancement of [^{3}H]-FNZ binding were about 0.3 μM. In cerebral cortex membranes, enrichment of cholesterol from 2.24 to 4.52 μmol cholesterol.mg protein^{-1} was associated with a 3.2-fold reduction in pregnanolone potency (0.506 ± 0.028 log. units; mean ± s.e.mean from 4 experiments with each pretreatment). In cerebellar membranes, enrichment of cholesterol from 1.95 to 3.62 μmol cholesterol.mg protein^{-1} was associated with no change in pregnanolone potency over most of the concentration range but concentrations of 0.1 and 0.3 μM gave a significant [^{3}H]-FNZ binding enhancement to 108.2 ± 1.4 % of control (mean ± s.e.mean, n=6). In spinal cord membranes, enrichment of cholesterol from 2.63 to 4.22 μmol cholesterol.mg protein^{-1} was associated with an 8.4-fold increase in pregnanolone potency (0.926 ± 0.052 log. units; mean ± s.e.mean from 3 experiments with each pretreatment).

Interpretation of these intriguing observations requires further studies with pure phosphatidylcholine liposomes and information on the disposition of the cholesterol enrichments of the membranes.

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295P IDENTIFICATION OF TWO COMPONENTS IN THE ENHANCEMENT OF [3H]-FLUNITRAZEPAM BINDING BY PREGNANOLONE

Y. Zhong & M.A. Simmonds, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

Early studies of neurosteroid potentiation of $GABA_A$ receptor responses yielded concentration dependencies that could be adequately represented by monophasic curves (e.g. Harrison & Simmonds, 1984). In two studies (Morrow *et al.*, 1990; Puia *et al.*, 1990), however, distinct biphasic neurosteroid concentration dependencies have been reported and in both cases the duration of tissue exposure to the neurosteroid was very short (5 s). We have investigated this two-component phenomenon further in experiments on the enhancement by pregnanolone (5β-pregnan-3α-ol-20-one) of [^{3}H]-flunitrazepam (FNZ) binding to $GABA_A$ receptors.

Membranes were prepared from the whole brains of adult male Wistar rats. A crude membrane fraction (Prince & Simmonds, 1993) was obtained and washed 3 times with a 5 mM Tris-HCl buffer containing 1 mM EDTA at pH 7.4 prior to storage at -20°C. For each binding assay, membranes were thawed and washed twice with distilled water and twice with the assay buffer (50 mM Tris, 150 mM NaCl, pH 7.4) at 4°C. In all experiments, 1 nM [^{3}H]-FNZ was incubated with membranes (0.4 mg protein.ml^{-1}) for 60 min at 4°C. The duration and conditions of exposure to pregnanolone were varied according to the experiment. The membranes were harvested by filtration and the bound radioactivity determined by liquid scintillation counting. Non-specific binding of < 5% was determined by addition of 10 µM unlabelled FNZ. Pregnanolone was initially dissolved in DMSO which was present at a final concentration of 0.45% in all assay tubes. Protein was assayed with the Biorad reagent.

Addition of 10 µM pregnanolone to the assay tubes for 1 to 60 min before the end of the incubation with [^{3}H]-FNZ gave a biphasic time profile for the enhancement of [^{3}H]-FNZ binding. An initial component reached a plateau at 5 and 10 min (8.4 ± 1.76 % increase in binding at 5 min; mean ± s.e.mean, n = 4) and the enhancement of binding then increased again with further exposure to pregnanolone to a second plateau between 30 and 60 min (21.1 ± 2.01 % increase in binding at 60 min; mean ± s.e.mean, n = 4). A concentration - effect relationship for a 5 min exposure to pregnanolone yielded a monophasic curve with a threshold concentration of 0.1 µM, an EC_{50} of 0.55 µM (-6.26 ± 0.18 log. units) and E_{max} of 112.3 ± 1.7 % control (mean ± s.e.mean, n = 6). Inclusion of 60 µM Δ^{16}-alphaxalone in the incubation for 55 min before and for the 5 min during exposure to pregnanolone failed to affect the concentration - effect curve for pregnanolone (log. EC_{50} -6.20 ± 0.31; E_{max} 110.4 ± 2.5 % control; mean ± s.e.mean, n = 4).

In experiments where the membranes were exposed to pregnanolone for the entire 60 min of [^{3}H]-FNZ incubation, a 10 min pre-incubation of the membranes with pregnanolone at 37°C was added since this doubles the enhancements of [^{3}H]-FNZ binding. The pregnanolone concentration - effect relationship determined under these conditions had a threshold of 0.1 µM and increased approximately linearly to a 52.2 ± 2.37 % enhancement of binding (mean ± s.e.mean, n = 8) at 30 µM pregnanolone. Inclusion of 60 µM Δ^{16}-alphaxalone throughout the incubation did not affect the lower part of the curve (up to 1 µM pregnanolone) but shifted the upper part of the curve to the right in a parallel manner by a factor of 1.9 (0.282 ± 0.013 log units; mean ± s.e.mean from 8 experiments for each curve).

These results suggest that pregnanolone has two components of action in enhancing [^{3}H]-FNZ binding to the $GABA_A$ receptor. The higher affinity component equilibrates more rapidly than the lower affinity component and only the latter can be antagonised by Δ^{16}-alphaxalone.

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296P ALPHA SUBUNITS INFLUENCE THE ACTION OF PENTOBARBITAL ON RECOMBINANT $GABA_A$ RECEPTORS

S.A. Thompson, P.J. Whiting & K.A. Wafford, (R.G. Hill) Merck Sharp & Dohme Research Labs., Terlings Park, Eastwick Rd., Harlow, Essex CM20 2QR, U.K.

$GABA_A$ receptors are a family of ligand gated ion channels formed from the co-assembly of different subunits. Various binding sites have been identified on the receptor which allosterically modify the action of GABA, including the barbiturate site. Barbiturates have been shown to have two mechanisms of action; a potentiation of the response to GABA, and at high concentrations, a direct activation of $GABA_A$ receptors.

In this study we have investigated the effect of pentobarbital (PB) on $GABA_A$ receptors containing different α subunits expressed in *Xenopus* oocytes using the two electrode voltage clamp method. PB activity consisted of the above mentioned two components on all the subunit combinations studied. Potentiation of the GABA response occurred before the direct effects of PB in $\alpha_1\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$ and $\alpha_5\beta_2\gamma_{2s}$ (2 log units) whereas on $\alpha_6\beta_2\gamma_{2s}$ both effects occurred at the same concentrations. On $\alpha_1\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$ and $\alpha_5\beta_2\gamma_{2s}$ the direct effect was small compared to the potentiation of the EC_{20} GABA response, however, on $\alpha_6\beta_2\gamma_{2s}$ the direct activation contributed greatly to the overall effect of PB. The affinity for the total effect (potentiation of GABA and direct effect) was between 15µM and 22µM on all subunits, however maximum potentiation (including the direct activation component) varied from 200% of GABA EC_{20} on $\alpha_1\beta_2\gamma_{2s}$ to 500% on $\alpha_6\beta_2\gamma_{2s}$.

The maximum direct effect elicited by PB alone on $\alpha_1\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$ and $\alpha_5\beta_2\gamma_{2s}$ was between 35 - 60% of the maximum response obtainable with GABA, whereas on $\alpha_6\beta_2\gamma_{2s}$, PB produced a maximum response nearly twice that of the maximum GABA response (180%). The affinity for PB on $\alpha_6\beta_2\gamma_{2s}$ was 40µM compared to between 250-400µM on $\alpha_1\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$ and $\alpha_5\beta_2\gamma_{2s}$. This direct action was blocked by picrotoxin and partially blocked by high concentrations of bicuculline, but not by the pure competitive antagonist SR95531. This indicates that the direct agonist activity was not being mediated via the GABA binding site.

The results from this study indicate that $GABA_A$ receptors containing α_6 subunits have both a higher affinity and efficacy for direct activation by PB. During anaesthesia PB reaches a concentration in the human brain of 187µM (Richards 1972). This concentration of PB corresponds to a near maximum direct effect on $\alpha_6\beta_2\gamma_{2s}$ with little direct effect on the other α subtypes. Although potentiation of GABA would be observed in all subtypes at 187µM the degree of efficacy and direct activation by PB would be much greater on $\alpha_6\beta_2\gamma_{2s}$. Since α_6 containing receptors are located primarily on cerebellar granule cells, it is likely that PB has much larger effects on $GABA_A$ receptors located in the cerebellum, compared to those in other regions of the brain.

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297P THE EFFECT OF CHRONIC AGONIST STIMULATION ON THE EXPRESSION OF $GABA_A$ RECEPTORS IN RAT CEREBELLAR GRANULE CELLS

D.C. SPENCER & D.R. BRISTOW, (introduced by F. Owen) Division of Neuroscience, School of Biological Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT.

Receptor desensitisation is the reduction in the transmembrane signal produced by a cell-surface receptor even though the stimulating agent continues to be present. One receptor at which this is known to occur is the γ-aminobutyric $acid_A$ ($GABA_A$) receptor in mammalian brain, a receptor for the important inhibitory neurotransmitter GABA. Chronic agonist stimulation results in a reduction in the $GABA_A$ receptor α_1-subunit mRNA (Montpied *et al.*, 1991; Hirouchi *et al.*, 1992). However, as yet, little is known about the effect of chronic agonist treatment on the protein expression of the $GABA_A$ receptor subunits and if this is linked to desensitisation. This study involves the investigation of chronic agonist treatment on the expression of two $GABA_A$ receptor subunits in rat cerebellar granule cells.

Cerebellar granule cells were prepared from 8 day postnatal rats according to an established procedure (Wilkin *et al*, 1976) and maintained in culture for 8 days prior to experimentation. Cells were treated with muscimol (10μM) or GABA (1mM) for 1-8 days before cellular RNA and protein were extracted. Total RNA was applied to nylon membranes by slot blot application and then hybridised at 43°C for 12 hours with ^{32}P-labelled (random primed) α_1 or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probes before undergoing autoradiography. Changes in protein expression were investigated using the techniques of SDS-PAGE and Western blotting and visualised by enhanced chemiluminescence. Resulting signals were quantitated using a flatbed scanner.

The results presented here show that mRNA expression of the α_1 subunit is significantly reduced ($p<0.05$, Students paired T-test, n=5) to 60 ± 13% (mean ± SEM) of control cells after 1 day treatment with 1mM GABA, returning to control levels by 4 days of treatment. In contrast, the mRNA expression for the 'housekeeping', enzyme G3PDH remains constant in control and GABA treated cells over the same time course.

Protein expression of the α_1 subunit in granule cells is significantly reduced ($p<0.01$, Wilcoxon Rank, n=7) to 75 ± 3.4% (mean ± SEM) of control after 2 days treatment with 10 μM muscimol, a $GABA_A$ receptor agonist, and to 48.7 ± 11.2% (mean ± SEM) of control after 6 days treatment with 10μM muscimol, returning to control levels by 8 days of treatment. Protein expression of the α_6 subunit is also reduced ($p<0.01$, Wilcoxon Rank, n=7) to 75 ± 6.2% (mean ± SEM) of control after 2 days treatment with 10μM muscimol but returns to control levels by 6 days.

The agonist-mediated reduction in the α_1 mRNA found here correlates with the down-regulation in protein expression observed at 2-6 days. However, muscimol does appear to have differential effects on the time course of α_1 and α_6 subunit protein expression. Thus, in rat cerebellar granule cells chronic treatment with $GABA_A$ receptor agonists induce changes in $GABA_A$ receptor expression. It is possible that these observed changes underlie the phenomena of agonist-induced desensitisation.

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298P THE EFFECT OF CHRONIC BENZODIAZEPINE TREATMENT ON $GABA_A$ RECEPTOR SUBUNIT PROTEIN EXPRESSION IN RAT CEREBELLAR GRANULE CELLS

M.J. BROWN and D.R. BRISTOW, (Introduced by F. Owen) Division of Neuroscience, School of Biological Sciences, Stopford Building, Oxford Road, University of Manchester, M13 9PT.

Prolonged benzodiazepine administration in man (Greenblatt and Shader, 1978) and in rat (Gallager *et al.*, 1984) results in tolerance to some of their effects. One possible mechanism for the development of tolerance to benzodiazepines may involve an alteration in the expression of the genes encoding the various subunits of the $GABA_A$ receptor. In particular, it has been reported that chronic benzodiazepine treatment causes the down-regulation of the $GABA_A$ receptor subunit α_1 (Heninger *et al.*, 1990; Hirouchi *et al.*, 1992) and γ_2 mRNAs (Kang and Miller, 1991; Zhao *et al.*, 1994). Regulation at the level of transcription could be occurring, since the benzodiazepine agonist, lorazepam is reported to decrease the transcriptional activity of the promoter construct of the α_1 subunit gene (Kang *et al.*, 1994).

In the present study, the effect of chronic treatment with the benzodiazepine agonist, flunitrazepam (1μM), on $GABA_A$ receptor subunit protein expression was examined in primary cultures of rat cerebellar granule cells. Cerebellar granule cells were prepared from post-natal day 8 rats and grown in supplemented BME for 8 days prior to treatment with 1 μM flunitrazepam (1-12 days). Cells were harvested, the proteins extracted and separated by 10% SDS-PAGE, followed by Western blotting onto nitrocellulose membranes. The membranes were probed with subunit-specific (α_1 and α_6) antibodies, incubated with HRP secondary antibody, and protein bands detected using ECL. The optical density of the protein bands was quantitated using a OmniMedia flatbed 600dpi scanner.

This study demonstrates that 1μM flunitrazepam induces a specific down-regulation of the $GABA_A$ receptor α_1 subunit protein of 40 ± 3 % (mean ± SEM, n=10, $p<0.01$, Wilcoxon Rank Test) following 2 days treatment, which recovers and remains at control values by 4 days through to 12 days of treatment. This flunitrazepam-induced down-regulation of the α_1 subunit protein was prevented by co-administration of the benzodiazepine antagonist, flumazenil (1μM). In contrast, there is no effect of 1μM flunitrazepam (1-12 days) treatment on the expression of the $GABA_A$ receptor α_6 subunit protein.

Studies to understand the molecular basis of the flunitrazepam-induced down-regulation of the $GABA_A$ receptor α_1 subunit protein, suggest an involvement of protein kinase C. The flunitrazepam-induced (2 days) down-regulation of the α_1 subunit protein in the presence of 0.3μM staurosporine was 3 ± 8 % (mean ± SEM, n=7), producing a complete inhibition of flunitrazepam effect.

The results presented here suggest that the response of the $GABA_A$ receptor to chronic benzodiazepine treatment involves a specific down-regulation of the $GABA_A$ receptor α_1 subunit protein after 2 days, and this process appears to involve protein kinase C. This modulation of $GABA_A$ receptor subunit protein expression may be involved in the development of tolerance to benzodiazepines.

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299P SCH 58261, A NEW POTENT AND SELECTIVE ADENOSINE A_{2a} RECEPTOR ANTAGONIST

C. Zocchi, E. Ongini,*P.G. Baraldi, S.Dionisotti. Research Laboratories Schering-Plough S.p.A., I-20060 Comazzo, Milan, Italy, *Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy.

Some non-xanthine heterocyclic compounds have been identified as antagonists of the adenosine A2a receptors. However, only a few chemical structures, such as the 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo-[1,5-c]quinazoline (CGS 15943) (Francis et al., 1988) and 5-amino-8-(2-fluorobenzyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo-(1,5-c)pyrimidine (8FB-PTP) (Gatta et al., 1993), show high affinity but they have little or no A2a vs. A1 selectivity. In order to enhance the A2a selectivity, we synthesized a series of 8FB-PTP derivatives and, in the present study, we characterized the *in vitro* pharmacological profile of one of them, the new potent and selective A2a antagonist 7-(2-phenylethyl-5-amino-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo-(1,5-c)pyrimidine (SCH 58261). A1 and A2a binding affinities were assayed on rat (whole brain and striatum) and bovine (cortex and striatum) brain tissues using [3H]CHA (Bruns et al., 1980) and [3H]CGS 21680 (Jarvis et al., 1989), respectively. The type of inhibition (competitive or noncompetitive) was also tested in rat striatum by saturation experiments at A2a receptors ([3H]CGS 21680, 1-128 nM) in the absence or presence of SCH 58261. Antagonism to A2a-mediated effects was tested on the antiaggregatory activity in rabbit platelets (Gatta et al., 1993) induced by the A2a agonist CGS 21680. Binding affinities at adenosine receptors are reported in the following Table (n=4-6; geometric mean, with 95% confidence limits in parentheses):

ANTAGONIST	RAT BRAIN			BOVINE BRAIN		
	A1 KI(nM)	A2a Ki(nM)	A1/A2a Ratio	A1 Ki(nM)	A2a Ki(nM)	A1/A2a Ratio
CGS 15943	3.0 (2.2-4.2)	1.6 (1.2-2.1)	1.9	2.4 (2.1-2.8)	0.95 (0.8-1.1)	2.5
8FB-PTP	3.3 (2.9-6.6)	1.2 (0.9-1.4)	2.8	2.0 (1.8-2.3)	0.07 (0.06-0.09)	29
SCH 58261	121 (103-104)	2.4 (2.0-2.7)	50	201 (160-251)	2.0 (1.7-2.3)	101

In rat striatum, SCH 58261 (3 nM) increased significantly ($p<0.01$; n=4) the Kd values of [3H]CGS 21680 binding, being 11.7 nM (10.8-12.7) or 31.0 nM (25.9-37.1) in the absence or presence of the antagonist. No changes were found in Bmax values, thus indicating a competitive inhibition at the A2a receptor. Inhibition induced by CGS 21680 of rabbit platelet aggregation, IC50 value of 4.19 μM (2.65-6.63), was antagonized competitively by SCH 58261 (0.03-0.1-0.3 μM) with pA2 and Schild plot values of 7.9 (6.8-8.9) and 1.1 (0.94-1.26) (n=4). SCH 58261 is therefore the first non-xanthine derivative which combines high affinity, good selectivity and competitive antagonism at A2a receptors.

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300P ROLE OF ADENOSINE A_{2A} RECEPTOR IN GABAERGIC SYNAPTIC TRANSMISSION IN THE STRIATUM

H. Kase, A. Mori, T. Shindou, H. Nonaka and M. Ichimura (Introduced by T.-P.D. Fan), Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shimotogari, Shizuoka 411, Japan

The adenosine A_{2A} receptor is highly enriched in striatal medium spiny neurons (MSNs)(Schiffmann et al., 1991), which constitute the majority of the neuronal population in the striatum and utilize γ -aminobutyric acid (GABA) as a neurotransmitter. However, little is known about synaptic regulation of the striatal neurones and still less of physiological role of the A_{2A} receptor. We report here that adenosine modulates the inhibitory synaptic transmission in the striatum through the presynaptic A_{2A} receptor.

We used both intracellular recording and whole-cell patch clamp recording in rat striatal slices, and examined the effect of selective agents for adenosine receptors on GABAergic synaptic transmissions in the MSNs. As an A_{2A} receptor selective antagonist, we employed a novel compound, KF17837 ((E)-8-(3,4- dimethoxystyryl)-1,3-dipropyl-7-methylxanthine) (Shimada et al., 1992, Nonaka et al., 1994).

Autoradiographic study of [^{3}H] KF17837 using rat brain sections demonstrated that the binding site was highly enriched in the striatal regions. Using the rat striatal slices, we observed that the A_{2A} selective agonist CGS21680 (1μM) reduced the amplitude of evoked $GABA_A$ -mediated inhibitory postsynaptic potentials (i.p.s.ps) (to 78±3% of control, n=12) and post synaptic currents (i.p.s.cs) (to 66±10% of control, n=7). These effects were dose dependent within the concentration range of adenosine-induced cAMP elevation in the striatal slices and was mimicked by cAMP analogues, 8-bromo- and dibutyryl-cAMP. KF17837 (1,10μM) blocked these actions but the A_1 selective antagonist DPCPX ((8-cyclopentyl)-1,3-dipropylxanthine) had no effect. CGS21680 decreased and KF17837 increased the mean frequency of spontaneous miniature i.p.s.cs without affecting the amplitude distribution. Futhermore, GABA-induced depolarization was not affected by CGS21680. These results indicate that adenosine acts on presynaptic A_{2A} receptors to suppress GABAergic transmission among the MSNs through a cyclic AMP signaling pathway.

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301P NEGATIVE ALLOSTERIC MODULATION AT GABA RECEPTORS AND SOMATOSTATIN SECRETION

H.E. Murray & G.E. Gillies,
Department of Pharmacology, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF.

Considerable evidence now suggests that in the CNS certain endogenously produced steroid hormones and/or their metabolites elicit rapid responses at the cell surface membrane which do not involve protein synthesis but an interaction at the $GABA_A$ receptor as a possible mechanism of action. Furthermore, we have recently demonstrated that GABA exerts a strong inhibitory influence on hypothalamic somatostatin (SRIH) secretion (Gillies & Davidson, 1992). Therefore, we have investigated the possibility that pregnenolone sulphate, a negative allosteric modulator at the $GABA_A$ receptor, can influence SRIH secretion. These studies have been performed on primary cultures of isolated hypothalamic neurones derived from rat hypothalami at 18 days' gestation and maintained in a serum-free defined medium containing low levels of progesterone (10^{-14}M). Both SRIH and GABA- producing neurones are present in such cultures and the latter appear to exert a tonic inhibitory influence on SRIH secretion via $GABA_A$ receptors as bicuculline has been shown to enhance SRIH secretion (Gillies & Davidson, 1992; Gillies et al., 1992).

When expressed as a percentage of basal secretion, pregnenolone sulphate caused a significant increase ($p < 0.05$, $n = 5$ Student's t test) in SRIH secretion at 10^{-12}M (144.0 ± 18.9%) and 10^{-10}M (159.2 ± 11.0%). Responses at 10^{-8}M were, however reduced (108.6 ± 10.3%). These effects were significantly reduced in the presence of a subthreshold concentration of muscimol(10^{-8}M) to 86.0 ± 8.1 and 120.8 ± 15.5% of basal secretion for 10^{-12} and 10^{-10}M pregnenolone sulphate respectively ($p < 0.05$). Conversely, the responses to pregnenolone sulphate, 10^{-12} and 10^{-10}M, were enhanced in the presence of bicuculline (defined as 100%) from 83.5± 15.4% and 156.5 ± 23.4% of the bicuculline response to 254.9 ± 36.7 and 466.5 ± 63.4%, respectively ($p < 0.025$ when the combined responses of the neuroactive steroid and antagonist are compared with the sum of the individual responses, Student's t test).

We conclude that pregnenolone sulphate influences somatostatin secretion and our evidence suggests that this is mediated via an effect at the $GABA_A$ receptor. This effect could be indirect e.g. via dopaminergic neurones, which are present in the cultures (Murray & Gillies, 1993) and can influence SRIH secretion. However, 10^{-12} and 10^{-10}M, pregnenolone sulphate had no effect on endogenous DA secretion (as measured by HPLC). Therefore as several reports suggest that $GABA_A$ receptor subunits are located on hypothalamic SRIH neurones, we propose that the pregnenolone sulphate may be acting on the SRIH neurones themselves. These results provide evidence for a role for rapidly acting neurosteroids in the regulation of neuroendocrine function and on growth hormone release in particular.

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302P DIFFERENTIAL EFFECTS OF THREE $GABA_B$ ANTAGONISTS ON GLUTAMATE AND GABA RELEASE FROM RAT SPINAL CORD

H. Teoh, L.J. Fowler and N.G. Bowery, Dept. Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

Autoreceptors for GABA in mammalian brain have been reported to be primarily of the $GABA_B$ subclass (Pittaluga *et al*, 1987; Waldmeier *et al*, 1988). However, $GABA_B$ receptors are not restricted to GABA nerve terminals but also appear to function as presynaptic receptors on other fibres. It has been suggested that the characteristics of these receptors may differ from those of autoreceptors (Bonanno & Raiteri, 1992). In the present study, we examined this possibility by comparing the effects of different $GABA_B$ receptor antagonists on the (–)baclofen-induced inhibition of endogenous GABA and glutamate release from rat isolated spinal cord. Dorsal horn slices (400μm) with roots attached were continuously perfused at $1ml.min^{-1}$ with Krebs' solution. The roots were isolated and laid over bipolar platinum electrodes. Consecutive three min fractions were collected after an hour of incubation. Two stimuli (S1 and S2), each of 20V, 1Hz, 0.5msec were applied for three min separated by an interval of nine min. Glutamate and GABA concentrations in the samples were determined by HPLC with o-phthaldialdehyde precolumn derivatisation and fluorimetric detection. Statistical analyses were accomplished with the Student's t test, ANOVA and Dunnett's test.

The mean basal concentrations of glutamate and GABA in the perfusates were 234.5 ± 34.8nM ($n = 6$) and 151.44 ± 10.6nM ($n = 3$). During S1, the concentration of glutamate increased to 510.71 ± 38.9nM ($p < 0.005$) whilst the GABA concentration rose to 243.26 ± 5.9nM ($p < 0.005$). The levels in S2 were lower for both glutamate and GABA with mean S2/S1 values of 0.72 ± 0.03 and 0.64 ± 0.02 respectively. (–)-Baclofen (0.1 - 1000μM), the $GABA_B$ agonist, dose-dependently inhibited the evoked but not basal release of glutamate and GABA. At 30μM, an average inhibition of 50% and 40% respectively was obtained. This effect was just submaximal and thus used subsequently for examining the effects of antagonists. Three $GABA_B$ antagonists, CGP36742, CGP55845A and CGP56999A (Bittiger *et al*, 1993; Bernasconi *et al*, 1994) were examined. None of them (0.01 - 30μM) affected the basal release of GABA or glutamate. Both CGP36742 and CGP55845A (1 - 30μM) significantly increased the evoked release of glutamate and GABA as well as reversed the inhibitory effect that (–)baclofen had on their outflow ($p < 0.05$). CGP56999A had no significant effect on the stimulated release of glutamate in the presence and absence of (–)-baclofen. However, this antagonist (0.1 - 10μM) reversed the inhibitory action of (–)-baclofen on the evoked release of GABA ($p < 0.01$).

This study supports the possible distinction between the $GABA_B$ autoreceptor and $GABA_B$ heteroreceptors which modulate the release of glutamate. Our results indicate that CGP56999A is a potent antagonist at the autoreceptor but appears to lack any influence on the control of glutamate release. We thank CIBA (Basle) for $GABA_B$ antagonists and (-)baclofen.

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303P LACK OF EVIDENCE FOR CHANGES IN CEREBROCORTICAL $GABA_B$ RECEPTORS IN RATS WITH GENETIC ABSENCE EPILEPSY

P. Mathivet, R. Bernasconi, H. Bittiger[1] , C. Marescaux, W. Froestl[1] & S.J. Mickel[1], Unité INSERM U-398, Strasbourg, France, [1]Ciba-Geigy, CH-4002 Basel, Switzerland

Previous studies in a genetic rat model (GAERS) have shown that $GABA_B$-receptors may play a central role in the control of absence epilepsy (Marescaux *et al.* 1992). In order to test whether $GABA_B$ receptors are also involved in the patho-genesis of absence epilepsy, we measured the affinity (Kd) and density (B_{max}) of $GABA_B$ receptors in cortical membranes prepared from GAERS and aged/ matched control rats using two radioligands: the agonist [^{3}H] CGP 27492 and the antagonist [^{3}H] CGP 54626 (Bittiger *et al.* 1992). These binding experiments revealed several binding sites. Two different affinity states for $GABA_B$ receptors were observed in saturation experiments with the antagonist radioligand. The high affinity site with a Kd of 0.12 ± 0.07 nM represents a small population (10 to 12%) of the total number of cortical receptors. The larger proportion of binding sites (B_{max} 2797 ± 137 fmol/mg protein) showed a Kd of 1.7 ± 0.2 nM. These parameters in GAERS were not significantly different from controls. The curves for the inhibition by the agonist R(-)baclofen of antagonist [^{3}H] CGP 54626 specific binding showed Hill coefficients (n_H) of 0.55 ± 0.04. The inhibition curves fitted best with three binding sites. The IC_{50} value in GAERS for the high affinity site was 4.5 ± 1.0 nM (11.4 ± 4.1%), for the medium site 198 ± 15 nM (50.3 ± 4.1%) and for the low affinity site 7.06 ± 1.00 µM (38.3 ± 4.6%). All binding parameters were similar in control animals. We also examined the regulation of $GABA_B$ binding by 5'-guanylimido-diphosphate [Gpp(NH)p]. The number of binding sites after the addition of Gpp(NH)p on the inhibition of [^{3}H] CGP 54626 binding by R(-)baclofen was decreased to two (nH = 0.59 ± 0.06): a high affinity site with an IC_{50} of 720 ± 120 nM (37.7 ± 4.6%) and a low affinity site with an IC_{50} of 26.3 ± 0.92 µM (62.3 ± 4.6%). Binding parameters observed in GAERS were not significantly different from controls. Concentrations of Gpp(NH)p from 0.1 nM to 1 mM inhibited the binding of the $GABA_B$ agonist [^{3}H] CGP 27492 in a dose-dependent and biphasic manner (nH = 0.31 ± 0.02), indicating two populations of binding sites with IC_{50}s of 31.9 ± 2.5 nM (63.6 ± 1.0) and 161 ± 33.8 µM (36.4 ± 2.9%), respectively. The two IC_{50} values suggest two different interactions between $GABA_B$ receptors and G-proteins. Inhibition of agonist [^{3}H] CGP 27492 binding by Gpp(NH)p was similar in GAERS and in controls. These results indicate that there is no alteration of the properties and coupling of $GABA_B$ receptors in GAERS and that changes in $GABA_B$ receptors are therefore probably not the reason for the occurence of absence seizures in GAERS.

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304P (-)BACLOFEN INHIBITS FORSKOLIN-STIMULATED CYCLIC AMP PRODUCTION IN VENTROLATERAL THALAMIC NUCLEUS OF EPILEPTIC AND NON-EPILEPTIC RATS

T. Lemos, K.P. Parry, D.A. Richards & N.G. Bowery, Dept. Pharmacology, The School of Pharmacy, London WC1N 1AX.

It has been suggested that dysfunction in $GABA_B$ receptor-mediated transmission is a possible mechanism underlying genetically determined spike and wave discharges (SWD) in GAERS (Genetic Absence Epilepsy Rats from Strasbourg) (Marescaux *et al.*, 1992). However, binding studies performed in vitro using tissue obtained from epileptic animals have indicated no change in the density or affinity of $GABA_B$ receptors compared to normal brain (Knight & Bowery, 1992). This suggests that functional changes in the events after receptor activation may be responsible for the generation of SWD. $GABA_B$ receptors in brain tissue are coupled via G-proteins to adenylyl cyclase.(Wojcik & Neff, 1984). Thus, changes in receptor function can be detected as an alteration in the production of cAMP.

Male GAERS and non-epileptic control rats (300-350g) were implanted with a microdialysis probe into the ventrolateral thalamic nucleus. 24 hours later they were subjected to microdialysis. The probe was perfused initially (40 min) with artificial cerebrospinal fluid (ACF) and followed by ACF+forskolin 100µM (160 min). Dialysate samples were collected at 40 min intervals. After collecting the third sample (at 120 min) some animals were injected intraperitoneally with (-)baclofen 2mg/kg and a further 2 samples were collected. Samples were analyzed by radioimmunassay (Amersham International plc). The response of isolated thalamic slices to forskolin alone or in combination with (-)baclofen was also examined. Thalami were cross-chopped (250x250µm), rinsed, preincubated for 10 min with (-)baclofen (0.3-300µM) and then incubated with forskolin (10µM for 15 min). Tissue cAMP levels were determined by the method of Brown et al (1972).

In vitro forskolin (10µM) increased the level of cAMP from 8.05±2.29pmol/mg protein to 111.43±14.39pmol/mg protein (n=3). (-)Baclofen inhibited the forskolin-induced cAMP production in a dose-dependent manner by a maximum of 42.7±9.3% at 100µM. Baclofen produced no effect on basal levels. *In vivo* no difference was observed in the basal levels of cAMP between GAERS (3.12±0.38fmol/20µl, n=10) and control rats (4.1±0.40fmol/20µl, n=8). Forskolin increased the level of cAMP to 23.08±3.01fmol/20µl in GAERS (n=5) and 16.0±2.17 fmol/20µl in the control group (n=4) and (-)baclofen (i.p) inhibited this stimulation to a similar extent in both animal groups (55.23±6.47% GAERS and 43.13±7.91% control). Whilst these results fail to demonstrate any significant difference between the epileptic and non-epileptic animals they do suggest that $GABA_B$ receptor activation can modify the production of cAMP in the thalamus both in vitro and in vivo.

We thank CIBA (Basle) for (-)baclofen.

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305P DOES BACLOFEN INHIBIT WATER INTAKE IN RATS BY STIMULATING CENTRAL $GABA_B$ RECEPTORS?

A.J. Houston, C.L.J. Wong & I.S. Ebenezer, Neuropharmacology Research Group, School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, PO1 2DT.

We have previously demonstrated that systemic administration of baclofen (BAC) inhibits water intake in rats in response to a variety of thirst provoking stimuli (Ebenezer *et al.*, 1992; Houston *et al.*, 1994), by an action that is behaviourally specific (Ebenezer, 1994). As BAC is a $GABA_B$ receptor agonist, the 1st experiment was conducted to confirm that this effect of BAC is mediated by $GABA_B$ receptors. Moreover, as the site of action is not known, the 2nd experiment was undertaken to determine the possibility that the adipsic effect of the drug is mediated centrally.

Experiment 1: 16h water-deprived male Wistar rats (n=8; b.wt. 350-450g) received s.c. injections of either saline (SAL) followed by SAL, SAL followed by BAC (2 mg kg^{-1}), CGP 35348 (a peripherally active $GABA_B$ receptor antagonist; 50 mg kg^{-1}) followed by SAL, or CGP 35348 (50 mg kg^{-1}) followed by BAC (2 mg kg^{-1}). The 2 injections were separated by 15 min. 15 min after the 2nd injection the rats were presented with water in 20 ml graduated water bottles, and cumulative water intake measured. A repeated measures design was used with each rat receiving all treatments with 2-3 days between successive drug trials. The data were analysed by Dunnett's t test.

Experiment 2: Male Wistar rats (n=5; b.wt. 390-490g) were surgically prepared under Equithesin anaesthesia for chronic intracerebroventricular (icv) administration of drugs into the lateral ventricle. The animals were allowed to recover for 2-3 weeks before experimentation. During experimental sessions, the rats that had been deprived of water for 16h were injected icv with either saline (control) or BAC (0.1, 0.5 or 2 nmol), and 15 min later presented with water. A similar protocol was used as in Experiment 1.

In both experiments, the rats consumed most of their water during the 1st 15 min after presentation. Analysis of the data from Experiment 1 revealed that while BAC (2 mg kg^{-1}) reduced water intake from a mean control value±s.e.mean of 8.9±0.6 ml to 3.6±1.2ml ($P<0.001$) during the 1st 15 min after presentation, pretreatment of the rats with the specific $GABA_B$ antagonist CGP 35348 abolished the inhibitory effect of baclofen (intake after CGP 35348 + BAC = 7.8±0.7 ml). CGP 35348 had no effect on water intake by itself (intake = 9.9±1.2ml). The results obtained in Experiment 2 show that icv administration of BAC (0.1-2 nmol) caused a dose-related inhibition of water intake in the water-deprived rats. Thus, 15 min after presentation of water to the animals, BAC 0.5 and 2 nmol reduced water consumption from a control value±s.e.mean of 9.9±1.2 ml to 4.1±2.2 ml and 0.1±0.1 ml respectively ($P<0.01$ in each case). The 0.1 nmol dose was without effect (intake = 10.0±1.9 ml). However, it is noteworthy that even after 60 min the dose-related depression in drinking was still apparent (data not shown).

The results obtained in this study thus indicate that systemic administration of BAC mediates its inhibitory effects on water intake by a $GABA_B$ mediated mechanism. Furthermore, as BAC can enter the CNS from the systemic circulation (Faigle and Keberle, 1972), and the icv doses required to inhibit water intake in this study are at least 1000 times lower than those required by systemic routes of administration (Ebenezer *et al.*, 1992), it is tempting to suggest that BAC stimulates $GABA_B$ receptors at a central site to exert its adipsic effects. Further support for this view comes from recent observations in our laboratory that systemic administration of 3-amino-propyl-phosphinic acid, a $GABA_B$ agonist that cannot cross the blood brain barrier, does not depress water consumption in rats.

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306P THE ANTI-TUSSIVE ACTION OF DEXTROPHAN-3-GLUCURONIDE IN CONSCIOUS GUINEA-PIGS

E.A. Laude, T.J. Grattan & A.H. Morice. Department of Medicine and Pharmacology, University of Sheffield, Beech Hill Rd, Sheffield S10 2RX

Dextromethorphan, a non-sedative, non-narcotic morphine analogue is widely used as an anti-tussive. Metabolites of morphine have been shown to vary in analgesic action, morphine 6-glucuronide is agonistic (Abbot & Palmour, 1988) and the 3-glucuronide antagonistic (Smith et al 1990). This study was designed as a five way crossover latin square, balanced for carry-over effects, to determine any anti-tussive action of dextrorphan-3-glucuronide, the principle metabolite of dextromethorphan (Chen et al 1990).

Guinea-pigs (340g–480g) n=7 were screened to determine the concentration of citric acid (300 or 500mM) which gave a reproducible cough response >10/10 min in a guinea-pig cough challenge model (Laude et al 1994). The three active treatments were oral dextromethorphan, 40mg.kg^{-1}, i.p. dextrorphan-3-glucuronide 46.8mg.kg^{-1} and i.p. dextrorphan 0.24mg.kg^{-1} (a dose equivalent to that present in the dextrorphan-3-glucuronide sample). Two controls (oral and i.p. saline) were included. A minimum 48 h washout period was allowed between treatments. Citric acid cough challenge was carried out 40 min post oral and 10 min post i.p. treatment

Oral dextromethorphan caused a significant ($P< 0.0005$) 44% reduction in the cough response from 14.6±1.4 (oral saline) to 8.3±1.6 (mean±sem). Dextrorphan-3-glucuronide i.p. also gave a significant ($P< 0.02$) 45% reduction from 14.0±2.0 (i.p. saline) to 7.7±1.7. Dextrorphan at the concentration present in the dextrorphan-3-glucuronide had no significant effect on cough frequency (Figure 1).

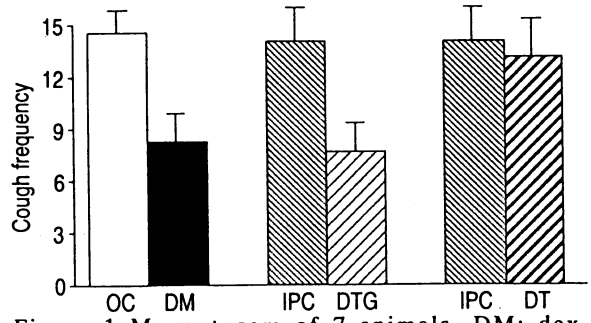


Figure 1 Mean ± sem of 7 animals. DM: dextromethorphan; DTG: dextrorphan-3-glucuronide; DT: dextrophan; OC: oral control; IPC: i.p. control.

In this study dextrorphan-3-glucuronide proved to be as potent an anti-tussive as dextromethorphan. On first pass metabolism dextromethorphan, at the doses used in cough remedies and in this study, is extensively metabolised to its polar metabolites. Dextrophan-3-glucuronide may, therefore, contribute significantly to the overall efficacy of dextromethorphan as an anti-tussive agent.

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307P THE EFFECT OF THE COOLING AGENT N-ETHYL-P-MENTHANE-3-CARBOXAMIDE ON CITRIC ACID-INDUCED COUGH IN GUINEA-PIGS

E.A. Laude, T.J. Grattan & A.H. Morice. Department of Medicine and Pharmacology, University of Sheffield, Beech Hill Rd, Sheffield S10 2RX.

N-ethyl-p-menthane-3-carboxamide (WS3, Sterling Organics, UK) was developed as a cooling agent with low volatility for use in the cosmetic industry. The aromatic vapours, menthol and camphor, also cooling agents, have been shown to have anti-tussive activity (Laude *et al*,1994). Watson *et al*,1978 found WS3 to be more potent than menthol as a cooling agent and this study was designed to investigate whether it also had antitussive activity.

Guinea-pigs (n=11) weighing 400-650g were exposed for 5 min to ultrasonically nebulised aerosols of WS3 (0.01, 0.03, 0.1 and 0.3mg/ml) 1 min before cough challenge with citric acid (500mM) (Laude *et al*,1994). WS3 was dissolved in 10% alcohol/saline. This concentration of alcohol had previously been shown to have no effect on citric acid induced cough. Control challenges (5 min vehicle exposure) were carried out 24 h pre and post WS3 treatment.

To investigate the duration of action, another group of animals (n=10) were cough challenged on separate days 30, 60 and 120 min after exposure to 5 min 0.3mg/ml WS3. Control challenges to vehicle were carried out at the same time points.

One min post exposure, 0.01 and 0.03 mg/ml WS3 had no significant effect on cough frequency, 0.1mg/ml produced a significant ($p<0.02$) 41% reduction from 14.0±2.4 (mean±sem) to 8.2±2.0 and 0.3mg/ml a significant (0.01) 60% reduction from 14.9±2.6 to 6.0±1.5 (Figure 1).

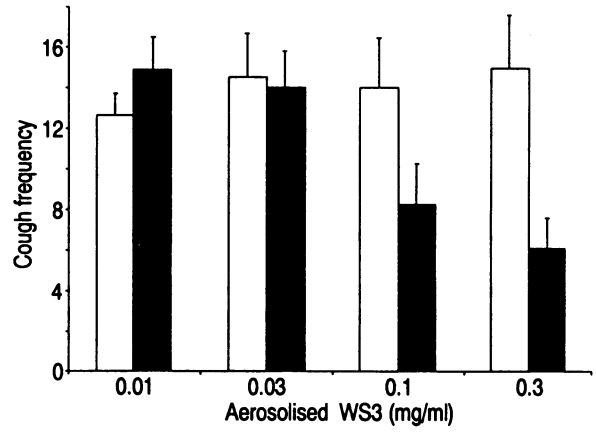


Figure 1 . Mean ± sem of 11 animals. □: 10% alcohol/saline; ■ : WS3.

Thirty min after exposure, there was a significant ($p<0.005$) 33% reduction in cough frequency from 14.4±1.6 to 9.7±1.9. There was no significant difference beween control and WS3, 60 and 120 min after exposure.

In this study WS3 was found to have significant anti-tussive activity which was still effective 30 min after exposure.

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308P THE ROLE OF PROTEIN KINASE C IN THE DESENSITISATION OF μ-OPIOID-INDUCED PHOSPHOLIPASE C ACTIVITY IN SH-SY5Y CELLS

D. Smart & D.G. Lambert (Introduced by S.R. Nahorski), Department of Anaesthesia, Leicester Royal Infirmary, Leicester LE1 5WW

We have recently reported that μ-opioids cause a rapidly desensitizing activation of phospholipase C (PLC) in SH-SY5Y cells (Smart *et al.*, 1994). PLC activation increases protein kinase C (PKC) activity, as well as $Ins(1,4,5)P_3$ formation (Berridge 1993), and acute PKC activation may inhibit further PLC activation. Therefore, we investigated the role of PKC in the rapid desensitization of μ-opioid induced $Ins(1,4,5)P_3$ formation. Whole cell suspensions (final volume 0.3ml) were preincubated at 37°C for 15min in Krebs buffer, with or without the PKC inhibitor Roche242a (R242a; 0.1nM to 10μM) or phorbol 12,13 dibutyrate (PDBu; 1μM). The cells were then incubated in the presence or absence of fentanyl (0.1μM) for 0-300s. $Ins(1,4,5)P_3$ was measured using a specific radioreceptor mass assay (Challiss *et al.*, 1988). Fentanyl (0.1μM) caused a transient, monophasic stimulation of $Ins(1,4,5)P_3$ formation (Table 1), as previously reported (Smart *et al.*, 1994). The PKC inhibitor R242a (1μM) had no effect on basal, but enhanced (1.6 fold at the peak) fentanyl-induced, $Ins(1,4,5)P_3$ formation (Table 1). R242a (1μM) also prolonged the duration of the fentanyl-induced $Ins(1,4,5)P_3$ response (Table 1). The enhancement was dose-dependent at both the peak (30s) (EC_{50} 20.0±4.0nM) and at 120s (EC_{50} 257.5±55.6nM). Furthermore, acute PDBu (1μM) had no effect on basal, but completely abolished fentanyl-induced, $Ins(1,4,5)P_3$ formation. These data suggest that PKC activity may be responsible for the desensitization of μ-opioid induced PLC activation in SH-SY5Y cells.

Table 1 The effect of the PKC inhibitor R242a (1μM) on fentanyl(0.1μM)-induced $Ins(1,4,5)P_3$ formation in SH-SY5Y cells

Time after fentanyl (s)	control	+R242a
0	9.7 ± 0.6	9.4 ± 0.4
15	18.2 ± 1.5	28.3 ± 3.2*
30	14.3 ± 0.6	33.4 ± 2.4*
60	12.5 ± 1.7	18.4 ± 1.3*
120	9.6 ± 0.8	12.7 ± 1.3*
300	9.6 ± 0.6	10.6 ± 0.8

(Data given as mean ± SEM pmol/mg/protein, n=7. *denotes $p<0.05$ (t-test) increased compared to control)

Financial support from The Wellcome Trust, and the gift of R242a from Roche (Herts. UK) are gratefully acknowledged.

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309P EFFECTS OF μ- AND δ-OPIOID RECEPTOR OCCUPATION ON INTRACELLULAR Ca^{2+} IN SH-SY5Y AND NG108-15 CELLS

A. Wandless., D. Smart. & D.G. Lambert. (Introduced by S.R. Nahorski) University Department of Anaesthesia, Leicester Royal Infirmary, Leicester. LE1 5WW.

In SH-SY5Y neuroblastoma cells μ- but not δ-opioids stimulate $Ins(1,4,5)P_3$ formation in a Ca^{2+} dependent manner. Activation of the μ-opioid receptor with fentanyl stimulated $Ins(1,4,5)P_3$ formation with an EC_{50} of 16nM (Smart *et al*, 1994a). Moreover, the response was blocked by nifedipine suggesting that fentanyl opened an L-type Ca^{2+} channel (Smart *et al*, 1994b). In this study we have examined the effects of fentanyl on intracellular Ca^{2+} ($[Ca^{2+}]_i$) in Fura2 loaded SH-SY5Y cells and compared this with previously reported δ-receptor stimulated increases in NG108-15 cells (Okajima *et al*, 1993).

Whole cell suspensions were loaded with 3μM Fura2/AM for 30 mins at 37°C in Krebs/HEPES buffer, pH 7.4. Loaded cells were postincubated for a further 20mins at room temperature. $[Ca^{2+}]_i$ was measured fluorimetrically according to Grynkiewicz *et al* (1985).

In 10/33(30.3%) batches of SH-SY5Y cells tested 0.3μM fentanyl increased $[Ca^{2+}]_i$ with a mean increase of 18.6±2.2nM (for responders). At 3μM fentanyl 32/52 (61.5%) batches increased $[Ca^{2+}]_i$ with a mean increase of 21.1±2.6nM (for responders). Dose-response analysis revealed an EC_{50} of 0.73μM. The response to 0.3μM fentanyl was reversed by naloxone (10μM). However, the response to 3μM fentanyl contained a significant non-opioid component. Moreover, fentanyl at 3μM inhibited the carbachol (5 & 50μM) stimulated plateau phase of $[Ca^{2+}]_i$. Surprisingly, DAMGO (1μM) and morphine (1μM) failed to increase $[Ca^{2+}]_i$. These data are at variance with those recently reported by Connor & Henderson (1994). δ-receptor stimulation in NG108-15 cells with DPDPE increased $[Ca^{2+}]_i$ at all concentrations (0.01-1μM) and in all batches tested. At 1μM the mean increase was 36.3±7.1nM and was fully naloxone reversible. In both cell types there was a significant Ca^{2+} entry component revealed by Ni^{2+} (2.5mM) pretreatment.

Taken at face value our data suggest that increased $[Ca^{2+}]_i$ in SH-SY5Y cells is not the mechanism by which μ-opioids activate phospholipase C. However, large increases in Ca^{2+} in the vicinity of the plasma membrane would be underestimated using whole cell population measurements. Further studies using subcellular fluoresence imaging will be required to answer these questions.

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310P NIRAVOLINE HYDROCHLORIDE, A NOVEL SELECTIVE κ AGONIST WITH MARKED AQUARETIC PROPERTIES BUT LOW ANALGESIC POTENCY

G. Hamon, F. Clémence, M. Fortin, O. Le Martret, S. Jouquey, J.C. Vincent, F. Petit and D.G. Bichet [1]. Centre de Recherches Roussel Uclaf, 111 Route de Noisy, 93235 Romainville cedex, France and [1] Hôpital du Sacré-Coeur, Université de Montréal, Québec H4J 1C5.

Most of the kappa agonists described up to now have been shown to possess both analgesic and diuretic properties, these two effects occuring in the same dose range. Here we describe the pharmacological profile of niravoline hydrochloride (RU 51599) [1S-trans)-N-[2,3-dihydro-2-(1-pyrrolidinyl)-1H-inden-1-yl]-N-methyl-3-nitro-benzeneacetamide, monohydrochloride] a new kappa agonist, which shows a dissociated aquaretic activity.

Binding studies on isolated membranes show that niravoline has a high selectivity for kappa receptor with respective IC_{50}'s of 1.83 ± 0.23 nM (n = 4)(guinea pig cerebellum) and 1141 ± 207 nM (n = 4)(rat brain) for kappa and mu receptors. Acute administration of niravoline in normally hydrated Sprague Dawley rats induced a dose-related increase in urine output from 0.1 mg/kg i.v. and 1 mg/kg p.o.. Either no change or only a very slight increase in urinary sodium excretion was observed and urine osmolality was decreased from 0.3 mg/kg i.v. and 3 mg/kg p.o. falling from 577 ± 62 (n = 9) to 141 ± 21 (n = 8) mOsm/kg H_2O at 10 mg/kg p.o.. Naloxone (5 mg/kg) markedly inhibited this increase in urine output. In dogs, deprived of water for 18 hours and receiving a continuous i.v. infusion of saline to compensate for the urine elimination, injections of niravoline induced a dose-dependent increase in urine output from 0.025 mg/kg i.v.. This effect peaked 30 to 60 minutes after injections, reaching 161, 341 and 321% of the initial value at 0.025, 0.05 and 0.1 mg/kg respectively. The decrease in urine osmolality reached 62 and 78% at 0.05 and 0.1 mg/kg. At the same time a slight but significant decrease in sodium excretion was observed. Free water reabsorption was markedly decreased by niravoline falling from 16.70 ± 3.33 to -18.83 ± 7.99 μl/kg/min (n = 8) at 0.1 mg/kg. These effects were sustained during a 4 hour infusion period at 0.0025 mg/kg/min. Furthermore at the end of a two week treatment period at 0.3 mg/kg bid in normal rat, the effects on urine output and osmolality were equal to those after a single injection, thus demonstrating the absence of tachyphylaxis under these experimental conditions.
Analgesic activity was explored in Swiss mice using the acetic acid-induced writhing and the hot plate tests. When injected 15 minutes before acetic acid, niravoline had no effect up to 1 mg/kg i.v. but displayed a significant analgesic activity at 3 mg/kg with 57% (n = 10) decrease in writhing. The reaction time to the heat stimulus (56°C) was prolonged (60%, n = 8) when niravoline was injected 15 minutes (but not 30 minutes) before the test at 3 mg/kg only.

These results indicate that niravoline is a selective kappa agonist with marked aquaretic properties but low analgesic potency.

311P THE EFFECT OF TESTOSTERONE ON THE ANTINOCICEPTIVE EFFECTS OF MORPHINE IN MICE

P.A. Sweeney, C.L.J. Wong & I.S. Ebenezer, Neuropharmacology Research Group, School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, PO1 2DT.

The degree and mechanism of antinociception induced by stress has been shown to be influenced by gender (Menendez *et al.*, 1994) and gonadal function (Wong, 1987). Antinociception induced by opioid agonists also fluctuates in response to alteration in ovarian steroid levels during the oestrous cycle (Molina *et al.*, 1990). In previous studies steroids were given in chronic dosage regimens. In the present study we investigated whether testosterone, administered in high doses in acute conditions, alters the antinociceptive effect of morphine.

CFLP mice weighing 20-25 g were used in groups of 10. The antinociceptive effects of morphine were assayed by tail immersion test and hot plate test. For the tail immersion test, the tip of the animal's tail was immersed in water at 50 ± 1°C with a 15 s cut-off point. Mice were injected s.c. with either testosterone (10 mg kg^{-1} or 20 mg kg^{-1}) or vehicle (2-hydroxy-propyl-β-cyclodextrin in saline, 10 ml kg^{-1}) 1 h prior to morphine administration. Morphine, 4 mg kg^{-1}, was given s.c. and the tail flick response measured at 0, 10, 20, 30, 45 and 60 min after morphine. For the hot plate test, the animal was placed on a hot plate at 55 ± 0.1° C. The time latency for lifting or licking the hind paw was measured with a cut off of 15 s. The animals received the same injections as outlined above. The response was measured at 0, 10, 20, 30 45 and 60 min after morphine. The dose of morphine used in the present study is approximately its ED_{50} in both the tail immersion test and the hot plate test. Antinociception was expressed as percentage increase over control. Results were analysed by two way ANOVA for repeated measures design and *post-hoc* analysis by Dunnet's *t*-test.

In both male and female animals the doses of testosterone used, by themselves, did not affect the response to either test. In the tail immersion test, the antinociceptive effects of morphine were observed in both males and females. The peak antinociceptive effect occurred at 30-45 min post morphine. There was no significant gender difference in the response to morphine. In males, pretreatment with testosterone dose-dependently enhanced morphine's antinociceptive effect. The % antinociception was 59 ± 11% (mean ± s.e. mean) and 75 ± 12% respectively for the low and high testosterone dose pretreated groups compared with 43 ± 11% for the vehicle pretreated controls ($P<0.05$). Potentiation was still observable 60 min after morphine. However, in the female animals testosterone pretreatment suppressed the antinociceptive effect of morphine. For the higher dose of testosterone the antinociceptive effect of morphine was reduced to 12 ± 3%. Such suppression was still apparent 60 min post morphine. In the hot plate test, testosterone pretreatment induced similar potentiation of morphine's action in the male mice. However, the degree of potentiation was less than that in the tail immersion test. The % antinociception was 52 ± 11% and 65 ± 9% respectively for low and high dose testosterone pretreated groups and the potentiation was statistically significant for the high testosterone dose group ($P<0.05$). In female mice, testosterone pretreatment did not significantly alter the antinociceptive activity of morphine.

We conclude that pretreatment with testosterone can alter morphine induced antinociception in a sex dependent manner.

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312P TREATMENT OF DIABETIC RATS WITH RECOMBINANT NERVE GROWTH FACTOR CORRECTS SUBSTANCE P LEVELS WITHOUT AFFECTING NERVE BLOOD FLOW OR CONDUCTION

K. Maeda, L.T. Diemel, P. Fernyhough, W.J. Brewster & D.R. Tomlinson, Department of Pharmacology, Queen Mary and Westfield College, London, U.K.

Diabetes mellitus is characterised by neurological defects which may derive from impaired neurotrophic support. This study examined effects of treatment with human recombinant nerve growth factor (NGF), given at three doses (0.2, 0.5 and 1.0 mg/kg s.c. 3 times per week for the last 4 weeks of 8 weeks streptozotocin-diabetes), on deficits in sciatic nerve laser Doppler flux (indicative of reduced nerve blood flow), motor nerve conduction velocity (MNCV) and substance P content. Functional measurements were made under alphaxalone/ alphadolone anaesthesia (without recovery) exactly as described elsewhere (Stevens *et al.*, 1994). Over the same period another diabetic group received continuous subcutaneous insulin for comparison. Data (mean ± 1SD) are given in the table.

Treatment	Doppler flux (arbitrary units)	MNCV (m/sec)	Substance P (pg/cm)
Control (7)	285±77	47.3±2.9	83±19
Diabetic+vehicle (7)	199±79	40.7±3.5	55±14
Diabetic + insulin (8)	322±147	44.9±6.2	87±12
NGF (0.2 mg/kg) (5)	161±25	38.4±3.1	111±46
NGF (0.5 mg/kg) (7)	175±42	41.7±3.7	127±11
NGF (1.0 mg/kg) (8)	209±64	40.6±1.9	144±39

Statistical analysis used one-way ANOVA, with Duncan's multiple range tests applied where the F-test gave $p<0.05$ and there was homogeneity of varainces (Cochran's test). Diabetic rats given only NGF injection vehicle showed deficits in nerve Doppler flux, MNCV and substance P content (70%, 86% and 66% respectively of values from untreated control rats; all $p<0.05$). Insulin treatment normalised glycated haemoglobin as well as nerve Doppler flux, MNCV and substance P. NGF treatment did not affect nerve Doppler flux or MNCV, but increased sciatic nerve substance P levels in a dose-related manner, giving values, with increasing dose, which were 202%, 231% and 262% of those measured in nerves of vehicle-treated diabetic rats ($p<0.05$); the values at the two highest doses being significantly ($p<0.05$) raised above those of controls. These findings indicate multiple causation of acute neurological/ neurochemical defects in streptozotocin-diabetic rats.

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313P EFFECT OF $CGRP_{8-37}$ AND CP-99,994 ON FELINE PIAL ARTERY DIAMETER RESPONSES EVOKED BY CAPSAICIN

K.J. Escott, H.E. Connor[1], S.D. Brain & D.T. Beattie[1]. Pharmacology Group and Vascular Biology Research Centre, King's College, Manresa Rd., London, SW3 6LX, U.K. and [1]Pharmacology II, Glaxo Research and Development, Park Road, Ware, SG12 0DP U.K.

Antidromic electrical or capsaicin stimulation of trigeminal nerves leads to plasma extravasation and vasodilatation within the dura mater which may contribute to the sensation of pain, or may sustain or amplify migraine headache. Local perivascular injection of capsaicin to pial arteries *in situ* activates primary sensory afferent neurones and thus stimulates neuropeptide release (Edvinsson *et al*, 1990). In this study, the effect of the selective CGRP receptor antagonist, $CGRP_{8-37}$ and the neurokinin-1 receptor antagonist, CP-99,994 on capsaicin-induced changes in pial artery diameter were investigated, using an on-line image analysis system *in situ* (Beattie *et al*, 1993), in chloralose anaesthetised cats. Test agents were administered by perivascular injection close to the pial artery under study and changes in pial artery diameter were recorded for up to 10 min following application. Results are expressed as % change in pial artery diameter from pre-injected levels, mean ± s.e.mean and n values quoted refer to the number of arteries studied. Comparisons between treatments were made by Student's unpaired t-tests and statistical significance was accepted when $p < 0.05$.

Perivascular injection of artificial cerebrospinal fluid (ACSF) or capsaicin vehicle (20% ethanol: 10% Tween 80: 70% ACSF, further diluted 10,000 fold in ACSF) produced little change in pial artery diameter (5.2 ± 1.4 %, n = 18 and 6.8 ± 2.0 %, n = 37 respectively). Perivascular injection of capsaicin (10^{-8} M to 10^{-5} M) produced a biphasic effect on vessel diameter. At 10^{-6} M, capsaicin produced an initial transient vasoconstriction (-50.5 ± 7.9 %, $p < 0.001$ compared to vehicle, n = 14) followed by a longer-lasting vasodilatation (28 ± 3.5 % $p < 0.001$, n = 14). The vasodilator component of the response to capsaicin was reduced in the presence of $CGRP_{8-37}$ (10^{-6} M) to 8.9 ± 3.3 % or CP-99,994 (10^{-6} M) to 6.9 ± 7.4 % ($p < 0.05$, n = 8), compared to capsaicin alone. However, neither of these antagonists had any effect on the vasoconstrictor response to capsaicin. The vasodilator response of feline pial arteries to CGRP (10^{-8} M) was significantly reduced in the presence of $CGRP_{8-37}$ by 73 % (10^{-6} M, $p < 0.05$, n = 6), compared to CGRP alone. CP-99,994 (10^{-6} M) reduced SPOMe-induced vasodilatation by 94 % (10^{-6} M, $p < 0.05$, n = 6), compared to SPOMe alone. These results provide evidence of a vasodilator role for both CGRP and substance P in feline cerebrovasculature following capasicin-induced activation of trigeminal nerves.

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314P GANGLION BLOCKADE INHIBITS NK RECEPTOR-DEPENDENT BLADDER CONTRACTIONS IN THE ANAESTHETISED GUINEA-PIG

M. Metcalfe, A.M. Naylor & M. Bushfield, Department of Discovery Biology, Pfizer Central Research, Sandwich, Kent, CT13 9NJ.

Rat and guinea-pig bladder are richly innervated by capsaicin-sensitive primary afferent neurones which are characterised by high levels of tachykinin immunoreactivity (Holzer et al, 1982) and NK_2 receptor binding sites (Carstairs et al, 1993). In the anaesthetised guinea pig, NK_2 receptor-dependent bladder contractions are indirect and are inhibited by pretreatment with atropine and [αβ-Me]ATP (Metcalfe et al, 1994). Therefore, the effects of NK_2 receptor stimulation on the bladder are mediated either by enhancement of efferent transmitter release or by activation of the micturition reflex arc. In the present study we have used ganglion blocking agents to explore further the site of action of the selective NK_2 receptor agonist, $[\beta ala^8]NKA_{(4-10)}$, in this system. Female Dunkin Hartley guinea pigs (350-450g) were anaesthetised with urethane (1.2g/kg i.p.), the carotid artery was cannulated for blood pressure recording and the left jugular vein cannulated for i.v. drug administration. After tying off the external urethra, a catheter was inserted via the dome into the bladder for intraluminal pressure recording. The guinea pigs were artificially ventilated and the tracheal insufflation pressure recorded.

Administration of the NK_2 receptor agonist $[\beta ala^8]NKA_{(4-10)}$ caused "all or nothing" biphasic contractions of the bladder, with the threshold dose being approximately 1 to 5μg/kg. Bladder or tracheal contractions were unaffected by the NK_1 receptor antagonist, SR140333 (10-1000μg/kg) but were abolished by pretreatment with the NK_2 receptor antagonist SR48968 (1-300μg/kg) at doses of 30μg/kg and above. Hexamethonium (0.1-30mg/kg) treatment 5 min prior to $[\beta ala^8]NKA_{(4-10)}$ (5μg/kg) had no effect on the increase in tracheal pressure. However, there was a dose-dependent inhibition of the bladder response, giving a maximal degree of inhibition of 80±6% at 30 mg/kg (mean±s.e.m., n=7). Similar results were seen with chlorisondamine (1-1000 μg/kg) treatment which produced dose-dependent inhibition of $[\beta ala^8]NKA_{(4-10)}$ induced bladder (ED_{50}=65.4 ±21.0μg/kg, mean±s.e.m., n=4) but not tracheal responses, giving a maximal degree of inhibition of 88±3% at 1 mg/kg (mean±s.e.m., n=7).

In conclusion, in the anaesthetised guinea pig $[\beta ala^8]NKA_{(4-10)}$ produces reflex contractions of the urinary bladder by an action on pre-ganglionic efferent or afferent NK_2 receptors. Since autoradiographic studies have identified NK_2 receptor sites on capsaicin-sensitive afferent nerves in the bladder mucosa (Carstairs et al, 1993), it is likely that activation of these neuronal NK_2 receptors underlies the stimulation of the micturition reflex by $[\beta ala^8]NKA_{(4-10)}$, seen in this study.

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315P BEHAVIOURS ELICITED BY INFUSION OF TACHYKININ RECEPTOR AGONISTS INTO MIDBRAIN DOPAMINE REGIONS IN RATS

N.M.J. Rupniak, S. Boyce, A. Jackson & D. Pearce, Neuroscience Research Centre, Merck Sharp & Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR.

Evidence that substance P may modulate cerebral dopamine function suggests possible neuropsychiatry utilities for tachykinin NK1 receptor antagonists. Elliott *et al* (1991) obtained evidence for a selective activation of the mesolimbic dopamine system (locomotor stimulation and an increase in dopamine turnover) following infusion of the NK1 receptor agonist, GR73632, into the ventral tegmental area (VTA) of rats. However, other findings suggest that locomotor stimulation is induced by intra-VTA infusion of NK3, but not NK1, receptor agonists (Stoessl *et al*, 1991). We have investigated whether methodological differences might explain these conflicing interpretations.

Sprague-Dawley rats were anaesthetised using isoflurane to permit insertion of a 25 gauge hypodermic needle to a depth of 4.5 mm at bregma for i.c.v. infusion of GR73632 (0.01-1nmol). Behaviours were observed for 10 min after recovery from anaesthesia. In separate rats, bilateral guide cannulae aimed at the VTA (AP -4.8 mm from bregma, ML ± 1.0 mm from midline, and DV -7.5 mm from skull) or the substantia nigra (SN; AP -5.3, ML ± 2.5 and DV -7.2) were implanted under anaesthesia. At least one week later, rats were habituated to automated photocell cages (30 x 20 x 30 cm) for 1 h prior to infusion of GR73632 (0.1-3 nmol) or the NK3 receptor agonist, senktide (0.5 nmol). Consecutive interruptions of the horizontal photocell beams were recorded as an index of locomotion, and the total number of interruptions of all photocells taken as a reflection of general activity, during the following 1 h. Data were subjected to ANOVA and Dunnett's t-tests where appropriate.

Infusion of GR73632 (0.03-1 nmol i.c.v.) induced a coarse tremor accompanied by catalepsy and profuse salivation, but did not increase locomotion. Intra-VTA or intra-SN infusion of senktide (0.5 nmol), but not GR73632 (1 nmol), increased the number of horizontal beam breaks, suggesting locomotor stimulation; both agonists increased the total number of beam breaks, reflecting general activity (Table 1). Observation of rats receiving intra-SN infusions conformed that senktide induced locomotion, sniffing and wet dog shakes, whilst GR73632 induced only grooming.

These findings and others suggest that the tachykinin receptors in the VTA and SN mediating hyperlocomotion are of the NK3, rather than the NK1, subtype. The proposal that NK1 receptors selectively modulate the mesolimbic dopamine system in rats is not supported by thest data.

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Table 1

	Consecutive Horizontal Beam Breaks/1h		Total Photocell Interruptions/1h	
	VTA	SN	VTA	SN
Vehicle	7.7 ± 1.7	9.5 ± 3.0	89.8 ± 24.0	118.6 ± 26.0
GR73632 (1nmol)	28.8 ± 11.0	17.0 ± 6.1	258.8 ± 67.9*	290.0 ± 73.3*
Senktide (0.5nmol)	54.1 ± 13.0*	34.8 ± 5.8*	322.9 ± 56.0*	400.5 ± 31.3*

*p < 0.05 compared with vehicle-treated control animals, Dunnett's t-test (n = 3-8).

316P THE ROLE OF NEUROKININ AND METABOTROPIC GLUTAMATE RECEPTORS IN THERMAL AND MECHANICAL PAW WITHDRAWAL RESPONSES IN NORMAL AND CARRAGEENAN-TREATED RATS

M.R.Young, S.M. Fleetwood-Walker, P.J. Birch[1], C. Bountra[2] & J. Bowers[2], Department of Preclinical Veterinary Sciences, University of Edinburgh; [1]Department of Molecular Science (Greenford), [2]Pharmacology I (Ware), Glaxo Group Research.

In view of previous electrophysiological evidence that NK_1, NK_2 and metabotropic glutamate (mGlu) receptors contribute to nociceptive inputs to dorsal horn neurons (Fleetwood-Walker *et al.* 1993; Thompson *et al.* 1993; Young *et al.* 1994), we assessed whether antagonists at these receptors were effective in normal and carrageenan-inflamed models of thermal and mechanical nociception. Lister-hooded rats (30-70g) were intrathecally (i.t.) injected (10µl) with vehicle (saline or 0.3% dimethylsulphoxide in saline) or drug, following measurement of baseline paw withdrawal latencies (PWL) for both noxious mechanical and thermal stimuli, applied to the dorsal hairy foot. A 100µl ipsilateral sub-cutaneous injection of 2% lambda-carrageenan was given under brief halothane anaesthesia. Paw withdrawal latencies measured 3 hours later showed an average decrease of approximately 50% from pre-carrageenan levels. Drug effects were then tested 1,3, and 5 mins after i.t. injection. In the non-inflamed state, the NK_2 receptor antagonist L659874 (3nmol, n=6) raised thermal (but not mechanical) PWL by 83±26% above pre-drug baseline levels ($P<0.01$ Mann-Whitney U-test, with respect to vehicle controls). In carrageenan-treated animals, L659874 (n=14) resulted in similar changes in thermal PWL (+91±21%, $P<0.01$). The mGlu receptor antagonist L-1-amino-3-phospho-propanoic acid (L-AP3; 60nmol, n=14) was ineffective, whereas (S)-4-carboxy-3-hydroxyphenylglycine ((S)CHPG; a second mGlu antagonist, 48nmol, n=14) showed increased mechanical and thermal PWLs in the non-inflamed group (+49±10%, $P<0.05$ and +40±8%, $P<0.01$, respectively). With carrageenan treatment, L-AP3 (n=14) was now found to increase PWL to both mechanical and thermal stimulation (+156±58, $P<0.05$ and +97±6%, $P<0.01$, respectively). The effects of L-AP3 were not mimicked by D-AP3. Subsequent to carrageenan inflammation, (S)CHPG (n=13) produced more profound increases in mechanical and thermal PWL (+130±31%, $P<0.01$ and +74±10%, $P<0.05$, respectively). The NK_1 receptor antagonist GR82334, at intrathecal doses (0.7-1 nmol), previously shown to effectively antagonize the caudally-directed biting and scratching evoked by the NK_1 agonist substance P methyl ester (Birch *et al.* 1991), was without effect on either mechanical or thermal PWL in untreated or carrageenan-treated animals under the present experimental conditions. Nevertheless NK_1 antagonists have been indicated to play a significant role in the enhanced nociceptive responses elicited by UV-light-induced inflammation or C-afferent conditioning stimulation (Thompson *et al.* 1994; Xu *et al.* 1992). These data are consistent with a role for NK_2 receptors in the transmission of brief thermal nociceptive inputs and for mGlu receptors in both mechanical and thermal nociceptive responses in this carrageenan model.

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317P EFFECT OF LAMOTRIGINE ON THERMAL HYPERALGESIA IN RATS WITH PERIPHERAL MONONEUROPATHY

S. Neale, A. Wyatt and M. Nakamura-Craig, Systems Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS.

Lamotrigine (Lamictal†), a new antiepileptic drug (Lees & Leach, 1993), has been shown to exert analgesic effects on mechanical hyperalgesia in the model sustained hyperalgesia induced by PGE_2 and in diabetic rats (Nakamura-Craig & Follenfant, 1994). We have now investigated the effect of lamotrigine on thermal hyperalgesia in rats with peripheral mononeuropathy. In addition the effects of carbamazepine are compared to lamotrigine in this model.

The peripheral neuropathy following sciatic nerve ligation was produced according to the method of Bennett & Xie (1988) in male Wistar rats (240-260g) under isoflurane anaesthesia. For intrathecal (IT) administration of drug, the IT cannulae were implanted 7 days after the sciatic nerve ligation, using a modified technique described by Yaksh & Rudy (1976). Drugs injected intrathecally were given 4 days after the IT surgery in 10 µl followed by 4 µl of saline. Hyperalgesia was determined by the paw withdrawal response (reaction time, s) to noxious radiant heat focussed on the subplantar surface of the paw using a modification of the technique of Hargreaves *et al* (1988).

The reaction time in control rats was 8.7 ± 0.2s (mean ± s.e.mean, n=20) and in rats with the sciatic ligation the reaction time was significantly reduced to 4.14 ± 0.1s ($P<0.001$, n=20). Oral administration of lamotrigine (10, 20 and 40 $mgkg^{-1}$), seven days after the sciatic ligation, produced analgesia (reaction times at 3h: 6.3 ± 0.5s , 7.6 ± 0.5s and 7.8 ± 0.4s, n=5, $P<0.001$, respectively) and was still effective at 5h with 20 $mgkg^{-1}$. Oral administration of carbamazepine (80 $mgkg^{-1}$) was ineffective (3h: 4.4 ± 0.3s, $P<0.5$). Intrathecal injection of lamotrigine (50 µg) also induced analgesia (at 3h: 7.8 ± 0.3s, n=5, $P<0.001$). This effect was still present 5h later. IT administration of carbamazepine (100µg) was ineffective (at 3h: 4.8±0.3s). A single dose of lamotrigine given orally (10 and 20 $mgkg^{-1}$) 1h before the ligation prevented the development of hyperalgesia (7 days post-surgery: 7.8 ± 0.7s and 7.4 ± 0.5s, n=5, $P<0.01$, respectively). In contrast carbamazepine (20, 40 and 80 $mgkg^{-1}$ p.o.), or a lower dose of lamotrigine (5 $mgkg^{-1}$ p.o.) were ineffective.

These results suggest a therapeutic role for lamotrigine in the treatment of neuropathic pain. Furthermore, lamotrigine may be effective as a pre-emptive treatment in conditions such as post-herpetic neuralgia.

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† This is a Trade Mark of Wellcome Group companies.

318P THE POSTNATAL DEVELOPMENT OF CALCIUM-DEPENDENT GLUTAMATE RELEASE FROM RAT CORTICAL SYNAPTOSOMES

Paul Jennings & Keith J. Collard, Physiology Unit, School of Molecular and Medical Biosciences, University of Wales College of Cardiff, P.O. Box 911, Cardiff CF1 3US.

Previous studies in this laboratory demonstrated that the release of glutamate in response to K^+ depolarisation in hippocampal and cortical synaptosomes varied considerably during postnatal development (Collard et al. 1993). The study did not however examine the Ca^{2+}-dependency of the release process at differing developmental ages. In order to further clarify the developmental changes in glutamate release, this study has investigated the Ca^{2+}-dependency of glutamate release from cortical synaptosomes of rats aged between postnatal day 5 (PND5) and adult. Using the same experimental techniques as used in the previous study (Collard et al. 1993), we have examined the release of [^{3}H]-glutamate from preloaded and superfused synaptosomes in the presence and absence of extracellular Ca^{2+}. The results of this study are shown in Table 1.

Table 1. The release of [^{3}H]-glutamate from preloaded and superfused synaptosomes in response to a 30 second depolarising pulse of 25mM K^+ (replacing Na^+) in the presence and absence of extracellular Ca^{2+}. Release is expressed as the percentage of tissue [^{3}H]-glutamate released above basal, caused by the application of the K^+ pulse. Results are given as the mean ± s.e. mean of 5-7 observations. Significant differences between Ca^{2+}-rich (1.0 mM) and nominally Ca^{2+}-free conditions is given by *P<0.01, **P<0.001 (Student's t test).

Age	PND5	PND7	PND10	PND15	PND30
Ca^{2+}-rich	0.918 ± 0.089	0.769 ± 0.180	0.923 ± 0.067	0.817 ± 0.076	2.156 ± 0.183
Ca^{2+}-free	0.808 ± 0.033	0.635 ± 0.054	0.571 ± 0.029*	0.374 ± 0.058*	0.766 ± 0.063**.

The results confirm the variations seen in the previous study with respect to the amount of transmitter released by depolarisation at different postnatal ages (Collard et al. 1993). Release at PND30 was very similar to that seen in the adult (Ca^{2+}-rich 2.359 ± 0.206, Ca^{2+}-free 0.745 ± 0.086; $P<0.01$). In addition, the study shows that depolarisation induced release of glutamate is not Ca^{2+}-dependent during early postnatal development. Significant differences between release in Ca^{2+}-rich and Ca^{2+}-free conditions was only apparent from PND10. The results indicate that the development of Ca^{2+}-dependent release correlates well with the appearance of synaptic vesicles in synaptosomes (Gonatas et al. 1971), and with the uptake of glutamate by synaptic vesicles (Kish et al. 1989).

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319P DIFFERENTIAL EFFECTS OF CNQX AND AP5 ON THE EXPRESSION OF *C-FOS*-LIKE IMMUNOREACTIVITY IN THE ISOLATED RAT SPINAL CORD

Li Ping Zhang, R. G. Williams J. Bagust, Department of Physiology and Pharmacology, Southampton University. Southampton, SO16 7PX.

There is increasing evidence of an involvement of NMDA receptors in the modulation of spinal responses to nociceptive stimuli (Urban *et al.*, 1994). The proto-oncogene *c-fos* is expressed in some dorsal horn neurones following noxious peripheral stimulation or high intensity afferent stimulation and we have previously demonstrated its localisation in mammalian isolated spinal cord preparations in response to high intensity dorsal root stimulation (Zhang *et al,* 1993). In this present study we have investigated the effects of the NMDA receptor antagonist AP5, and the non-NMDA receptor antagonist CNQX, on *c-fos* expression in an isolated preparation of juvenile rat spinal cord.

Spinal cords were removed from 18-20 day old rats weighing 30-40gm and maintained *in vitro* at 27°C. After an incubation period of 2 hours field potentials were recorded from the dorsal horn of the lumbar cord using blunt microelectrodes and the threshold stimulus for dorsal root stimulation determined. 50μM CNQX or 50μM AP5 was added to the bathing medium and trains of electrical stimuli (0.5ms pulses, 10Hz for 10 min) were delivered to a lumbar dorsal root at an intensity twenty times the threshold stimulus. Control cords were treated in the same way, but no AP5 or CNQX was added. After a further two hours incubation cords were processed for *c-fos* like immunoreactivity and counts made of the number of dorsal horn cells expressing *c-fos* like activity on the stimulated side of the lumbar cord. Cell counts were also obtained from cords incubated in medium in which the calcium was replaced by manganese (2mM) to block synaptic activity.

In control cords dark staining cells expressing *c-fos* like immunoreactivity were present in the the outer layers of the dorsal horn (Laminae I-IV). The mean number of positive cells per section was (53.7, ± 3.4, S.E.M, n=42, 2 experiments). In 3 cords incubated in manganese no *c-fos* positive cells were found.

Cords incubated in the presence of 50μM CNQX to block non-NMDA excitatory amino acid receptors displayed a band of positive staining cells in the dorsal horn. The mean cell count per section (51.2 ± 2.0, S.E.M, n=91, 4 experiments), was not significantly different from the control data ($p>0.1$, t-test). Incubation of cords in 50μM AP5 caused a significant reduction in the number of dorsal horn cells showing *c-fos* like immunoreactivity (22.0 ± 1.25, S.E.M, n=103, 4 experiments), when compared to the control data, ($p<0.001$, t-test).

These results support the concept that NMDA receptors might be involved in modulation of the processing of nociceptive information in the mammalian spinal cord.

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320P THE PHARMACOLOGICAL PROFILE OF THE GLYCINE/NMDA RECEPTOR ANTAGONIST, L-701,324

K.Saywell, L.J.Bristow, A.C.Foster, S.Grimwood, R.G.Hill, P.H.Hutson, J.Kulagowski, P.D.Leeson & M.D.Tricklebank, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR.

4-Hydroxyquinolones substituted in the 3 position are high affinity glycine/NMDA receptor antagonists (Kulagowski *et al.*, 1994). Here we describe some of the in vitro and in vivo properties of one of these compounds, L-701,324 (7-chloro-4-hydroxy-3-(3-phenoxy)phenylquinolin-2-(1H)-one).

Affinity for the strychnine-insensitive glycine site *in vitro* was determined by displacement of [^{3}H]L-689,560 binding to rat cortical/hippocampal or human cortical membranes (Grimwood, *et al.*, 1992). L-701,324 was also examined for its ability to interact with sites labelled by [^{3}H]dizocilpine, [^{3}H]AMPA, [^{3}H]kainate and [^{3}H]strychnine (Foster et al., 1992). Anticonvulsant studies were carried out in audiogenic seizure prone DBA/2 mice or against seizures induced by NMDLA in Swiss Webster mice with concomitant assessment of motor coordination using a rotarod apparatus (Tricklebank *et al.*, 1994).

L-701,324 displaced [^{3}H]L-689,560 binding with an IC_{50} (geometric mean (- s.e. mean, + s.e. mean, n = 4)) of 1.97 (1.83, 2.13) nM and 2.55 (1.54, 4.22) nM in rat and human brain membranes respectively but was without activity at amino acid receptors labelled by [^{3}H]AMPA, [^{3}H]kainate or [^{3}H]strychnine (IC_{50} > 100 μM). Under non-equilibrium conditions, L-701,324 completely inhibited [^{3}H]dizocilpine binding (EC_{50} = 3.4 (3.2, 3.6) nM), consistent with an antagonist action at the glycine site. In the DBA/2 mouse, L -701,324 given 30 min before test antagonised audiogenic seizures with ED_{50}s of 0.9 mg/kg, i.p. and 1.8 mg/kg, p.o. Increasing the pretreatment time to 10 h increased the oral ED_{50} less than four fold to 7.1 mg/kg. L-701,324 also antagonised seizures induced by NMDLA (ED_{50} = 3.4 mg/kg, i.v. and 18 mg/kg, p.o. given 15 and 30 min beforehand respectively). Pretreatment with D-serine (100 μg/mouse, i.c.v.) immediately after L-701,324 (5 mg/kg, i.v.) and 15 min before NMDLA, decreased the latency to convulse from 26.5 ± 1.6 min (mean ± s.e. mean, n = 8 per group) to 10.7 ± 0.8 min ($p < 0.05$, Dunnett's t test), consistent with the anticonvulsant effects being mediated by blockade of the glycine receptor. The minimum effective dose of L-701,324 to induce a significant decrease in latency to fall from the rotarod was 3 mg/kg, i.v. and 20 mg/kg, p.o. in the Swiss Webster mouse, whilst in DBA/2 mice significant motor incoordination in general occurred at doses approximately two fold greater than the ED_{50} to block audiogenic seizures

Thus, L-701,324 is an orally active and long acting anticonvulsant with high affinity and selectivity for the glycine/NMDA receptor.

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321P THE GLYCINE/NMDA RECEPTOR ANTAGONIST, L-701,324, BLOCKS THE NEUROCHEMICAL AND BEHAVIOURAL EFFECTS OF AMPHETAMINE IN RODENTS

L.J. Bristow, K.L. Flatman, L. Young, L. Thorn, P.H. Hutson & M.D. Tricklebank, Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Rd., Harlow, Essex, CM20 2QR.

We have previously shown that R-(+)-HA-966, a low efficacy partial agonist at the glycine/NMDA receptor complex, blocks amphetamine-induced activation of mesolimbic dopamine systems (Hutson *et al.* 1991). In the present study, we have examined whether the glycine/NMDA receptor antagonist, L-701,324 (7-chloro-4-hydroxy-3(3-phenoxy)phenyl-2(H) quinolone), will attenuate the increase in dopamine synthesis and the hyperactivity or stereotyped behaviour induced following amphetamine injection in rodents.

Male BKTO mice (20-30g) were orally dosed with L-701,324 (5 mg/kg) or vehicle (0.5% methylcellulose; 10 ml/kg) followed by d-amphetamine sulphate (2.5 mg/kg, s.c.) or saline (10 ml/kg) 20 min later. After a further 20 min, mice received the DOPA decarboxylase inhibitor, NSD 1015 (100 mg/kg, i.p.), were killed 30 min later and the nucleus accumbens and striatum dissected and analysed for DOPA content using HPLC. Locomotor activity was recorded in mice and in male Sprague Dawley rats (250-320g) implanted with bilateral guide cannulae for intra-accumbens injections (AP +1.7mm, L ± 1.5mm, V -5.5 mm from dura, Paxinos and Watson). Animals were habituated to appropriate photocell activity cages and then orally dosed with L-701,324 (0.3-10 mg/kg in mice or 5 mg/kg in the rat) or vehicle (3 ml/kg in rats, 10 ml/kg in mice). Sixty min later, mice received a subcutaneous injection of d-amphetamine (5 mg/kg) and rats were infused with either amphetamine (5 µg/0.5 µl/side) or saline (0.5 µl/side) directly into the nucleus accumbens. Locomotor activity was recorded in 10 min intervals for up to 2h post-injection. Stereotyped behaviour was examined in rats implanted with bilateral guide cannulae for microinjection into the striatum (AP + 0.2mm, L ± 3.4mm, V-6mm from dura) and was defined as the time spent sniffing + the time spent licking and/or biting. Animals were habituated to individual observation boxes, infused with d-amphetamine (40 µg/µl per side) 60 min after pretreatment with L-701,324 (30 mg/kg, p.o.) or vehicle (3 ml/kg, p.o.) and observed for the following 30 min.

In mice, pretreatment with L-701,324 (5 mg/kg) significantly attenuated amphetamine-induced DOPA accumulation in both the nucleus accumbens (mean DOPA concentration (ng/g) ± s.e.mean (n = 5/6 mice/group): veh/sal = 1481 ± 103*, veh/amph = 2379 ± 92; 324/sal = 1518 ± 73*, 324/amph = 1503 ± 95*; *P<0.05 compared to veh/amphetamine treated mice, ANOVA + Tukeys t test) and striatum (veh/sal = 1205 ± 91*, veh/amph = 2859 ± 195; 324/sal = 1117 ± 72*, 324/amph = 1599 ± 196*). Oral administration of L-701,324 also dose dependently attenuated amphetamine-induced hyperactivity in the mouse (ED_{50} = 1 mg/kg, n = 8 mice/group) and blocked the marked increase in cage crossings recorded following amphetamine infusion into the rat nucleus accumbens (veh/saline = 24 ± 5*; veh/amphetamine = 280 ± 31; 324/saline = 17 ± 4*; 324/amphetamine = 60 ± 13*, *P<0.05 compared to veh/amphetamine treated rats, ANOVA + Dunnetts t test). In contrast, the marked stereotyped behavioural response observed following amphetamine infusion directly into the rat striatum was not significantly altered in rats pretreated with a high dose (30 mg/kg, p.o.) of L-701,324 (mean duration of stereotyped responding (sec) ± s.e.mean: veh/amph = 1848 ± 1134; 324/amph = 1708 ± 133).

In conclusion, these results demonstrate that the glycine/NMDA receptor antagonist, L-701,324, attenuates amphetamine-induced activation of mesolimbic dopaminergic systems in rodents.

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322P L-701,324, A GLYCINE/NMDA RECEPTOR ANTAGONIST, ATTENUATES ACTIVATION OF THE MESOLIMBIC DOPAMINE SYSTEM BY PHENCYCLIDINE

P. H. Hutson, L. Thorn, L. J. Bristow and M. D. Tricklebank Merck,Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow , Essex CM20 2QR.

Previous studies have demonstrated that activation of mesolimbic dopamine (DA) metabolism and motor activity by phencyclidine (PCP) was prevented by pretreatment with R(+)-HA-966, a low efficacy partial agonist at the glycine/NMDA receptor complex (Bristow et al.,1993). We have now examined the effects of L-701,324, (7-chloro-4-hydroxy-3-(3-phenoxy)phenyl quinolin-2-(1H)-one), a selective and high affinity antagonist at the glycine/NMDA receptor (Saywell et al.,1994) on the PCP induced increase of mesolimbic DA metabolism and locomotor activity in rodents.

Male Sprague-Dawley rats (250-300g) were pretreated with either vehicle (0.5% methylcellulose,1 ml/kg i.p.) or L-701,324 (5 mg/kg i.p.).Twenty min. later animals received either vehicle (0.9%NaCl,1 ml/kg i.p.) or PCP hydrochloride (10 mg/kg i.p.) and were killed after a further 30 min.Brains were removed, dissected into n.accumbens (n.Ac.) and striatum and analysed for DA and its metabolites DOPAC and HVA by HPLC.The ratio [DA]/[DOPAC]+[HVA] provided an index of DA metabolism. Male BKTO mice(20-30g) were habituated to photocell cages (23x28x21 cm) for 2h before pretreatment with either vehicle (25% PEG 300 in saline;10ml/kg i.p.) or L-701,324 (5mg/kg i.p.). Thirty min later, mice received either vehicle (0.9% NaCl;10ml/kg i.p.) or PCP (10mg/kg i.p.).Locomotor activity (cage crosses) was recorded for 10 min periods upto 2h.

Consistent with previous studies (Bristow etal.,1993), PCP (10mg/kg i.p.) increased DA metabolism in n.Ac. but not striatum.In contrast, L-701,324 (5mg/kg i.p.) did not affect DA metabolism in either brain region but significantly attenuated the increase of n.Ac. DA metabolism by PCP(table 1).In the mouse , PCP (10mg/kg i.p.) but not L-701,324 (5mg/kg i.p.) markedly increased the number of cage crossings and the increase induced by PCP was significantly attenuated by pretreatment with L-701,324 (table 1).

In conclusion, L-701,324 attenuated the neurochemical and behavioural effects of PCP providing further support for a role of NMDA receptors in the regulation of mesolimbic dopaminergic function.

Bristow,L.J. *et al.* (1993) Br. J. Pharmacol. 108,1156-1163.
Saywell *et al.* (1994) Br. J.Pharmacol. This meeting.

Table 1

treatment	[DA]/[DOPAC]+[HVA] striatum	n.Ac	cage crossing No. in 1 h
vehicle /vehicle	0.21 ± 0.02	0.25 ± 0.03 *	7 ± 7 *
vehicle/PCP	0.21 ± 0.01	0.39 ± 0.01	346 ± 67
L-701,324/vehicle	0.23 ± 0.01	0.26 ± 0.01 *	4 ± 2 *
L-701,324/PCP	0.20 ± 0.01	0.30 ± 0.02 *	129 ± 34 *

Results are means± s.e.mean.*P<0.05 compared with vehicle/PCP group by ANOVA and Tukey's (neurochemistry, n=5/6/group) or Dunnett's t test (behaviour, n=8/group).

323P THE GLYCINE/NMDA RECEPTOR ANTAGONIST, L-701,324, REVERSES ISOLATION-INDUCED DEFICITS IN PREPULSE INHIBITION OF THE ACOUSTIC STARTLE RESPONSE IN THE RAT

L.J. Bristow, L. Landon, K.L. Saywell & M.D. Tricklebank, Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Rd., Harlow, Essex, CM20 2QR.

The inhibition of the acoustic startle response by pre-exposure to a non-startle eliciting stimulus (prepulse inhibition, PPI) is significantly attenuated in schizophrenic patients and in rats following systemic injection of dopamine receptor agonists. Impaired PPI has also been reported in rats reared in social isolation following weaning, environmental conditions which induce both neurochemical and behavioural abnormalities suggestive of a physiologically-induced dopaminergic hyperactivity (Geyer et. al., 1993). Given that the glycine/NMDA receptor antagonist, L-701,324 (7-chloro-4-hydroxy-3(3-phenoxy)phenyl-2(*H*)quinolone), attenuates psychostimulant-induced activation of mesolimbic dopamine systems (Bristow et. al., this meeting), we have determined whether L-701,324 will also reverse isolation-induced deficits in PPI in the rat.

Male Lister rats (aged 21/22 days) were housed singly or in groups of 5 and kept in the same temperature and humidity controlled room for 8 weeks prior to testing. Startle responses were recorded 10 min after injection of either L-701,324 (0.5 or 1 mg/kg, i.p.) or vehicle (25% PEG 300; 1ml/kg, i.p.) using 6 SR-LAB (San Diego Instruments) stabilimeter chambers. Following an initial 5 min acclimation period to background noise (65dB), rats were exposed to 15 repetitions of 4 different trial types; i) a 30 msec, 80 dB prepulse, ii) the same prepulse followed 100 msec later by a 40 msec, 140 dB tone (prepulse + pulse), iii) the 140 dB stimulus alone (pulse) and iv) a period when no stimulus was presented. Stimuli were presented in a random order for each subject (n = 11/12 rats per group) and PPI calculated as the % reduction in the magnitude of the startle response (i.e. [pulse - (prepulse + pulse)]/pulse x 100) and analysed by 2 way analysis of variance with simple main effect contrasts.

The startle amplitude of vehicle-pretreated, group-housed rats was reduced by 59 ± 5 % when a 140 dB stimulus was preceded by an 80 dB prepulse (mean startle amplitude ± S.E.M. in arbitary units: pulse = 243 ± 20; prepulse + pulse = 96 ± 13). In contrast, PPI was significantly ($P<0.05$) reduced to 42 ± 4 % in rats reared in social isolation (pulse = 262 ± 27; prepulse + pulse = 150 ± 16*, *$P<0.05$ compared to prepulse + pulse responding in vehicle-pretreated, group-housed rats). In the absence of any effect in group-housed animals (mean PPI ± S.E.M.: veh = 59 ± 5 %; 0.5 mg/kg L-701,324 = 61 ± 2 %; 1 mg/kg L-701,324 = 61 ± 3 %), pretreatment with L-701,324 dose-dependently and significantly reversed the isolation-induced deficits in PPI (veh = 42 ± 4 % *; 0.5 mg/kg L-701,324 = 45 ± 4 % *; 1mg/kg L-701,324 = 56 ± 3 %, *$P<0.05$ compared to vehicle-pretreated, group-housed rats). This effect resulted from a marked reduction in the increased startle response to the prepulse + pulse stimuli seen in isolates pretreated with vehicle (veh = 150 ± 16*; 0.5 mg/kg L-701,324 = 112 ± 11; 1 mg/kg L-701,324 = 84 ± 9, *$P<0.05$ compared to vehicle-pretreated group-housed rats). In contrast, pretreatment with L-701,324 did not significantly alter startle responses to the 140 dB pulse, the prepulse-alone or cage displacement recorded when no stimulus was presented in either group-housed or isolated rats.

In conclusion, the present study shows that L-701,324 reverses isolation-induced deficits in PPI. Given that this effect may result from the physiological induction of dopaminergic hyperactivity, these results provide further evidence that glycine/NMDA receptor antagonists attenuate the activation of mesolimbic dopamine systems in the rat.

Geyer, M.A. et. al., (1993) Biol. Psychiatry, 34, 361-372.

324P INHIBITION OF SOUND-INDUCED SEIZURES AND *C-FOS* EXPRESSION IN DBA/2 MICE BY AN ANTISENSE PROBE TO NMDA-R1 RECEPTOR SUBUNIT

A.G. Chapman, V.L. Woodburn[1], G.N. Woodruff[1] & B.S. Meldrum, Department of Neurology, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF, UK, and [1]Parke-Davis Neuroscience Research Center, Addenbrooke's Hospital, Cambridge CB2 2QB, UK

The NMDA receptor complex is comprised of a heteromeric combination of NMDA-R1 (NR1) and any of NMDA-2A/2B/2C/2D subunits. The NMDA receptor subunits have different developmental profiles, and different anatomical distribution, and the recombinant subunits differ in their pharmacology. All sub-classes of NMDA antagonists have potent anticonvulsant activity in experimental seizure models, but the NMDA antagonists so far tested in man have caused adverse side effects at therapeutic doses. Subunit-selective antagonists may have a better therapeutic index. The currently available NMDA antagonists show only modest preference for specific subunit recombinations. NMDA subunit expression can also be modified by antisense probes specific for subunit mRNA. Pretreatment with an NR1 antisense probe can reduce the infarct volume due to MCA occlusion in rat (Wahlestedt et al., 1993). We have utilized the same antisense oligodeoxynucleotide (an 18-mer, NR1AS/c, corresponding to nucleotides 4-21, directly following translation initiation code) to protect against seizures in DBA/2 mice.
DBA/2 mice were pretreated with 2 x 1 μg NR1 antisense or NR1 nonsense (scrambled deoxynucleotide sequence) probes by repeated (-29 and -7 hrs before testing for seizure response) intracerebroventricular (icv) 5 μl injections under light ether anaesthesia. Mice in the control group received 2 x 5 μl saline icv (n = 14-15 per group). DBA/2 mice were tested for sound-induced clonic seizures by exposure to a 108 dB stimulus.

Table 1
Protection against clonic seizures in DBA/2 mice by NR1 antisense pretreatment (-29 and -7 hrs; 2 x 1 μg, icv)

	Clonic seizures
2 x 5 μl vehicle	14/14 (100%)
2 x 1 μg NR1 antisense	1/14 (7%)
2 x 1 μg NR1 nonsense	14/15 (93%)

Repeated (but not a single administration at -24 hrs) icv administration of NR1 antisense therefore provided almost complete anti-convulsant protection in DBA/2 mice.
NMDA receptor levels were assessed by receptor autoradiography ([^{3}H]-MK 801) in the same groups of mice, and significant reduction (-20%) was observed in cerebral cortex. Expression of *c-fos* that was detected in thalamic nuclei and inferior colliculus of vehicle and NR1 nonsense-treated mice was completely blocked by NR1 antisense pretreatment.

Wahlestedt et al. (1993) Nature 363, 260-263

325P EFFECT OF CORTICAL ADENYLYL CYCLASE-COUPLED METABOTROPIC GLUTAMATE RECEPTOR AGONISTS ARE REVERSED BY 4-CARBOXY DERIVATIVES OF PHENYLGLYCINE COMPOUNDS

M.C. Kemp, D.E. Jane, H-W. Tse, P. J. Roberts and J. C. Watkins, Department of Pharmacology, University of Bristol, Bristol BS8 1TD.

L-2-amino-4-phosphonobutyric acid (L-AP4) and (2S,3S,4S)-α-(carboxycyclopropyl)-glycine (L-CCG-1) have been demonstrated to have agonist effects at metabotropic glutamate receptors negatively coupled to adenylyl cyclase present in rat cortical slices (Kemp *et al.*, 1994). L-AP4 and L-CCG-1 have also been demonstrated to depress the monosynaptic excitation of neonatal rat motoneurones (Evans *et al.*, 1982; Ishida et al., 1992) presumably by a presynaptic mechanism. This depression can be blocked by phenylglycine derivatives (Birse et al., 1993). Previously we have reported the effects of a series of 3-carboxyphenylglycine derivatives; we now present the effects of a representative series of 4-carboxy analogues.

The adenylyl cyclase present in adult rat cortical slices (0.3 x 0.3mm, preloaded with [^{3}H]adenine) was stimulated with 30μM forskolin in the presence of 10μM L-AP4 or 0.3μM L-CCG-1 (corresponding to EC_{50} values for cyclic AMP inhibition in this system), potential antagonists, plus adenosine deaminase to remove any adenosine produced. Accumulated tritiated cyclic AMP was isolated by ion exchange chromatography and estimated by liquid scintillation counting. Results were expressed as a percentage of maximum stimulated cyclic AMP above basal. Reversal of L-AP4 and L-CCG-1 agonist effects by six 4-carboxy derivatives of phenylglycine are presented as EC_{50} values in table 1.

These data suggest that 4-carboxy derivatives of phenylglycine compounds may discriminate mixed populations of mGluRs and that these compounds may be used as a basis for further more specific ligand development.

Birse E.F. *et al.*, (1993) *Neuroscience* 52, 481-488.
Evans R.H. *et al.*, (1982) *Br. J. Pharmacol.* 75, 65-75.
Ishida M. *et al.*, (1992) *Soc. Neurosci. Abst.* 18, 650.
Kemp M.C. *et al.*, (1994) *Br. J. Pharmacol.* 113, 147P.

Table 1. EC_{50} (μM) ± SEM values for test compounds against 10μM L-AP4 or 0.3μM L-CCG-1

	L-AP4	L-CCG-1
(RS) α ethyl-4-carboxyphenylglycine ((RS)αE4CPG)	18.4± 9.5	1.63±0.54
(RS) α methyl-3-chloro-4-carboxyphenylglycine ((RS)αM3Cl4CPG)	76.6 ±14.7	> 100
(RS) α methyl-4-carboxy-3-hydroxyphenylglycine ((RS)αM4C3HPG)	> 100	83.2±40.3
(RS) α methyl-4-carboxymethylphenylglycine ((RS)αM4CMPG)	376.9 ± 37.6	197.1±128.4
(RS) 2-iodo-4-carboxyphenylglycine ((RS)2I4CPG)	> 100	> 100
(S) 4 - carboxyphenylglycine ((S)4CPG)	> 100	> 100

326P THE EFFECTS OF METABOTROPIC GLUTAMATE RECEPTOR ACTIVATION ON SECOND MESSENGER FORMATION IN HUMAN BRAIN

D.A.Kendall, P.J.Millns and J.L.Firth[1], Depts. of Physiology and Pharmacology and Neurosurgery[1], Queens Medical Centre, Nottingham NG7 2UH, U.K.

We have previously described the effects of metabotropic glutamate receptor (mGluR) agonists on the activity of second messenger generating systems in guinea-pig and rat brain slices (Cartmell *et al.*, 1993). These data showed that there were species differences in the responses so we have extended these studies to human brain slices taken in the course of neurosurgical procedures.

Brain slices were prepared from different areas of cerebral cortex taken from patients undergoing a variety of trans-cortical procedures. The tissue was tumour-free by visual inspection. The mean age of patients was 57 years (range 4-78) and there were approximately equal numbers of males and females. Accumulations of [^{3}H]inositol phosphates and [^{3}H]cAMP from [^{3}H]inositol and [^{3}H]adenine pre-labelled slices were measured as previously described (Kendall & Firth, 1990; Kendall *et al.*, 1992).

1S,3R-ACPD (1-aminocyclopentane-1,3-dicarboxylate) produced only a small accumulation of [^{3}H]inositol phosphates (E_{max} 150 ± 18% basal, EC_{50} 76 ± 20μM, n=3). Forskolin (10μM) increased [^{3}H]cAMP accumulation by 6 ± 2 fold (n=6) and this was unaltered by either 1S,3R-ACPD (30μM) or DCG IV ((2S,1R,2R,3R)-2-(2,3-dicarboxycyclopropyl) glycine, 300nM) (n=3). L-CCG-I (2S,3S,4S)-α(carboxycyclopropyl) glycine, 300uM) (n=3) enhanced the forskolin response by 1.8 ± 0.2 fold. However, forskolin-stimulated [^{3}H]cAMP accumulation was inhibited by 57 ± 16% and by 62 ± 5% in the presence of L-AP4 (2-amino-4-phosphonobutyrate) and L-serine-O-phosphate respectively (both 1mM, n=3). L-AP4 also inhibited the [^{3}H]cAMP response to 10μM NECA (5'-N-ethylcarboximidoadenosine) with an IC_{50} of 700μM (n=2).

These data are in marked contrast to those generated in experiments using rat and guinea-pig cerebral cortex in which 1S,3R-ACPD, DCG-IV and L-CCG-I all produced marked inhibitions of forskolin-stimulated cAMP formation and this calls into question the presence of functional receptors of the $mGluR_2$ and $mGluR_3$ classes in human cortex. However the effectiveness of L-AP4 and L-SOP suggests that $mGluR_4$ or $mGluR_7$ subtypes might be represented.

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327P EFFECTS OF CHRONIC ETHANOL TREATMENT ON KAINATE BINDING SITES IN CEREBRAL CORTEX AND HIPPOCAMPUS

Rabbani M. and Little H.J. Department of Pharmacology, School of Medical Sciences, University Walk, Bristol BS8 1TD.

After withdrawal from chronic ethanol ingestion, behavioural hyperexcitability is seen, and the number of NMDA receptor-ion channel complexes in CNS is increased (Grant et al., 1990). We have also previously shown an increase in the density of AMPA receptors after chronic ethanol treatment (Rabbani and Little, 1994). In the present work we investigated the state of the kainate receptors after chronic ethanol treatment.

Male rats, Wistar strain, 150-200g, were given ethanol as a liquid diet, with 3.5% v/v ethanol for two days, and 7% v/v ethanol for a further ten days. Control rats received isocalorific control diet. Brains were removed and dissected and the tissues frozen at -25°C. At 0, 7 and 24h from cessation of chronic ethanol treatment, cortical and hippocampal membranes were prepared. One measurement of binding was then made at each withdrawal time, plus controls, per experimental day. Tissues were homogenised in Tris buffer and [^{3}H]-kainate binding measured using the method of Honoré and Drejer (1988) with 1 mM glutamate to estimate non-specific binding. Incubation was terminated after 45 min by rapid filtration through Whatman GF/B filters. The data from each binding experiment was fitted into the binding isotherm equation using the non-linear curve fitting program GraphPAD Inplot.

Values in table 1 are means ± s.e.m., (n=5 per treatment group). After chronic ethanol treatment the mean Bmax or Kd values were not changed in cortex or hippocampus at any of the time intervals, compared with controls (P>0.1, Student's t-test or ANOVA). We have previously shown a lack of changes in convulsion thresholds to kainate in mice undergoing withdrawal from chronic alcohol (Watson and Little, 1994). These results extend our previous work and indicate that there is little role for kainate receptors during withdrawal from ethanol.

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Honoré,T. and Drejer, J. (1988) J. Neurochem. 52, 457-461
Rabbani, M. and Little, H.J. (1994) Br. J. Pharmacol. 112, 481P
Watson, W.P. and Little, H.J. (1994) Alcohol Clin. Exp. Res. 18, 7.15

Table 1

	CORTEX		HIPPOCAMPUS	
Treatment	Bmax (fmol/mg protein)	Kd (nM)	Bmax (fmol/mg protein)	Kd (nM)
Controls	1039 ± 62	19.6 ± 3.5	369 ± 85	15.4 ± 4.0
Ethanol, 0h withdrawal	915 ± 59	18.0 ± 2.2	446 ± 55	14.7 ± 1.8
Ethanol, 7h withdrawal	1067 ± 64	20.6 ± 2.9	506 ± 17	17.8 ± 3.8
Ethanol, 24h withdrawal	1035 ± 89	23.6 ± 6.6	465 ± 39	13.3 ± 0.6

328P LOCAL APPLICATION OF A COMPETITIVE NMDA ANTAGONIST IN THE MEDIAL PREFRONTAL CORTEX INCREASES *IN VIVO* DA BUT NOT NA RELEASE

M.G.P.Feenstra, W.van der Weij, M.H.A.Botterblom, M.Bubser, R.P.W.Heinsbroek, Graduate School Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam ZO, The Netherlands

Activation of N-Methyl-D-Aspartate (NMDA)-type glutamate receptors has been shown to increase the release of various neurotransmitters, e.g. dopamine (DA) and noradrenaline (NA) measured in vitro and in vivo. Antagonists of these receptors, however, also activate mesocortical DA metabolism (Deutch et al, 1987) and release (Nishijima et al, 1994). In addition these antagonists may increase neuronal activity of the mesocortical DA neurons in the ventral tegmental area (VTA) and also increase behavioural activity. It is not clear how these various effects are related, and where the NMDA-antagonists act to elicit the behavioural and neurochemical effects but it has been suggested that cortical NA is not activated by NMDA-antagonists (Tanii et al, 1990).

We have studied in vivo extracellular concentrations of DA and NA in the medial prefrontal cortex (mPFC) using microdialysis in freely moving animals. The NMDA-antagonist D-AP5 (D(-)-2-amino-5-phosphonopentanoic acid) was applied locally in the dialysis fluid in increasing concentrations (0.1, 0.3, 1 mmol/l). One group of rats was used to determine the effects on NA concentrations, another group for effects on DA and for video registration of behaviour.

DA and NA extracellular concentrations were shown to be Calcium- and TTX-dependent, suggesting an origin of active, neuronal release. D-AP5 dose-dependently increased DA to 149%, 204% and 286%, respectively (n=7; paired Dunnett's t-test: $p<0.05$ or <0.01) and (to a lesser extent) DA metabolite concentrations, but did not significantly affect NA (113%, 120% and 128%, respectively; n=6; $p>0.05$). The time that the rats were awake (activity level 1) and the time that the rats showed motor activity (activity level 2) were also dose-dependently increased from 5% to 99% and from 0% to 11%, respectively (median values; n=6; Wilcoxon matched-pairs signed ranks test: $p<0.05$).

These results show that NMDA-receptor blockade in the mPFC a) strongly activates DA release in the mPFC; b) differentially regulates DA and NA release in the mPFC; c) induces behavioural activation. The latter effect confirms previous reports of activation after local microinjections (Crawley et al, 1992). The differential effects on DA and NA show that the mechanism by which D-AP5 acts in the mPFC is not a simple presynaptic one but probably involves either a local multisynaptic pathway or a selective circuit involving mPFC and the ventral striatum (VS) and VTA, which both receive mPFC projections. To further elucidate this, studies of local NMDA-antagonist applications and DA measurements in the VS and VTA are in progress.

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329P AMPA RECEPTOR DESENSITIZATION REGULATES DA RELEASE IN THE HIPPOCAMPUS *IN VIVO*

P.S. Whitton[1], S. Maione[2], F. Rossi[2], C.S. Biggs[1] and L.J. Fowler [1]
[1] Department of Pharmacology, The School of Pharmacy, London and [2] Istituto Di Farmacologia E Tossicologia, Universita Degli Studi Di Napoli. Napoli, Italy.

Recent evidence has suggested that release of DA within the CNS may be under glutamatergic control (Westerink et al., 1992). Using microdialysis these authors observed that infusion of α-amino-3-hydroxy-5-methyl-4-isozoxazole-propionate (AMPA) into rat caudate increased DA release. A characteristic of the AMPA receptor is rapid desensitization in response to prolonged agonist stimulkation (Mayer and Vyklicky, 1989). In the present study we have investigated the effects of infusing AMPA on dialysate concentration of DA in rat hippocampus, and whether this is influenced by AMPA receptor desensitization *in vivo*.

Male Wistar rats (280-350g) were implanted, under chloral hydrate anaesthesia (400mg/kg), with concentric dialysis probes into the ventral hippocampus. The following day rats were dialysed with artificial cerebrospinal fluid (0.5 µl/min). Four 30 min samples were collected prior to infusion of AMPA alone, or in combination with either the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3,-dione (CNQX; 200µM) diazoxide (500µM) or tetrodotoxin (TTX; 1µM). In some experiments cromakalim (100µM), an activator of ATP-dependent K^+ channels was infused. DA concentration was determined using HPLC with electrochemical detection. Basal dialysate concentration of DA was 57 ± 4 (n = 40).

Hippocampal DA release was highly sensitive to AMPA stimulation. At 1 and 10 µM AMPA, DA release was increased, by up to 253 ± 30 and 792 ± 303 % of basal respectively (mean ± s.e.mean, n = 6-8). When 100 µM AMPA was infused dialysate DA was significantly decreased to around 50 % of basal for 3 h. The maximal decrease was 53 ± 7 % below basal. This indicated possible desensitization of the hippocampal AMPA receptor (cf. Mayer and Vyklicky, 1989). When 100µM AMPA was infused with diazoxide DA release was increased by 331 ± 39 %, and diazoxide alone increased dialysate DA significantly. Responses to AMPA and/or diazoxide were blocked by CNQX. CNQX alone decreased DA release by up to 47 ± 6 %. Since diazoxide can activate ATP-dependent K^+ channels, we infused cromakalim, a drug which shares this action, but to our knowledge has no reported effect at the AMPA receptor. Cromakalim induced a sustained decrease (about 50 %) in hippocampal DA release. In this instance the maximal decrease in extracellular DA was 57 ± 9 % below basal. Responses to AMPA were abolished by co-infusion with TTX, and basal DA release was reduced by 20 ± 3 % by this drug. TTX significantly reduced extracellular DA for 3 h following infusion of the drug.

Collectively these data indicate that hippocampal DA release is under tonic glutamatergic control via AMPA receptors. Furthermore it appears that this tone is maintained in part by the number of AMPA receptors in a desensitized state *in vivo*.

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330P S 17625, A SELECTIVE AND COMPETITIVE NON-NMDA ANTAGONIST: CEREBRAL ANTI-ISCHAEMIC EFFECTS IN RODENTS

P. MORAIN, P. LESTAGE, A. ROGER, C. BOBICHON, P. DESOS, A. CORDI and J. LEPAGNOL (Introduced by M. SPEDDING) , IdRS Neurosciences Research Center, 125 Chemin de Ronde, 78290 Croissy-sur-Seine (Paris, France).

Competitive AMPA antagonists are potentially useful for treatment of cerebral ischaemia. In a new chemical series of quinolone derivatives. S 17625 (6.7-dichloro-2-(1H)-quinolinone-3-phosphonic acid) was selected for its *in vitro* AMPA and kainate antagonism properties and consequently studied in models of global (Kirino, 1982) and focal ischaemia (Welsh *et al.*, 1987).
In Xenopus oocytes injected with rat cortex mRNA, S 17625 inhibited AMPA-and kainate- induced currents with IC50s of 5 and 2 µM respectively. In contrast, S 17625 was devoid of inhibitory effects on NMDA-induced responses (IC50>100 µM). The parallel and rightward shift of the AMPA dose-response curve with no change in maximum response indicated the competitive nature of the S 17625- induced inhibition. These effects were confirmed in rat hippocampal slices by recording field e.p.s.p. in the CA1 region after stimulation of the Schaffer collateral-commissural projection. In the presence of Mg^{++} (1 mM), the inhibitory effect (IC50 = 2.5 µM) of S 17625 on the non-NMDA-mediated e.p.s.p. was very close to that obtained in oocyte experiments. As expected, in absence of Mg^{++} (NMDA-mediated e.p.s.p.), S 17625 showed a very weak inhibitory potency (IC50>300 µM).

The *in vivo* neuroprotective effects of S 17625 were studied in models of cerebral ischaemia. In gerbils, transient (5 min.) global ischaemia was induced by bilateral carotid occlusion and delayed hippocampal neuronal loss in CA1 region was histologically determined 4 days later (Kirino, 1982). Post-ischaemic treatment (30 and 90 min. after ischaemia) with S 17625 at 10 mg/kg IP, exhibited a complete protection from neuronal death in 40 % of animals ($p<0.05$; Wilcoxon rank-sum test). In mice, permanent focal ischaemia was induced by occlusion of the left middle cerebral artery and the cortical infarct volume was histologically evaluated 24 hours later (Welsh *et al.*, 1987). S 17625 treatment (30 mg/kg IP, 5, 60 and 120 min. after occlusion) resulted in 23 % reduction of infarct volume ($p<0.05$; Anova + Newman-Keuls).
These results with S 17625, a new competitive non-NMDA antagonist, provide support for its potential usefulness in acute treatment of stroke and prompt its clinical evaluation.

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331P NEUROPROTECTIVE EFFICACY OF THE ION CHANNEL MODULATOR LIFARIZINE (RS-87476) IN A RODENT MODEL OF GLOBAL CEREBRAL ISCHAEMIA

D.E. McBean, V. Winters, A.D. Wilson, C.B. Oswald & J.M. Armstrong, Department of Pharmacology, Syntex Research Centre, Riccarton, Edinburgh, EH14 4AP, Scotland.

Lifarizine (RS-87476)(1-[(2-(4-methylphenyl)-5-methyl)-1H-imidazol-4-yl-methyl]-4-diphenyl-methyl-piperazine) is an ion channel modulator which has been shown to inhibit both Na^+ and Ca^{2+} channels. It has a broad neuroprotective profile in both global (rat 4 vessel occlusion) and focal (cat middle cerebral artery occlusion) models of cerebral ischaemia (Alps, 1992). The aim of the present study was to test the efficacy of lifarizine in the global rat 2 vessel occlusion model of ischaemia.

The experiments were carried out using male Sprague-Dawley rats (267-350g). Anaesthesia was induced with 5% halothane in oxygen (O_2) and maintained at 2% halothane in O_2. Both common carotid arteries were exposed and the rat was fitted with a tightly fitting face mask and put on a ventilator. This gave ventilatory support during the occlusion period. The halothane concentration was increased to 5% with a resulting decrease in blood pressure (BP). When the mean BP had dropped below 50mmHg microvascular clips were applied to both common carotid arteries for a period of 12min. The mean BP was maintained at 50mmHg or less throughout the 12min. occlusion period. 5min. post-occlusion each rat received an i.a. dose of either 0.4ml/kg vehicle (n=12) or 100µg/kg lifarizine (n=11). 15min. post-occlusion each rat received an i.p. dose of either 2ml/kg vehicle or 500µg/kg lifarizine. Each rat was subsequently dosed twice daily for 72h with i.p. injections of either 2ml/kg vehicle or 500µg/kg lifarizine. After 72h, each rat was subjected to perfusion-fixation under terminal anaesthesia with 10% neutral buffered formal saline. The brains were removed, processed, sectioned and stained to allow assessment of ischaemic damage. A parallel control group (n=11) was also processed to give normal values. In the hippocampal CA_1 sub-field the damage was assessed in terms of the number of intact cells per mm of CA_1, and presented as a percentage of those found in normal control rats. In the other brain areas the level of damage was assessed by means of a grading system from 0 (no damage) through to 6 (complete neuronal death, status spongiosus and vacuolation).

Lifarizine treatment resulted in significant reductions in damage, compared to controls, in the CA_1 (4.1±0.3 to 2.8±0.6), the anterior (2.0±0.2 to 1.2±0.2) and posterior (1.5±0.2 to 1.0±0.2) cortices, thalamus (1.5±0.2 to 0.8±0.2) and the cerebellar brain stem (0.9±0.2 to 0.4±0.1)($P<0.05$; Student's t-test).The model used produces a very severe insult to the cerebral circulation, since not only is the carotid flow to the brain removed for a period of 12min., but collateral flow through the other vessels is also markedly reduced by the induction of hypotension. Lifarizine, when administered post-occlusion as above, was able to achieve highly significant reductions in damage in the CA_1, the cortex, thalamus and cerebellar brain stem. The activity of lifarizine in the rat 2 vessel occlusion model suggests potential as a cerebral anti-ischaemic agent.

Alps, B.J. (1992) *Br.J.Clin.Pharmac.*34, 199-206.

332P THE ION CHANNEL MODULATOR LIFARIZINE (RS-87476) REDUCES THE SIZE OF THE INFARCT PRODUCED IN THE RAT BY THE ROSE BENGAL PHOTOCHEMICAL FOCAL MODEL OF CEREBRAL ISCHAEMIA

D.E. McBean, V. Winters, A.D. Wilson, C.B. Oswald & J.M. Armstrong, Department of Pharmacology, Syntex Research Centre, Riccarton, Edinburgh, EH14 4AP, Scotland.

Lifarizine (RS-87476)(1-[(2-(4-methylphenyl)-5-methyl)-1H-imidazol-4-yl-methyl]-4-diphenyl-methyl-piperazine) is an ion channel modulator which has been shown to inhibit both Na^+ and Ca^{2+} channels. It has been shown to have a broad neuroprotective profile in both global (rat 4 vessel occlusion) and focal (cat middle cerebral artery occlusion) models of cerebral ischaemia (Alps, 1992). The aim of the present study was to study the efficacy of lifarizine in the focal photochemical rose Bengal model of ischaemia in the rat (Grome et al.,1988).

The experiments were carried out using male Sprague-Dawley rats (230-350g). Anaesthesia was induced with 5% halothane in oxygen (O_2) and maintained at 2% halothane in O_2. The femoral vein were cannulated to allow administration of the histological dye rose Bengal. A midline head incision was made and the skull exposed. The skull was marked at the bregma and a fibre optic light source (Osram Xenophot 250W lamp, passed through a heat filter, with a 3mm diameter tip) was positioned over the right cerebral cortex at the level of the bregma. Rose Bengal (15mg/kg) was injected via the femoral vein over 2min. On completion of the rose Bengal administration, the light source was switched on and the skull illuminated for 20min. 5min. post-illumination each rat received an i.v. dose of either 0.4ml/kg vehicle (n=11) or 100µg/kg lifarizine (n=12). 15min. post-occlusion each rat received an i.p. dose of either 2ml/kg vehicle or 500µg/kg lifarizine. The anaesthetic was discontinued and the rats were allowed to recover consciousness in a warm environment.
Each rat was subsequently dosed twice daily for 72h with i.p. injections of either 2ml/kg vehicle or 500µg/kg lifarizine. Following this dosing regimen, each rat was killed by an overdose of halothane anaesthesia and decapitated. The brains were rapidly removed and frozen in isopentane at -42°C. Coronal sections, 20µm thick, were cut on a cryostat, fixed in FAM and stained with cresyl violet. The lesioned area on each section is able to be clearly delineated on the image analyzer. Volume of infarct was then calculated for each brain.

Following rose Bengal photochemical insult the area of cresyl violet deficit stain, and hence the area of lesion, was evident only in the right hemisphere of the brain. This indicates the focal nature of the lesion, since at no time was there any evidence of damage in either the ipsilateral subcortical or contralateral hemisphere brain regions. Lifarizine treatment resulted in significant reductions in the volume of infarct produced by rose Bengal photochemical insult from 49.1±5.4 to 32.0±3.5mm^3 (-35%)($P<0.05$, Student's t-test). The model used produces a very severe, localized insult where the damage was limited to the cortex underlying the area of light. Lifarizine, when administered post-occlusion at a dose of 100/500 µg/kg i.v./i.p. was able to produce significant reductions in both volume of infarct and level of oedema. These results support the use of lifarizine as a neuroprotective agent in human cortical stroke.

Alps et al.(1992) Br.J.Clin.Pharmac. 34, 199-206.
Grome et al.(1988) J.Cereb.Blood Flow Metab. 8:89-95.

333P ANTAGONISM BY FELBAMATE OF MUSCARINIC AND METABOTROPIC RECEPTOR-MEDIATED RESPONSES IN GUINEA-PIG OLFACTORY CORTEX NEURONES *IN VITRO*

V. Libri & A. Constanti. *Department of Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N.*

Felbamate (FBM) possesses anticonvulsant and neuroprotectant properties in a variety of experimental models (White *et al.*, 1992). FBM blocks high-frequency repetitive firing in mammalian spinal cord neurones by acting on voltage-dependent sodium channels (White *et al.*, 1992). However, it is possible that FBM may also interact with other voltage-sensitive ion channels (e.g. K^+ or Ca^{2+}). We have recently reported that some olfactory cortical neurones respond to muscarinic or metabotropic glutamate receptor activation with a slow and prolonged membrane depolarization, an increase in membrane resistance and an intense and sustained repetitive firing. A large depolarizing stimulus applied during muscarinic/metabotropic activation was able to generate a slow post-stimulus afterdepolarization (sADP) associated with a *decrease* in membrane input conductance, which was proposed to represent the decay of a novel Ca^{2+}-sensitive K^+ current (termed I_{ADP}) (Constanti *et al.*, 1993; Libri *et al.*, 1994). In the present study, we were interested to know whether FBM could affect I_{ADP} generation, and therefore influence the prolonged muscarinic/metabotropic agonist response profile.

Transverse slices of guinea-pig olfactory cortex were prepared as previously described (Constanti *et al.*, 1993). Stable intracellular recordings were made from neurones in the pyramidal cell layer (II-III) using microelectrodes filled with 4 M potassium acetate (50-70 MΩ). Bath-application of FBM (100 μM; n=4) had no effect on membrane properties or action potential duration. On increasing the dose to 500 μM (n=4), FBM induced only a small hyperpolarization (< 2 mV) and a slight increase in membrane input resistance (4 ± 2%). In contrast, bath-application of the muscarinic receptor agonist oxotremorine-M (OXO-M; 10 μM; 2min; n=5) or the metabotropic-glutamate receptor agonist 1-aminocyclopentane-1S-3R-dicarboxylic acid (1S-3R-ACPD; 10 μM; 2 min; n=5) induced a strong membrane depolarization (9.2 ± 0.6 mV), an increase in input resistance (14 ± 4%, measured at -70 mV) and a sustained neuronal discharge. A large (1.5 s) depolarizing stimulus applied during the action of either of the two drugs revealed a sADP which was insensitive to 1 μM tetrodotoxin. Pre-incubation with FBM (100 μM; 15 min; n=4) markedly reduced the neuronal excitability produced by OXO-M or 1S-3R-ACPD, and also the amplitude of the post-stimulus sADP. In order to determine whether underlying Ca^{2+} currents might be affected by FBM, cells were recorded with 2M CsCl-filled electrodes, which induced a prominent spike prolongation and a large Ca^{2+}-dependent tail afterdepolarization. These effects were clearly reduced after 15 min exposure to 100-500 μM FBM, which also produced a sustained increase in the input resistance (94 ± 8%) of the cells (n=4).

The present data show that the excitatory effects of muscarinic or metabotropic receptors activation in olfactory cortical neurones can be antagonized by the anticonvulsant drug felbamate at micromolar concentrations. In addition, FBM did not produce any noticeable changes in fast sodium spikes, but was able to reduce the duration of presumed Ca^{2+} action potentials revealed by Cs^+ loading. Although the decrease in amplitude of the sADP could be most readily explained by a reduction in Ca^{2+} influx, an additional interaction of FBM with the muscarinic/metabotropic response generation mechanism cannot presently be excluded.

Felbamate was kindly provided by Dr. E. Ongini, Schering Plough, Comazzo, Milan, Italy.

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Constanti, A. *et al.*(1993). *Neuroscience* **56**, 887-904.
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334P MUSCARINIC RECEPTORS INVOLVED IN BLOOD PRESSURE CHANGES INDUCED BY THE ELECTRICAL STIMULATION OF CENTRAL AMYGDALOID NUCLEUS

N.Aslan, Z.Gören, F. Onat, Ş.Oktay, University of Marmara, School of Medicine, Department of Pharmacology, Haydarpaşa, 81326, İstanbul, Turkey.

There is vast literature concerning the blood pressure increase by direct or indirect activation of central muscarinic receptors[1]. Several nuclei and areas of the brain are known to take place in this effect. On the other hand, the amygdala which is a part of limbic system, is thought to have a function in the coordination of behavioral and autonomic responses to the environmental stimuli. Neuroanatomical studies have indicated that the amygdala receives projections from cortex and subcortical structures responsible for sensory integration and also extensive projections from the amygdala reach widespread brainstem structures involved in the central regulation of the cardiovascular functions. It has been reported that electrical stimulation of central amygdaloid nucleus (CAN) led to pressor and tachycardic responses in the conscious rats, while reverse of these responses were observed in the anesthetised rats[2]. Along with these findings, autoradiographic studies imply the presence of muscarinic receptors in the amygdala[3].

This study was undertaken to investigate the role of muscarinic receptors in the CAN on the pressor effect in response to the electrical stimulation of this area. Female Sprague-Dawley rats were used. The electrical stimulation and microinjections were done via a cannula-electrode placed unilaterally by using a stereotaxic frame under ketamine (50 mg/kg, i.p.) anesthesia. Rats were prepared for blood pressure monitoring 2-3 days after this operation and continuous blood pressure recording was performed in conscious rats via a pressure transducer connected to a polygraph. The placement of the cannula-electrode was verified histologically after each experiment.

As a result of the electrical stimulation (80Hz, 1 ms, 200-300 μA, 30 sec.) of CAN, reproducible increases in MAP (14.9± 0.1 mmHg) and behavioral changes such as rotation of the body and jaw movements were observed. Atropine pretreatment blocked these increases in a dose-dependent manner (ID_{50}: 1.16 nmol). The inhibition was 56.4±4.9, 71±5.1, 99±0.9 % by 1, 10 and 100 nmol, respectively. Atropine at the highest dose (100 nmol) also completely blocked the behavioral changes occured during the electrical stimulation of the CAN.

These results indicate the involvement of the muscarinic receptors in the blood pressure increases and behavioral changes induced by the electrical stimulation of the CAN.

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[2] Gelsema, A.J., McKitrick, D.J., Calaresu, F.R. (1987) Am.J. Physiol. 253 (Reg. Comp. Physiol. 22), 712-718.
[3] Quirion, R., Aubert, I., Araujo, D.M., et al. (1993) Prog. Brain Res. 98: 85-93.

335P A BEHAVIOURAL COMPARISON BETWEEN THE ANTICHOLINESTERASE DRUGS TACRINE AND E2020

D. L. Kirkby, D. N. C. Jones. & G. A. Higgins, Glaxo Unit of Behavioural Psychopharmacology, Division of Biosciences, University of Hertfordshire, Hatfield, Herts, AL10 9AB.

The anticholinesterase drug tacrine has hepatotoxic effects which limit its use as a treatment for Alzheimer's disease. Newer anticholinesterase drugs have been developed which are reportedly non-hepatotoxic. One such drug is E2020, a purported brain selective anticholinesterase (Rogers, 1991). There is little comparative data between tacrine and E2020 in preclinical models, therefore the present study compared these drugs in terms of potency to (1) produce cholinomimetic effects, (2) produce disruptions of operant responding for food reward under a variable interval schedule of 20s (VI20), and (3) reverse a scopolamine-induced deficit in a test of working memory, the operant delayed-match-to-position task (DMTP; Dunnett, 1985).

Male, hooded lister rats (350-450g) were used throughout. To assess cholinomimetic effects, animals were injected with either tacrine (3-30mg/kg sc) or E2020 (1-10mg/kg ip) and assessed at 15min intervals for the occurrence of secretory signs (salivation/lacrimation) and tremor; pupil diameter and body temperature were also recorded. Following this study, these animals were trained to respond for food reward in standard operant chambers up to a VI20 schedule. In the DMTP task, a separate group of rats were trained to respond to a level of >85% accuracy at all delays (delay range 1-24s).

Both drugs produced clear cholinomimetic effects i.e secretory signs, hypothermia, tremor at the dose range tested, although E2020 was more potent e.g minimal effective dose (MED) hypothermia; E2020 3mg/kg (35.9±0.4°C cf 37±0.2°C for vehicle; $p<0.05$), tacrine 10mg/kg (34.2±0.3°C cf 37.1±0.2°C for vehicle; $p<0.01$). Only tacrine produced miosis. Both E2020 and tacrine (MED 3mg/kg and 10mg/kg respectively) significantly reduced response rates in the VI20 schedule e.g E2020 study:- vehicle: 1334±217 responses, E2020 3mg/kg: 588±175 responses; $p<0.01$; tacrine study:- vehicle: 1347±217 responses, tacrine 10mg/kg: 793±107 responses; $p<0.05$. In the DMTP paradigm, both E2020 and tacrine attenuated a scopolamine (scop; 0.075mg/kg sc)-induced deficit in correct responding, however the MED for each drug was identical (1mg/kg) e.g E2020 study:- vehicle 88±2%, scop 76±2%, E2020 1mg/kg + scop 81±2%; tacrine study:- vehicle 95±1%, scop 78±3%, tacrine 1mg/kg + scop 84±4%; $p<0.05$ anticholinesterase/scop vs. scop alone. Taken as a whole, with the exception of miosis, the results show similar profiles for E2020 and tacrine in all models. With each drug, the dose separation between cognitive and cholinomimetic effects was around 3-10 fold.

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336P THE LACK OF EFFECT OF NICOTINE IN THE CONDITIONED PLACE PREFERENCE PARADIGM IN THE MOUSE

I. Ali, M.E.Kelly & J.W.B.Marshall[1] Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, West Yorkshire, BD7 1DP and [1]MRC Comparative Cognition Team, Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge, CB2 3EB.

Studies of the reinforcing properties of nicotine using the conditioned place preference (CPP) paradigm have yielded a conflicting picture. For example Fudala et al. (1985) demonstrated a dose dependent CPP with nicotine in rats whereas Clarke & Fibiger (1987) failed to obtain this response. The present study examined the effects of nicotine on CPP responding in the mouse. Additionally, effects on locomotor activity (LMA) were examined since it has been suggested that drugs which modify LMA may influence CPP in the absence of reinforcing effects (Swerdlow & Koob, 1984).

For CPP experiments male adult BKW mice (30-48g) were administered saline or nicotine (0.25, 0.5 and 1.0mg/kg, i.p, n=10) according to the protocol and using the apparatus previously described (Marshall et al., 1992). For the LMA experiments the apparatus consisted of 10 clear plexiglass cages (10x24x14cm) each fitted with two photocell units located 2.5cm above the floor of the cage and 3cm from the long sides. The mice (n=10) were administered saline or nicotine (0.25, 0.5 and 1.0mg/kg, i.p.) and placed immediately into the individual photocell cages. Interruptions of the light beams were recorded and summed over 30 min. This procedure was repeated intermittently on four occasions to mimic CPP conditioning. Data were analysed by 2 way analysis of variance followed by post-hoc t-test analysis.

None of the mice showed a significant effect of conditioning although, there was a general tendency for a decrease in the post-conditioning time spent (s) in the drug paired side (saline from 299.5±14.9 s to 270.5±24.3 s, 0.5 and 1.0mg/kg nicotine from 298.5±16.4 s to 263.1±20.2 s and 299.6±12.9 s to 259.2±23.7s, respectively). However, treatment with nicotine (0.25mg/kg) failed to modify time spent in the drug-paired chamber (301.3±24.4 s and 313.9±28.2 s). There was a significant decrease in LMA (counts/30min) on day 3 and day 4 of testing when comparing the saline control group and the group receiving nicotine (1.0mg/kg), from 382.2±39.8 to 240.0±26.1 ($P<0.05$) on day 3 and from 452.8±34.7 to 277.6±35.9 ($P<0.05$) on day 4.

These results suggest a failure to demonstrate CPP with nicotine in BKW mice and whilst in contradiction with the findings of Fudala et al. (1985), support the results of Clarke & Fibiger (1987). The decrease in LMA is opposite to the findings of Jerome & Sanberg (1987) who reported an increase in LMA in rats following nicotine treatment. These data may simply reflect species differences but it cannot be excluded that the decrease in LMA may have influenced the outcome of the CPP study.

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Marshall, J.W.B. et al. (1992) J. *Psychopharmacology* Abstract A80, 318
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337P EFFECTS OF 6-HYDROXYDOPAMINE LESIONS IN THE VENTRAL TEGMENTAL AREA OR SUBSTANTIA NIGRA ON DOPAMINE RELEASE IN SUBREGIONS OF THE RAT STRIATUM

M.M. Iravani, S. Khan, A. Michael-Titus, R. Whelpton & Z.L. Kruk. Department of Pharmacology, Queen Mary & Westfield College, Mile End Road, London E1 4NS

Dopamine (DA) projections to the striatum arise in the substantia nigra (SN; area A9) and ventral tegmental area (VTA; area A10). In earlier studies, it has been shown that there are significant differences in DA release in subregions of the caudate putamen (CPu) and nucleus accumbens (NAc) (Patel et al 1992; Davidson & Stamford 1993), and it was speculated that A9 or A10 innervation to the subregions may account for the observed differences. The aim of this investigation was to define the relative contribution of A9 and A10 innervation to DA release in subregions of the striatum. Two groups of 7 albino Wistar rats received unilateral stereotaxic injections of 8 µg 6-OHDA (Ungerstedt, 1971) in either the VTA or SN. One month later, 350 µm coronal slices incorporating lesioned and non-lesioned striatum (1.6-1.2 mm anterior to bregma) were set up in a superfusion chamber. Slices were superfused with oxygenated artificial cerebrospinal fluid at 32° C, and DA release was measured using fast cyclic voltammetry (Trout & Kruk 1992) following 20 pulse 50 Hz (20 V; 0.1ms pulse width) electrical stimulation at sites a-e (see Fig 1) in lesioned and non-lesioned striatum. DA release following electrical stimulation at sites a-e in lesioned and non-lesioned striata is summarised in Table 1.

At the end of each experiment slices were divided into 4 segments consisting of lesioned and non-lesioned CPu and NAc, homogenised in ice-cold perchloric acid, and DA content was assessed by HPLC-ED. In VTA lesioned rats, DA in CPu was not significantly affected (unlesioned: 184 ± 38ng DA/mg protein; lesioned: 130 ± 46ng DA /mg protein; n=7), but DA in NAc was significantly lower (unlesioned: 244 ± 38 ng DA/mg protein; lesioned: 133 ± 28ng DA/mg protein, n=7, P<0.05). In the SN lesioned rats, DA content of CPu was significantly reduced in the lesioned side (unlesioned: 300 ± 48 ng DA/mg protein; lesioned: 124 ± 18 ng DA/mg protein, n=7, P<0.05); DA content of NAc was unaffected in the lesioned side (unlesioned: 194 ± 19 ng DA/mg protein; lesioned: 170 ± 20 ngDA/mg protein). After VTA lesions, DA release in both the core and shell of the NAc (sites d and e respectively) was significantly reduced, whereas SN lesions did not affect DA release in the NAc. These results are consistent with the predominantly A10 innervation of NAc. In the CPu, DA release in site a was significantly reduced following SN lesioning. The finding that VTA lesions reduced DA release in sites b and c, suggests that there is a significant A10 innervation at these sites. This study provides further evidence of regional heterogeneity of DA innervation in the caudate putamen of the rat.

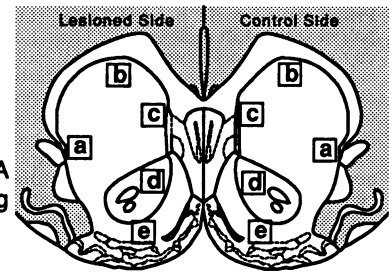


Figure 1. Location of recording sites in the CPu & NAc.

Table 1. Peak DA release (nM) following electrical stimulation.

Sites:	a	b	c	d	e
Control	37±6	73±22	261±54	326±45	288±35
VTA lesion	31±10	28±14*	143±52*	198±53*	137±53*
Control	169±34	62±18	322±55	357±62	404±59
SN lesion	104±24*	50±25	220±57	300±66	422±78

(* P < 0.05, n=7, ANOVA) all values are mean ± s.e.m.

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338P SUBSTANCE P FRAGMENTS (1-7) AND (5-11) INCREASE STRIATAL DOPAMINE RELEASE

S. Khan, R. Whelpton & A.T. Michael-Titus, Department of Pharmacology, Queen Mary & Westfield College, Mile End Road, London E1 4NS.

Substance P (SP) and its fragments SP(1-7) and SP(5-11) have been shown to modulate striatal dopamine (DA) release after nigral administration (Reid *et al.*, 1990). Previously, we investigated the effects of SP(1-7) and SP(5-11) (10^{-8}-10^{-5}M) on endogenous DA release in striatal slices. No significant modulation was observed (Khan *et al.*, 1994). The present study has continued the investigation over a lower concentration range of these peptides (10^{-11}-10^{-8}M). The NK1 agonist [Sar^9-Met $(O_2)^{11}$]SP (10^{-9}-10^{-6}M) was tested, and the NK1 antagonist WIN51708 (2.5 x 10^{-7}M) has been tested against one dose of peptides (10^{-9}M).

Striatal slices (400-500 µm) obtained from adult male Wistar rats (150-200 g), 2 per chamber, were superfused with oxygenated Krebs-Ringer solution at 34°C. After 60 min incubation, fractions were collected over 2 min. After 4 min basal release, peptide solutions alone, or in the presence of WIN51708 or [Sar^9-Met $(O_2)^{11}$]SP, were infused for 10 min and samples collected for a further 10 min. DA was quantified by HPLC-ED. Changes in DA release were expressed as percent of basal release, representing area under the curve.

Basal DA release (± s.e.m, n=36) was 0.48 ± 0.03 pmol/ml/mg protein. One way ANOVA showed a significant effect on DA release for the three peptides (F(4,43) = 103, 22.5, 31.3; P<0.05 for SP, SP(1-7) and SP(5-11), respectively) (Figure 1). [Sar^9-Met$(O_2)^{11}$]SP increased DA release from basal at 10^{-7}M (+160%) and 10^{-8}M (+249%) (F(4,43) = 11.2; P<0.05). Two way ANOVA showed that WIN51708 decreased basal DA release (-94%) and reversed peptide stimulated release of DA to below basal for SP and SP(5-11) (F(1,32) = 24.3 and 6.02; P<0.05, respectively) (Figure 2). The antagonist did not reduce the effect of SP(1-7)(F(1,32)=1.07; ns).

This study is the first to show local modulatory effects of SP fragments on endogenous DA release in the striatum. The data suggest that different receptor types may be involved in the effect of SP(1-7) and SP(5-11) on striatal DA release.

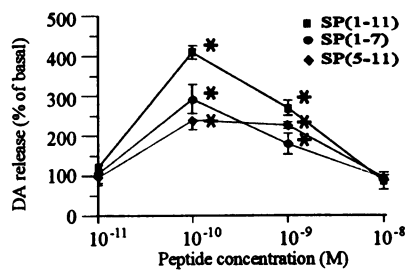


Figure 1. Effects of SP, SP(1-7) and SP(5-11) on DA release from striatal slices. Means ± s.e.mean of six determinations. *P<0.05, Bonferroni's test versus respective non-treated controls.

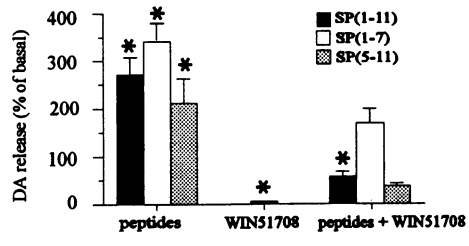


Figure 2. Effect on WIN51708 (250 nM) on peptide (1 nM) stimulated DA release from striatal slices. Means ± s.e.mean of 6 determinations. *P<0.05, Bonferroni's test versus respective non-treated controls.

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339P COCAINE-INDUCED RELEASE OF DOPAMINE IN THE NUCLEUS ACCUMBENS DECREASED BY PERINATAL COCAINE EXPOSURE

A. Giustino & C.A. Marsden, Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham University, Nottingham, NG7 2UH

There is increasing use of cocaine in pregnant women and, since this drug easily crosses the placenta and rapidly achieves high brain concentrations (Chasnoffi *et al.*, 1992; Spear *et al.*, 1989; Goeders & Smith, 1983), *in vivo* studies of the neurochemical alterations of its potential principal targets, such as the mesolimbic dopaminergic system, are required. In this study we have used microdialysis to investigate the effects of perinatal cocaine treatment on the mesolimbic dopaminergic system of young-adult rats.

Primiparus female hooded Lister rats, weighing 200-230 g, were paired with adult male rats and day 1 of gestation (E1) was defined as day of copulatory plug detection. Subcutaneous injections of 0.9% saline or cocaine (20 mg/kg) were started on E10 and continued every other day until weaning of the offspring (25 days after birth). Three weeks after weaning both the pretreated and the saline injected male rats were anaesthetised under halothane anaesthesia and a microdialysis probe stereotaxically implanted into the nucleus accumbens. Probes were continuously perfused with artificial cerebrospinal fluid (aCSF). Animals were returned to their home cage and allowed at least 20 hr to recover from surgery with food and water available *ad libitum*. During the experiment, the microdialysis probe was perfused with a CSF at a flow rate of 1.0 ul/min and three 20 min samples were collected before administration of cocaine (15 mg/kg i.p.). Extracellular dopamine in the dialysate samples was determined by high performance liquid chromatography with electrochemical detection.

The results show an evident, but not significant, difference in basal dopamine levels between the rats given perinatal cocaine (n = 7) or saline (n = 6). In the saline pretreated rats basal dopamine levels were 47.7 ± 4.1 fmols/10 µl (mean ± S.E.) and in perinatally cocaine treated animals 26.1 ± 1.8 fmols/10 µl. Acute administration of cocaine to the rats born to mothers given saline showed a marked and significant increase in extracellular dopamine (max increase 293.6 ± 25.3 %) compared to pre-injection values ($p < 0.01$); the increase was significantly greater than that observed in the rats exposed to perinatal cocaine (max increase 179.1 ± 15.6 %) (Fig 1).

A two-way ANOVA demonstrates a significant difference between groups ($F = 108.8$; $df = 1/77$; $p < 0.001$), between challenges ($F = 14.7$; $df = 6/77$; $p < 0.001$) and between groups x challenges ($F = 5.17$; $df = 6/77$; $p < 0.002$) in the extracellular dopamine between the two groups of rats following a single dose of cocaine.

The results clearly demonstrate that the increase in extracellular mesolimbic dopamine produced by a single injection of cocaine, in young adult rats is reduced by cocaine administration to the mothers during pregnancy and lactation. The change in dopamine response could be due to prolonged desensitization produced by perinatal cocaine. Alternatively, exposure to cocaine *in utero* and during lactation may results in altered development of the mesolimbic dopaminergic system.

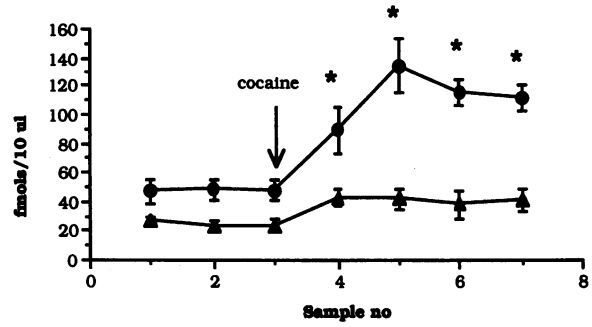


Figure 1: Effect of acute cocaine (15 mg/kg i.p on extracellular dopamine in the nucleus accumbens in rats exposed to either perinatal saline (●) or cocaine (▲).. * $p < 0.01$ (Duncan's test).

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340P RELEASE OF DOPAMINE FROM THE STRIATUM BY NICOTINE IS TTX-SENSITIVE: *IN VIVO* EVIDENCE OF "PRETERMINAL" nAChRs?

D.L. Marshall, P. H. Redfern & S. Wonnacott[1], School of Pharmacy & Pharmacology and [1]School of Biology & Biochemistry, Bath University, Claverton Down, Bath BA2 7AY.

Nicotine (Nic) stimulates nicotinic acetylcholine receptors (nAChRs) to increase neurotransmitter release. *In vivo* studies (Nisell *et al.*, 1994) showed that the increase in dopamine (DA) release caused by subcutaneous Nic is due to an action on cell body nAChRs. The physiological significance of tetrodotoxin (TTX)-sensitive "preterminal" nAChR's (Lena *et al.*, 1993) and TTX-insensitve "presynaptic" nAChR's (Wonnacott *et al.*, 1990) is more controversial. Here, we have examined the effect of locally-administered Nic on DA release from corpus striatum by *in vivo* microdialysis in conscious male Sprague-Dawley rats.

Microdialysis probes were flushed for 1 hr with artificial CSF (4µl/min) before 15 min samples were collected and analysed, using HPLC, for DA content. After collection of 3 samples (the average of which constituted basal release) a pulse of Nic was given by addition to the artificial CSF (t=0-15 min). Nic caused a dose-dependent increase in DA levels (n=4-8), peaking at t=0-15 min and returning to basal over the next hour (table 1). The recovery of Nic across the probe *in vivo* was 0.68% (n=2).

After 90 min pretreatment with 10^{-3}M mecamylamine in the artificial CSF, $3x10^{-3}$M Nic (n=7) failed to evoke any increase in DA levels, whereas $3x10^{-1}$M Nic increased DA release to 185±86 fold of basal (n=3). In a similar experiment, 10^{-5}M TTX abolished basal DA release and the subsequent addition of $3x10^{-3}$M Nic had no effect (n=6), whereas $3x10^{-1}$M Nic increased DA levels to 359±18 fold of basal (n=3).

Our data are compatible with the existence of "preterminal" nAChRs on striatal DA axons which are stimulated by lower concentrations of Nic to release DA.

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Table 1: Effect of Nicotine On Striatal DA Levels

Nic [M]	0-15min	15-30min	30-45min	45-60min	60-75min
Control	0.8±0.1	0.7±0.1	1.1±0.3	1.0±0.2	1.4±0.4
10^{-3}M	1.2±0.1	0.9±0.1	0.6±0.1	0.7±0.1	0.8±0.1
$3x10^{-3}$M*	3.3±1.0	1.2±0.2	1.0±0.2	1.1±0.3	0.8±0.2
10^{-2}M*	8.3±2.8	4.8±0.8	2.0±0.4	1.9±0.8	1.9±0.7
$3x10^{-2}$M*	105±29	29±8.0	3.1±1.5	0.8±0.2	0.9±0.3
10^{-1}M*	260±103	100±41	8.0±2.1	1.6±0.4	1.1±0.5
$3x10^{-1}$M*	265±120	88±29	9.1±3.0	1.2±0.4	0.5±0.1

Data is shown as fold of basal DA values (mean ± s.e. mean). *$p<0.05$, significance of difference from control, two-way ANOVA for repeated measurements.

341P PHARMACOLOGICAL CHARACTERISATION OF DOPAMINE-STIMULATED [^{35}S]GTPγS BINDING MEDIATED BY HUMAN DOPAMINE D_2 RECEPTORS

F. Emms, A.E. Fletcher and S.B. Freedman. Neuroscience Research Centre, Merck Sharp & Dohme Research Laboratories, P.O Box 1675, Harlow, Essex. CM20 2QR.

We have investigated the binding of the stable GTP analogue [^{35}S]-GTPγS to hD2 (short) CHO stable cell lines, as an indirect means of measuring G-protein mediated responses following receptor activation.

Membranes containing the hD2 receptor expressed in CHO cells were prepared and assayed by a modification of the method described by Lazareno and Birdsall (1993).

Dopamine induced a dose dependent (2.5 fold) increase in [^{35}S]-GTP γ S binding with an EC_{50} of 380nM. Data obtained for a number of dopamine receptor agonists is shown in Table 1. All maximal responses are expressed relative to that observed for 100μM dopamine (100%) Similar maximal responses were observed for quinpirole and (+)-PHNO. In contrast, lower maximal responses were observed for 5,6 ADTN and PD 128907 (54 ± 3.2 and 65 ± 2.2% respectively). In comparison with dopamine, 7-OH DPAT (EC_{50} 70nM) displayed a maximal response equivalent to only 24% of dopamine. The dopamine receptor antagonists haloperidol and clozapine failed to evoke any increase in the basal levels of binding. However, both antagonists dose-dependently shifted the dopamine response curve to the right. Subsequent Schild analysis revealed pA2 values of 9.22 and 7.15 for haloperidol and clozapine respectively.

The reduced maximal response of 7-OH DPAT observed in this study is the first demonstration that this compound may have partial agonist activity at this receptor, an important observation when evaluating functional data obtained for this compound. Furthermore the similarities between the EC_{50} values and previously published affinities (Freedman *et al* 1994, Patel *et al* 1994) of the compounds tested suggest that this model may provide a useful means of evaluating agonist affinity and selectivity.

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Table 1

Compound	EC_{50} nM	% maximal response
Dopamine	380 (327,441)	100
Quinpirole	453 (417,493)	91 ± 3.2
(+) PHNO	5.8 (5.2,6.5)	88 ± 21
Apomorphine	13 (12,16)	71 ± 1.2
6,7-ADTN	22 (18,28)	70 ± 2.4
PD 128907	230 (201,263)	65 ± 2.2
5,6-ADTN	392 (276,557)	54 ± 3.2
7-OH DPAT	70 (64,77)	24 ± 3

EC_{50} values are geometric mean with 95% confidence limits

% max values are arithmetic mean ± s.e. mean (n=3-4)

342P ESTIMATION OF EFFICACY OF DOPAMINE AGONISTS ACTING AT RAT D_{2SHORT} DOPAMINE RECEPTORS EXPRESSED IN CHO-K1 CELLS

B. Gardner & P.G. Strange, Research School of Biosciences, The University of Kent, Canterbury, Kent, CT2 7NJ, UK.

Dopamine receptors are members of the G-protein coupled receptor superfamily. For an agonist to elicit a tissue response at a G-protein coupled receptor the agonist must first form an agonist/receptor complex which activates the interacting G-protein. The activated G-protein then interacts with the appropriate second messenger system and this leads to the tissue response.

The affinity of agonist for receptor, K_i, uncoupled from G-protein, is obtained when radioligand binding assays are carried out in the presence of guanine nucleotides, this is analogous to conditions within a whole cell. Under these conditions the agonist/receptor/G-protein complex is a continuously formed but transient intermediate which never accumulates to an extent measurable by binding assays (Sibley *et al.*, 1984).

A functional assay is required to measure activation of G-protein by the agonist/receptor complex. One method of observing G-protein activation is to follow the binding of a non-hydrolysable analogue of GTP such as [^{35}S]guanosine 5'-[γ-thio]triphosphate, ([^{35}S]GTPγS). It has been shown that the rate of [^{35}S]GTPγS binding in membranes from CHO-K1 cells expressing rat D_{2short} dopamine receptors is increased in a dose dependent manner by dopamine (Gardner & Strange, 1994). This allows EC_{50} values for agonists to be determined. The EC_{50} is defined as the concentration of agonist that produces a response that is 50% of maximal. The EC_{50} values for the dopamine agonists tested here were as follows (-)-quinpirole 417±108nM, dopamine 1400±300nM and R(-)-apomorphine 177±29nM (mean ± S.E.M. (n≥3)). In the present study the K_i values for the dopamine agonists tested in ligand binding assays versus [^{3}H]spiperone in the presence of 0.1mM GTP were as follows (-)-quinpirole 7.3±1.7μM, dopamine 9.3±2.7μM and R(-)-apomorphine 0.27±0.03μM (mean ± S.E.M. (n≥3)).

The ratio of agonist K_i, in the presence of 0.1mM GTP, divided by the EC_{50} is a measure of receptor reserve for that agonist and can be directly related to efficacy of the agonist (Kenakin (1987)). Application of this may allow ranking of agonists in order of their efficacy at a G-protein coupled receptor. For the rat D_{2short} dopamine receptor the agonists tested rank in the following order (-)-quinpirole (17.5)> dopamine (6.6)> R(-)-apomorphine (2.3). The value in parentheses after each agonist is the observed K_i/EC_{50} ratio. This determination of efficacy can be applied to other second messenger functional responses eg. inhibition of adenylyl cyclase by D_{2long} dopamine receptor (Chio *et al.*, 1994). Application of this analysis may allow the degree of amplification of agonist signal within a tissue to be quantified.

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343P ANTIPSYCHOTIC ACTIVITY IN A MODEL OF HEMISPHERIC LATERALITY IN THE RAT

A.G.Smith , A.M. Domeney[1], R.J. Naylor , B. Costall .
Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford, West Yorkshire BD7 1DP and [1] Battelle, 7, Route de Drize, CH-1227 Carouge- -Geneva.

Previous research has shown that bilateral injection of DiMe-C7 (the stable analogue of the neuropeptide substance P, obtained as [p-Glu^5, Me-Phe^8, Sar^9]-substance P(5-11)) into the ventral tegmental area (VTA) of the rat brain produces a dose-related increase in locomotor activity (Eison et al, 1982) corresponding to increases in mesolimbic catecholamine levels, particularly in the right amygdala (Hagan et al, 1990). The aim of the present studies was to utilise stereotaxically directed intracerebral injections into multiple sites to enable studies of the actions of antipsychotic agents on the hyperactivity response induced by acute unilateral injection of DiMe-C7 (2μg) into the VTA of rats. Prior to surgery Sprague - Dawley rats (Bantin & Kingman, n=5-7) were assessed for postural-motor asymmetry in turn preference (Bradbury et al, 1985). Animals categorised for right hemispheric dominance were subjected to stereotaxic surgery for dual implantation of chronically indwelling guide cannulae directed at the ventral tegmental area (VTA) and the amygdala centralis (ACE) . A minimum recovery period of 14 days was allowed following surgery.

Bilateral intraVTA injection of DiMe-C7 2μg was found to produce a significant ($p<0.001$) locomotor hyperactivity response (994±16.6 activity counts/60 min); unilateral injection of DiMe-C7 (2μg) into the VTA of the right dominant hemisphere also produced a significant ($p<0.01$-0.001) increase in locomotor activity (663±75.6 counts/60 min compared to 367±47.7 for vehicle treated animals, assessed in photocell cages). Unilateral injection of DiMe-C7 (2μg) into the VTA of the left sub-dominant hemisphere failed to increase locomotor activity (423±60.8 counts/60mins).Preliminary studies (data not shown here) involving administration of neuroleptic agents into the right ACE prior to right intraVTA DiMe-C7(2μg) challenge enabled selection of doses of agents capable of maximal antagonism of the hyperactivity response to DiMe-C7. Unilateral right intraACE injection of fluphenazine (10ng) and clozapine (50ng) prior to DiMe-C7 (2μg) injection into the right VTA was found to significantly ($p<0.001$) attenuate the hyperactivity response (308±50.9 and 328±75.6 counts/60min respectively compared to 994±16.6 activity counts/60 min for the DiMeC7 control). Activity for the fluphenazine and clozapine control groups (189±49.9 and 197± 27.9 respectively) was not significantly different from the vehicle treated group (227±42.6).

In these investigations the behavioural hyperactivity resulting from disruption of intrinsic hemispheric asymmetry of dopaminergic mechanisms has been successfully antagonised by discrete administration of antipsychotic agents into limbic nuclei in the dominant hemisphere.This may provide the basis for an aetiological model of laterality in the mesolimbic system.
These findings may further elucidate the hypothesis that lateralised dopamine dysfunction in the dominant temporal lobe in schizophrenia may lead to behavioural consequences which might be inhibited by the action of typical and atypical antipsychotic agents in the mesolimbic system.

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344P DUK 25: A CHO CELL LINE IN WHICH THE D_3 DOPAMINE RECEPTOR IS NEGATIVELY COUPLED TO ADENYLYL CYCLASE

D.A. Hall & P.G. Strange, Research School of Biosciences, The University, Canterbury, Kent CT2 7NJ

Molecular biological studies have indicated that the pharmacologically defined D_2 dopamine receptor (DAR) can in fact be divided into several subtypes: D_{2short}, D_{2long}, D_3 & D_4. The sequences of these receptors suggest that they are members of the G-protein-linked-receptor superfamily. Indeed the binding of agonists to the D_{2short}, D_{2long} and D_4 receptors has been shown to be modulated by guanine nucleotides supporting this proposal. These receptor subtypes have also been shown to couple to second messenger systems (e.g. inhibition of adenylyl cyclase) which are normally associated with G-protein-coupled receptors. However, information relating to the D_3 DAR is less clear cut. Whilst some groups have reported no modulation of agonist binding by GTP (e.g. Sokoloff, 1990), this has been demonstrated by others (e.g. Castro & Strange, 1993), although these shifts do tend to be relatively small perhaps suggesting weak or qualitatively different coupling to G-protein. Studies of the coupling of D_3 DARs to functional responses has been similarly contradictory (e.g. Freedman *et al.*, 1994; Chio *et al.*, 1993).

In this report we present data showing that a chinese hamster ovary (CHO) cell line which heterologously expresses the D_3 DAR, and which shows modulation of agonist binding by GTP (DUK 25; Castro & Strange, 1993), also shows dose-dependent inhibition of forskolin (10 μM)-stimulated cyclic-AMP accumulation in response to dopamine. No such dose-dependent inhibition was seen in the parental CHO-DUK cell line. The pD_2 value of dopamine for this inhibition was 8.60 ± 0.12 (s.e.m.; n = 4) (EC_{50} = 2.5 nM) and the maximal extent of inhibition was 50 %. This inhibition was antagonised by (+)-butaclamol at least 1000x more strongly than by the (-)-isomer, this strong stereoselectivity being characteristic of DARs of the D_2-like family.

Thus we report a CHO cell line in which the D_3 DAR is negatively coupled to adenylyl cyclase via G-protein and which should allow the further functional characterisation of this D_2-like receptor subtype.

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345P PHOSPHORYLATION- AND VOLTAGE-DEPENDENT INHIBITION OF CALCIUM CURRENTS IN TRANSFECTED NEUROBLASTOMA-GLIOMA HYBRID NG108-15 CELLS

Nicola A. Brown, & Guy R. Seabrook. Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR.

This study investigated the mechanism(s) by which dopamine receptors inhibit ω-conotoxin GVIa-sensitive calcium currents in transfected neuroblastoma cells. Calcium currents were recorded from differentiated NG108-15 cells that had been transfected with short isoform of the human D_2 dopamine receptor, using conventional whole cell and nystatin-perforated patch clamp recording (Seabrook *et al.*, 1994).

In nystatin-perforated patch clamp recordings, activation of D_2 receptors with exogenously applied quinpirole (10 nM) caused a maximal 55.3 ± 4.0% (n = 21) depression of the high-threshold calcium current (100 ms voltage steps from -60 to +10 mV). This effect did not involve $[Ca^{2+}]_i$, as assessed by its persistence following intracellular dialysis with the calcium chelator EGTA (10 mM), and following the use of extracellular Ba^{2+} ions (5mM) as the charge carrier. The effect of D_2 receptor activation was rendered irreversible following dialysis of cells with the non-hydrolysable GTP analogue GTP-γ-S (100 μM, n = 5), and was completely prevented following preincubation of cells with 1 μg/ml of pertussis toxin (2.5 ± 1.3%, n = 4). The phosphatase inhibitor okadaic acid (100 nM) precluded the sustained inhibition of the calcium current but not the slowing in the rate of current activation. Inhibition of calcium currents did not involve cGMP-dependent protein kinase as the effect of quinpirole was not mimicked by the membrane permeant cGMP analogue Sp-8-bromoguanosine-3',5'-cyclic monophosphorothioate (100 μM, n = 5). However, inhibition of both the peak and sustained components of the calcium current by quinpirole (10 nM) was enhanced following intracellular dialysis with cyclic AMP (100 μM); up to 72.8 ± 9.8% and 70.4 ± 14.8% depression respectively (n = 5). The sustained inhibition of calcium currents was voltage-resistant, whereas following abolition of the sustained depression with okadaic acid (100 nM) the residual kinetic slowing was reversed by depolarising prepulses to +100 mV that immediately preceded the test pulse.

These data demonstrate that pertussis toxin sensitive G-proteins liberated upon activation of human $D_{2(short)}$ dopamine receptors modulate high-threshold calcium currents by two distinct mechanisms. The decrease in rate of calcium current activation occurred via a voltage-dependent pathway that was resistant to okadaic acid, whereas the sustained inhibition of calcium channels was voltage-resistant and mediated by cAMP-dependent protein kinase, the inactivation of which involved okadaic acid sensitive protein phosphatases 1/2a.

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346P COMPARISON OF THE EFFECTS OF SIBUTRAMINE AND D-AMPHETAMINE ON EXTRACELLULAR DOPAMINE LEVELS IN RAT NUCLEUS ACCUMBENS: AN *IN VIVO* MICRODIALYSIS STUDY

K.F. Martin, S.D. Hannon, C. Czudek, D.J. Heal & W.R. Buckett, Boots Pharmaceuticals Research Department, Nottingham NG2 3AA.

Sibutramine HCl, a novel monoamine reuptake inhibitor (Buckett *et al.*, 1988), dose-dependently improves weight loss of patients on restricted calorie diets (Weintraub *et al.*, 1991). Previous studies have indicated dopamine (DA) is not a pharmacological target for sibutramine (Heal *et al.*, 1992). We have further investigated this question using intra-cerebral microdialysis.

Concentric microdialysis probes (Carnegie Medecin) were stereotoxically implanted into the n. accumbens of choral hydrate (600 mg/kg ip and 30 mg as required) anaesthetised male Wistar rats (280 - 300 g; Olac, Bicester). The probes were perfused (1 μl/min) with an artificial cerebrospinal fluid. Perfusates were collected every 20 min and analysed for DA by HPLC-ECD. After a 2h stabilisation period, 3 x 20 min baseline samples were taken before ip injection of drugs and the collection of 9 further samples.

Dose-related increases in dialysate [DA] were evoked by d-amphetamine and, to a much lesser extent, sibutramine (Table 1). In addition, sibutramine's times of maximum effect at 1 and 3 mg/kg were significantly later than d-amphetamine's. Therefore, at doses which inhibit food intake by 50% (sibutramine 2 mg/kg; d-amphetamine 1.5 mg/kg; Halford *et al.*, 1994), sibutramine produces only a 2-fold increase in limbic DA, compared to d-amphetamine which evokes an estimated 14-fold increase. Furthermore, the results show that thermogenic doses of sibutramine (3 and 10 mg/kg; Connoley *et al.*, 1994) are similarly associated with relatively small enhancements of limbic DA. Taken together with previous data (Buckett *et al.*, 1988; Heal *et al.*, 1992), these results demonstrate a minor role for dopamine reuptake inhibition in the pharmacological profile of sibutramine.

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Table 1. The effects of sibutramine and d-amphetamine on dialysate DA levels

Treatment	Dose (mg/kg)	n	Max % increase from vehicle Mean	(95% C.I.)	Median time to max. (min)
Sibutramine	1	7	76*[a,b,c]	(28, 141)	140[a,b,c]
Sibutramine	3	7	111*[a,b,c]	(53, 192)	100[a,b,c]
Sibutramine	10	5	409*[b,c]	(253, 633)	100
d-Amphetamine	0.3	7	268*	(168, 405)	40
d-Amphetamine	1	7	833*	(576, 1191)	40
d-Amphetamine	3	7	2536*	(1805, 3548)	40

[a,b,c] = P<0.01 from d-amphetamine 0.3, 1 or 10 mg/kg, respectively (Neuman-Keuls test). * P<0.01 from vehicle (Williams' test).

347P *IN VITRO* CHARACTERISATION OF [3H]PD128907 AS A SELECTIVE RADIOLIGAND FOR THE DOPAMINE D3 RECEPTOR

Smita Patel, Shil. Patel, R. Marwood, K. L. Chapman, F.Emms & S. B. Freedman, Merck Sharp & Dohme, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR.

The dopamine D3 receptor is structurally and pharmacologically similar to the dopamine D2 receptor subtype (Sokoloff *et al.*,1990) Recently, PD128907 was described as a D3 agonist with 1800 fold selectivity over D2 receptors (Dijkstra *et al.*, 1993). We have examined the binding characteristics of [^{3}H] PD128907 (110Ci/mmol, Amersham,U.K) on human embryonic kidney (HEK) cells stably transfected with the human D3 receptor and rat brain for use as a selective D3 receptor ligand.

Radioligand binding studies were performed on cell lines stably expressing the human or rat D2 and D3 receptors as described previously (Freedman *et al.*,1994). For saturation studies, D3HEK membranes were incubated with either [^{3}H] PD128907 (0.1- 5nM) or [^{3}H] spiperone (0.01-0.6nM) and the affinity (Kd) and maximum number of receptors bound (Bmax) was determined for each radioligand. For receptor autoradiography, rat brain sections were preincubated in assay buffer for 1h, followed by 5nM [^{3}H] PD128907 for 2h. 1μM haloperidol was used to define non specific binding. Sections were washed, dried and exposed to [^{3}H] Ultrafilm (Amersham,U.K)for 2 weeks.

PD128907 displaced [^{3}H] spiperone binding to D3 receptors with high affinity (Ki(nM): human 1.7, rat 0.84) compared to D2 receptors (Ki(nM): human 179, rat 770). [^{3}H] PD128907 bound to D3 receptors with a Kd of 1.4 (1.3,1.4)nM and a Bmax of 1035±110 fmol/mg. [^{3}H] Spiperone also showed specific and saturable binding to D3HEK cells with comparable binding capacity (Kd = 0.25 (0.19,0.25)nM; Bmax = 910±200 fmol/mg). Dopamine receptor agonists and antagonists inhibited [^{3}H] PD128907 binding to D3HEK cells in a concentration dependent manner and a rank order of affinity indicative of D3 receptor pharmacology: Ki(nM) geometric mean with low and high errors of mean in parentheses, PHNO 0.11 (0.07;0.19), PD128907 0.51 (0.2;1.2), apomorphine 2.2 (1.3;3.9), dopamine 2.3 (1.8;2.7), quinpirole 2.6 (1.5;4.5), domperidone 2.6 (2.0;3.4), haloperidol 3.0 (1.0;8.9), (-)-sulpiride 88 (53;146) and clozapine 155 (71;340). In rat forebrain [^{3}H] PD128907 labelled D3 receptors primarily in the islands of Cajella and shell region of the nucleus accumbens.

The selectivity of PD128907 for the D3 over D2 receptor appeared species dependent (110 and 940 fold: human and rat respectively) although this may be a consequence of the differing proportions of high affinity sites in these cloned receptors. [^{3}H] PD128907 can be used to selectively label D3 receptors and is particularly useful for receptor autoradiograhy, where D3 receptors can be visualised within 2 weeks, an improvement over currently available tritiated D3 radioligands.

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348P DIADENOSINE POLYPHOSPHATES EVOKE CALCIUM SIGNALS IN SYNAPTOSOMAL PREPARATIONS OF VARIOUS REGIONS OF THE GUINEA-PIG BRAIN

J. Pintor, C.H.V.Hoyle[1], M. Abal, J. Gualix, J.A. Puche & M.T. Miras-Portugal, Dept. Bioquimica, Facultad de Veterinaria, Universidad Complutense, Madrid 28040 and [1]Dept. Anatomy, University College London, London WC1E 6BT

The presence of diadenosine tetra- and penta-phosphate (Ap4A and Ap5A) in rat brain synaptosomes, their calcium-dependent release, the characterisation of specific binding sites for them, and the fact that they stimulate calcium entry into synaptosomes, increases the physiological relevance of these compounds as neurotransmitters or neuromodulators in the central nervous system. In order to extend these findings the present study examined the possible extracellular signal role of a series of P^1,P^n-bis(5'-adenosine)oligo-phosphates, where n ranges from 2 to 6 (Ap_2A to Ap_6A) in synaptosomal preparations of the guinea-pig brain.

Synaptosomes prepared from the cerebral cortex, the diencephalon plus midbrain, and the cerebellum were incubated with FURA 2-acetoxymethylester (5 μM) at 37°C for 30 min at a protein concentration of 1 mg.ml^{-1}. The synaptosomes were washed in Elliott's buffer and placed in a cuvette in a fluorimeter set to measure fluorescence at 510 nm. After 1 min a dinucleotide (100 μM) was added to the cuvette, and 1 min later KCl (60 mM) was added. The signal for each preparation was calibrated by adding EGTA (25 mM) and triton-X100 (20% v/v) (zero Ca^{2+}-signal) followed by $CaCl_2$ (10 mM). In synaptosomes from the cortex the compounds were equi-effective (Table 1), however, in the cerebellum and diencephalon/midbrain there was a distinct rank order of effectiveness, which was similar in both these regions (Table 1), with Ap_2A being the most potent and Ap_5A being the least potent compound tested. The mean resting calcium concetration was 115 ± 3 nM, and in all cases the increase was statistically significant ($P < 0.05$, Student's *t*-test). These results indicate that diadenosine polyphosphates may function as physiological signal molecules in the guinea-pig brain. Whether or not the dinucleotides are acting on their own receptor is currently the subject of further investigation.

Table 1. Increase in synaptosomal Ca^{2+}-concentration (nM) induced by Ap_nA. Mean ± s.e. mean (n=3).

	Ap_2A	Ap_3A	Ap_4A	Ap_5A	Ap_6A
Cerebellum	24.2 ± 1.41	27.7 ± 1.13	28.8 ± 3.53	21.8 ± 2.30	23.8 ± 2.61
Cortex	36.1 ± 0.92	21.0 ± 1.13	25.7 ± 1.20	17.1 ± 0.67	28.1 ± 7.20
Diencephalon/Midbrain	32.8 ± 1.34	22.8 ± 1.41	29.6 ± 2.33	20.7 ± 5.10	27.8 ± 3.10

349P CHARACTERISATION OF NEUROTENSIN AND ITS METABOLITES IN RAT BRAIN, USING HPLC AND RADIOIMMUNOASSAY

S.H. Moss, C.D. Forster, G.W. Bennett & C.A. Marsden, Dept. of Physiology & Pharmacology, Nottingham University Medical School, Queens Medical Centre, Nottingham, NG7 2UH

We have previously investigated the neurotensin-like immunoreactivity (NT-LI) developmental patterns in various regions of the foetal rat brain, by radioimmunoassay (RIA), using an N-terminally directed antibody (Moss et al., 1994). Immunocytochemical studies, (Sato et al., 1990) were consistent with these results showing early transient NT increases in some brain areas, implicating a developmental role for NT. The RIA measured, in addition to NT, N-terminal NT metabolites, which are of physiological relevance in the adult brain since they are formed on proteolytic inactivation of NT by various peptidases located in the vicinity of the NT receptors (Kitabgi et al., 1992). It is therefore of interest to examine NT degradation during brain development. In this study, high pressure liquid chromatography (HPLC) was combined with the established RIA, providing a specific and highly sensitive (< 1 fmol/tube) method for separate measurement of NT and its metabolites in brain tissue.

HPLC separation of synthetic NT, NT1-11 and NT1-8 standards was achieved, using a Beckman System Gold made up of a (507) auto injector, a (168) solvent delivery system and a 125 x 4.6 mm (internal diameter) 5m Nucleosil RP18 300Å column. Elutions were carried out at room temperature by means of a 30 minute linear gradient from a 30% to a 60% methanol solution, acidified with 0.02% trifluoroacetic anhydride (TFA), at a flow rate of 1.5ml/min. Absorbance was monitored using photodiodarray detection at 220nm with a band width of 5nm.

The HPLC-RIA method was used to quantify NT and metabolites in hypothalamus and striatum of adult female Lister Hooded rats (200-250g). On injection of brain sample extracts, fractions were collected at one minute intervals and NT-LI per fraction subsequently measured by RIA. Results showed negligible quantities of NT1-8 and NT1-11 in hypothalamus and striatum, but indicated significant amounts of two additional N-terminal NT fragments. In the absence of purified standards, their identity was predicted to be NT1-12 and NT1-10 (and possibly NT1-7), by calculating their expected retention times (Meek and Rossetti, 1981), where contribution to retention of each amino acid and end group of each peptide was summed. In the hypothalamus approximate ratios of NT-LI (0.56 ±0.09 fmol/µg protein) to NT1-12-LI (0.21 ±0.03 fmol/µg protein) and NT1-10 (0.32 ±0.06 fmol/µg protein) of 2.5:1 and 1.75:1, respectively occurred, whilst striatal NT-LI (0.046 ±0.003 fmol/µg protein) was present in a ratio of 4:1 and 2:1, to NT1-12-LI (0.012 ±0.001 fmol/µg protein) and NT1-10-LI (0.023 ±0.004 fmol/µg protein), respectively. Further clarification of the identity of metabolites will occur following availability of the relevant synthetic NT metabolites.

In future studies this method will provide a means of measuring NT and its metabolites in foetal tissue, in order that changes in peptide metabolism can be evaluated during development, and may also provide a means of selectively measuring such peptides in microdialysis samples.

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350P THE ANALGESIC ACTIVITY OF THE NK_1 RECEPTOR ANTAGONIST CP99,994 IN MODELS OF CHRONIC INFLAMMATORY AND NEUROPATHIC PAIN IN THE RAT

E.A. Campbell, A. Davis, A. Dray, C. Gentry, S. Kane, S. Patel, M.N. Perkins and L. Urban, Sandoz Institute for Medical Research, 5 Gower Place, London, WC1E 6BN.

There are now several reports showing analgesic efficacy of NK_1 receptor antagonists (Boyce et al, 1992; Garret et al, 1991; Birch et al, 1992) but few studies have determined their effect in models of chronic inflammatory pain, or in neuropathic pain. We therefore determined the efficacy of the potent NK_1 receptor antagonist CP99,994 in chronic inflammatory and neuropathic pain in rats.

Mechanical hyperalgesia was induced following chronic inflammation produced by either intra-articular injection of 100µl of Freunds complete adjuvant into one knee or injection of turpentine (50µl of 50%turpentine/50%paraffin oil) into one paw. In these models the effect of CP99,994 was studied 3 days post induction of inflammation. A neuropathic pain model was established by ligation of 1/2-1/3 of the left sciatic nerve using 7-0 silk suture under enflurane anaesthesia and the animals allowed to recover (Seltzer et al, 1990). Mechanical and thermal hyperalgesia develops in the ligated paw over several days. CP99,994 was studied in these animals 2 weeks post-ligation.

In the dose range 0.1-10mg/kg CP99,994 did not fully reverse the hyperalgesia, in particular in the turpentine and neuropathic models the response appeared to plateau at 1mg/kg. In the Freunds adjuvant model the peak reversal of hyperalgesia showed a similar dose response relationship when CP99,994 was administered s.c. or p.o. (71.3 ± 10.4% and 64.7 ± 5.84 reversal respectively at 10mg/kg, n = 8/group). However, the duration of action was longer following s.c. administration (at 10mg/kg s.c. > 5h, compared to 2h at the same dose p.o.). Based on the mean percentage reversal which takes into account peak effect and duration, an ED_{50} of 8.73mg/kg s.c. could be calculated. CP99,994 p.o. reversed the mechanical hyperalgesia induced by turpentine at a dose of 1 and 10mg/kg with no significant difference in efficacy between the two groups (52.1 ± 12.2% and 65.26 ± 12.3% reversal respectively, n = 10/group). In the neuropathic animals oral activity was apparent up to 4h post administration when both 1 and 10mg/kg produced a partial reversal of mechanical (30.7 ± 10.8% and 27.6 ± 10.9% reversal respectively, n = 8/group) and thermal (74.3 ± 15.9 and 51.9 ± 8.26% reversal respectively, n = 6/group) hyperalgesia. A lower dose (0.1mg/kg) had no significant effect at any time.

The data show clear analgesic activity of the NK_1 receptor antagonist, CP99,994 in both inflammatory and neuropathic pain models and supports the therapeutic use of such an agent in chronic pain conditions.

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351P COMPARISON OF SPECIES DIFFERENCES BETWEEN AFFINITY FOR NK_1 RECEPTORS *IN VITRO*

Dacquet, C., Ribault, C., Cogé, F., Lechevallier, S.P. & Spedding, M. Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy-sur-Seine (Paris, France).

Prediction of species differences is particularly important when assessing the potency of neurokinin antagonists on tachykinin receptors, so we have characterised five *in vitro* radioligand binding assays of NK_1 receptors, using a wide range of agonists and antagonists : 1) the human NK_1 receptor was transfected into chinese hamster ovary cells as previously described (NK_1-CHO ; Cogé & Regoli, 1994), 2) the human cell line IM9 was grown in culture (Payan et al. 1984), binding was assessed in homogenates of 3) rat cerebral cortex, 4) mouse cerebral cortex, 5) gerbil cerebral cortex. Radioligand binding conditions were : $[^3H]$-$Sar^9,Met(O_2)^{11}$ substance P (1 nM) as the ligand, in the presence of membrane preparations (0.2 mg/ml) or cells (NK_1-CHO 4.10^5 cells/ml, IM9 3.10^6 cells/ml). Non specific binding was defined in the presence of 1 µM of substance P in the incubation medium.

These results show an excellent correlation between pKi values obtained in rat and mouse cerebral cortex (r=0.96) but there is little correlation between rat and mouse cortex and gerbil cortex (respectively, 0.14 and 0.10). On the contrary, the binding of tachykinin ligands on the human NK_1 receptor was cell correlated between transfected CHO cells and IM9 cells (r=0.85) and the correlation was also excellent between pKi values in the cerebral cortex of the gerbil and human NK_1 receptor on CHO or IM9 cells (r=0.94 with the two cell lines). In a large series of new molecules the correlation between affinities for human and gerbil NK_1 receptors was 0.85.

The results suggested that, like the guinea-pig NK_1 receptor, the pharmacological NK_1 receptor profile in the gerbil is very close to that of the human NK_1 receptor . Furthermore both NK_1-CHO and IM9 assays systems are well correlated. This receptor seems to be different from those of rat and mouse cerebral cortex confirming the observations of Beresford et al. 1991.

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Table 1: Inhibitory potency of tachykinin receptor agonists and antagonists on NK_1 receptors from different species

	Rat	*Mouse*	*Gerbil*	*NK1-CHO*	*IM9*
Substance P	9.13±0.14	9.15±0.07	8.95±0.15	9.64±0.12	9.33±0.10
$[Sar^9,Met(O_2)^{11}]$SP	8.60±0.49	8.78±0.67	8.95±0.11	9.85±0.30	8.88±0.40
CP99994 (±)	6.52±0.52	6.62±0.47	9.22±0.06	9.30±0.15	9.60±0.08
CP96345 (±)	7.14±0.21	6.61±0.51	8.66±0.59	9.52±0.10	9.60±0.90
RP67580	7.58±0.29	7.78±0.30	6.53±1.01	7.51±0.06	7.08±0.27
L732138	5.75±0.51	5.97±0.30	7.68±0.83	------	8.72±0.05
NKA	6.48±0.29	6.50±0.46	6.67±0.84	6.4[illegible]0.05	7.45±0.80
SR48968	<4	<5	5.66±0.41	5.56±0.05	<4
Senktide	<5	<4	5.11±1.37	<4	<4

Affinities are expressed as pK_is (-log Ki) and were the means of independent experiments (n=3) each done in duplicate.

352P INFLUENCE OF THE TACHYKININ NK_1 RECEPTOR ANTAGONIST, SR-140333, AND OF COLCHICINE ON FUNCTIONS MEDIATED BY AFFERENT C-FIBRES IN THE RAT

I. Juránek & F. Lembeck, Department of Experimental and Clinical Pharmacology, University of Graz, Universitätsplatz 4, A-8010 Graz, Austria.

The inhibition of function of substance P-containing afferent C-fibres was investigated in the rat (1) following colchicine pretreatment which unspecifically inhibits the axonal transport of neurotransmitters by using the method of Moussaoui *et al.* (1993) and (2) following administration of the non-peptide NK_1 receptor antagonist SR-140333 which inhibits the effect of substance P released from peripheral nerve terminals (Emonds-Alt *et al.*, 1993).

Plasma protein extravasation into the skin (Emonds-Alt *et al.*, 1993) induced by antidromic sciatic nerve stimulation (180 ± 12 µg Evans blue g^{-1} wet wt), mustard oil application (150 ± 10 µg g^{-1}) and i.v. injection of substance P (94 ± 13 µg g^{-1}) were strongly reduced by SR-140333 (10-40 µg kg^{-1}, i.v.) ($P<0.001$, $P<0.01$ and $P<0.05$, respectively). Colchicine (120 µg kg^{-1}, i.p., daily for 3 days) inhibited the first two effects ($P<0.01$ and $P<0.05$), but not that of substance P.

Vasodilatation in the skin was measured (Hajos *et al.*, 1988) by the increase in temperature on the ventral side of the fore paws (ΔT = 2.3 ± 0.4 °C, n=6) following i.v. injection of capsaicin (200 µg kg^{-1}) in rats sedated by diazepam (25 mg kg^{-1}). While SR-140333 (100 µg kg^{-1}, i.v.) completely blocked the rise in skin temperature ($P<0.001$), colchicine had no significant effect (ΔT = 1.9 ± 0.4 °C, n=6). The reduction of the capsaicin-induced increase vasodilatation in the paw by SR-140333 but not by colchicine indicates that substance P, released by capsaicin, is mainly released from peripheral nerve terminals and that the vasodilatation is not based on a reflex heat loss reaction (Donnerer & Lembeck, 1983).

Depressor reflexes which are known to be mediated by capsaicin-sensitive afferent neurones such as those elicited (a) by a stimulating dose of 30 ng capsaicin i.a. (fall in mean arterial pressure: -29 ± 2 mmHg, n=6), (b) by short distension (40 mbar) of the ascending colon (-17 ± 2 mmHg, n=6) and (c) by afferent sciatic nerve stimulation (-21 ± 2 mmHg, n=6) were used. Colchicine significantly reduced the depressor reflexes evoked by (a) and (b) but not those induced by (c). SR-140333 in doses up to 100 µg kg^{-1}, i.v., 5 min before did not inhibit any of the depressor reflexes.

The effects of SR-140333 and colchicine on peripheral functions of afferent C-fibres confirm the efficacy of the treatments. The effects of both treatments on centrally mediated functions are controversial. None of the depressor reflexes were affected by SR-140333, indicating either a major contribution of transmitters other than substance P or an insufficient entrance of SR-140333 into the CNS. Two of the depressor reflexes, (a) and (b), were reduced by colchicine suggesting a reduced axonal transport of neurotransmitters in the central terminal of afferents, in the CNS or in efferent fibres. The depressor reflex evoked by afferent sciatic nerve stimulation (c) was not reduced by colchicine which has to be investigated further.

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353P EFFECT OF A TACHYKININ NK_1 RECEPTOR ANTAGONIST SR140333 ON OEDEMA FORMATION INDUCED IN RAT SKIN BY VENOM FROM THE *PHONEUTRIA NIGRIVENTER* SPIDER

R. Palframan, P. Wilsoncroft, E. Antunes[1], G. de Nucci1 & S.D. Brain. Pharmacology Group and Vascular Biology Research Centre, Divison of Biomedical Sciences, King's College, Manresa Road, London SW3 6LX. U.K. [1]Dept of Pharmacology, Faculty of Medical Sciences, UNICAMP, PO Box 61111, 13081 Campinas, Sao Paulo, Brazil.

Venom from the *Phoneutria nigriventer* spider induces oedema formation when injected intradermally (i.d.) in rabbit and rat skin (Antunes *et al.*, 1992). In this study we have investigated whether the oedema formation observed in rat skin has a neurogenic component, by investigating the ability of the selective non-peptide tachykinin NK_1 receptor antagonist SR140333 (Emonds-Alt *et al.*, 1993) to modulate responses. The dorsal skin of male Wistar rats (200-250g), anaesthetised with sodium pentobarbitone (50mg kg^{-1}, i.p.), was shaved and oedema formation induced by agents made up in a modified Tyrode solution and injected i.d. (0.1 ml/site). The response was measured at skin sites over 30 min by the extravascular accumulation of i.v. ^{125}I-albumin (Antunes et al., 1992).

The selective NK_1 agonist GR73632 (30 pmol/site, Beresford *et al.*, 1991) and histamine (10nmol/site) induced oedema when injected i.d. with the vasodilator CGRP (10pmol/site). SR140333 (1nmole/site, co-injected at different sites in the same animal) significantly ($p<0.01$, modified t-test) inhibited oedema induced by GR73632 + CGRP whilst having no effect on oedema induced by histamine + CGRP. Results as follows: control sites (Tyrode i.d.) 12.6 ± 1.6µl, GR73632 + CGRP 71.2 ± 12.3 µl, histamine + CGRP 76.0 ± 11.7 µl, SR140333 alone 7.9 ± 0.6 µl, GR73632 + CGRP + SR140333 24.1 ± 3.0 µl, histamine + CGRP + SR140333 81.0 ± 9.1 µl oedema, mean ± s.e. mean, n=6. *Phoneutria nigriventer* venom induced oedema formation in a dose-related manner as follows; 0.03 µg/site 19.99 ± 2.8µl, 0.1 µg/site 35.5. ± 4.5µl, 0.3 µg/site 51.5 ± 6.4 µl, n=6. SR140333 (1 nmol/site co-injected) significantly reduced oedema formation at all doses as follows: 0.03 µg/site 11.6 ± 1.1µl, 0.1 µg/site 11.4 ± 1.4 µl, 0.3 µg/site 30.3 ± 3.1 µl, n=6. The results also indicate that *Phoneutria nigriventer* venom induces release of oedema-inducing amounts of a NK_1 agonist in rat skin.

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354P CHARACTERISATION OF SUBSTANCE P-INDUCED ^{111}In-NEUTROPHIL ACCUMULATION IN GUINEA-PIG SKIN

D.T.Walsh, T.J.Williams & S.Nourshargh, Dept. Applied Pharmacology, National Heart and Lung Institute, London, SW3 6LY.

Substance P (SP) has been implicated as a major mediator of neurogenic inflammation (Lembeck & Holzer, 1979).We have previously shown that i.d. SP induces ^{111}In-eosinophil accumulation and oedema formation in guinea-pig skin, via two distinct mechanisms (Walsh *et al.*, 1994). We have now extended these studies to investigate the profile of ^{111}In-neutrophil accumulation induced by SP.

Accumulation of ^{111}In-neutrophils was measured as previously described (Sanz *et al.*, 1994). Using a 2h accumulation period, i.d. SP caused a dose dependent ^{111}In-neutrophil accumulation. Significant accumulation was only reached at the top two doses tested (10^{-10} and 10^{-9} moles/site producing 1293 ± 269 and 1720 ± 196 cells/site respectively compared to 814 ± 144 cells/site with i.d. saline/BSA $p<0.05$). Maximal ^{111}In-neutrophil accumulation occurred within the first hour and remained elevated during the 2h test period.Using two specific NK_1 receptor antagonists, CP-96,345 (Snider *et al.*, 1991) and RP-67,580 (Garret *et al.*, 1991) we have studied the role of NK_1 receptors in the ^{111}In-neutrophil accumulation. CP-96,345 (1mg/kg i.v.) did not affect ^{111}In-neutrophil accumulation significantly (10^{-9}moles/site SP inducing 1720±196 and 1404±88 cells/site in control and CP-96,345 treated animals respectively, n=7). The isomer CP- 96,344, which has no effect on NK_1 receptors, had no effect on cell accumulation. RP-67 580 when co-injected i.d. with SP (10µg site) had no effect on neutrophil accumulation.

The specific H_1 receptor antagonist chlorpheniramine (10µg/site), whilst totally inhibiting the oedema induced by i.d. histamine (10^{-8}moles/site) had no effect on SP (10^{-9}moles/site)-induced ^{111}In-neutrophil accumulation (1277±143 and 1255±153 cells/site in control and chlorpheniramine sites respectively, n=6). The PAF antagonist UK-74,505 (Parry *et al.*, 1994 2.5mg/kg i.v.) also had no effect on ^{111}In-neutrophil accumulation (10^{-9} moles/site SP induced 2066±327 and 2012±356 cells/site in control and UK-74,505 treated animals respectively, n=6) whilst abolishing the neutrophil accumulation induced by PAF (10^{-9}moles/site). Administration of an anti-CD18 monoclonal antibody, 6.5E $F(ab')_2$ (Andrew *et al.*, 1993, 2.5mg/kg i.v), abolished SP-induced ^{111}In-neutrophil accumulation (e.g. accumulation induced by 10^{-9}moles/site was reduced from 1420±480 to 480±81 cells/site $p<0.01$ n=6).

These results suggest SP-induced neutrophil accumulation is a mechanism independent of NK_1 receptors but dependent on the CD18 adhesion molecule.

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355P NK_1 AND CGRP RECEPTOR-MEDIATED DILATATION OF THE CAROTID ARTERIAL BED IN THE ANAESTHETISED RABBIT

Marion J. Perren, H.E. Connor & D.T. Beattie, Pharmacology 2, Glaxo Research and Development Ltd, Park Road, Ware, Herts SG12 0DP, U.K.

Substance P and CGRP relax cranial blood vessels via activation of NK_1 and $CGRP_1$ receptors respectively (Stubbs *et al.*, 1992; Jansen, 1992). In the present study, changes in carotid arterial blood flow (CF) and vascular resistance (CVR) have been measured in response to intra-carotid administration of the selective NK_1 receptor agonist, substance P methyl ester (SPOMe), and human α and βCGRP. Responses were characterised using the selective NK_1 and CGRP receptor antagonists, CP99,994 and human $\alpha CGRP_{8-37}$ respectively (McLean *et al.*, 1993; Jansen, 1992).

Adult rabbits (NZW, 2.5-3.5kg) of either sex were anaesthetised with sodium pentobarbitone (45mgkg^{-1} i.v. followed by 90mgkg^{-1} s.c. every 90min) and artificially respired with room air supplemented by oxygen (50 strokes min^{-1}, 5mlkg^{-1}). Arterial blood pressure (BP) was recorded from a femoral artery and right CF was measured using a Doppler flow probe (Iowa 545C-4). CVR was determined from the BP and CF signals. Two consecutive two-point dose-response curves were constructed to SPOMe, α or βCGRP, injected into the carotid arterial bed via the right lingual artery (15min between each dose). Receptor antagonists, or vehicle, was then injected i.v. via a femoral vein and agonist dose-response curves repeated. Data are expressed as arithmetic (±s.e. mean) or geometric means (range) as appropriate.

SPOMe (0.014 and 0.14ngkg^{-1}) and α or βCGRP (both 3.8 and 38ngkg^{-1}) caused dose-related, reproducible increases in CF as a consequence of reductions in CVR, with no change in BP. SPOMe (0.14ngkg^{-1}), α and βCGRP (both 38ngkg^{-1}) reduced CVR by 45±4, 53±3 and 53±6% respectively (n=3-6). Changes in CVR and CF evoked by SPOMe were transient (duration approximately 90s at 0.14ngkg^{-1}), while those produced by α and βCGRP were more prolonged (duration approximately 8min at 38ngkg^{-1}). CP99,994 (100μgkg^{-1} i.v.) and $\alpha CGRP_{8-37}$ (1.3mgkg^{-1} i.v.) had no effect on BP, CF or CVR in their own right. CP99,994 (100μgkg^{-1} i.v.) caused a parallel rightward shift of the dose-response curve to SPOMe (CVR dose-ratios were 57 (42-76) and 26 (14-48), 15 and 75min following antagonist administration respectively (n=3)). CP99,994 (100μgkg^{-1}) had no effect on α or βCGRP (both 38ngkg^{-1})-induced changes in CF and CVR. $\alpha CGRP_{8-37}$ (1.3mgkg^{-1} i.v.) inhibited, with similar potency, the α and βCGRP-induced changes in CVR; 5min after antagonist administration dose-ratios were 12 (8-15) and 31 (20-41) respectively (n=3 for each). After 65min, responses to both agonists had almost recovered to pre-antagonist levels; dose-ratios were 2 (2-3) and 1 (0.7-3) for α and βCGRP respectively. $\alpha CGRP_{8-37}$ (1.3mgkg^{-1} i.v.) had no effect on SPOMe (0.14ngkg^{-1})-induced changes in CF or CVR.

It is concluded that intra-carotid arterial injection of NK_1 and CGRP receptor agonists in the anaesthetised rabbit results in an increase in CF and reduction in CVR, indicative of a dilatation of the carotid arterial bed. The model used in the study could be useful for determining the potency and duration of action of NK_1 and CGRP receptor antagonists *in vivo*.

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356P (–)-PINDOLOL INCREASES EXTRACELLULAR 5-HT LEVELS IN RAT VENTRAL HIPPOCAMPUS AND REDUCES THE EFFECTS OF THE $5\text{-}HT_{1A}$ AGONIST 8-OH-DPAT: A MICRODIALYSIS STUDY

M.B. Assié & W. Koek, Neurobiology Division II, Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81106 Castres Cedex, France

The β-blocking agent pindolol has substantial affinity for $5\text{-}HT_{1A}$ and $5\text{-}HT_{1B}$ receptors. It has been shown to antagonise the decrease of 5-HT release in rat hippocampus, induced by the $5\text{-}HT_{1A}$ receptor agonist 8-OH-DPAT (Sharp & Hjorth, 1990). The present experiments were aimed at characterising further the profile of (-)-pindolol alone, and in combination with 8-OH-DPAT, on 5-HT levels in ventral hippocampus of anaesthetised rats using *in vivo* microdialysis.

Male Sprague Dawley rats (260-340 g) were anaesthetised with chloral hydrate. A microdialysis probe (CMA/12, 2mm length) was implanted into the left hippocampus and was continuously perfused (1μl/min) with artificial CSF (Sharp *et al.*, 1989). Starting 2h after implantation, perfusates were collected every 20min and analysed for 5-HT content using HPLC-EC. Four samples were collected to determine the basal level, after which the animals were given (-)-pindolol or vehicle (s.c., or through the probe during the remainder of the experiment), 2 samples were collected before the injection of either 8-OH-DPAT (0.31 mg/kg) or saline (i.p.), and additional samples were collected for a further 140min.

(-)-Pindolol, after systemic administration, dose-dependently increased 5-HT levels in the hippocampus (Table 1); infusion of (-)-pindolol (10μM) through the probe induced an effect comparable to a systemic dose of 10 mg/kg. (-)-Pindolol inhibited the significant decrease of 5-HT levels induced by 8-OH-DPAT, and did so at a dose lower than those needed to increase 5-HT levels when given alone (Table 1).

Table1. Effects of (-)-pindolol alone and in combination with 8-OH-DPAT on 5-HT levels in rat ventral hippocampus. Data are expressed as percent AUC for 140 min after injection of 8-OH-DPAT or saline compared to pre-injection values (100%). Number of animals in brackets; * significantly different from dose 0, Dunnett's test.

Dose (mg/kg)	alone	+8-OH-DPAT
0	76.7 ± 4.6 (5)	33.8 ± 5.3 (5)
0.16	87.9 ± 6.3 (5)	52.2 ± 5.0 (5)
0.63	97.5 ± 8.9 (5)	72.7 ± 11.4 (5)*
2.5	109.7 ± 9.2 (5)*	81.0 ± 6.1 (5)*
10	104.3 ± 5.4 (5)*	65.3 ± 8.5 (5)*
10 μM (probe)	107.4 ± 9.8 (3)	58.3 ± 6.3 (3)

Although the results obtained with a low dose of (-)-pindolol are consistent with its $5\text{-}HT_{1A}$ antagonist properties, at doses only slightly higher, the ability of (-)-pindolol to diminish the effects of 8-OH-DPAT may represent antagonism of $5\text{-}HT_{1A}$ and $5\text{-}HT_{1B}$ receptors.

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357P EFFECTS OF ENDOGENOUSLY RELEASED 5-HT ON THE ACTION OF 8-HYDROXY-DPAT AT 5-HT$_{1A}$ AUTORECEPTORS

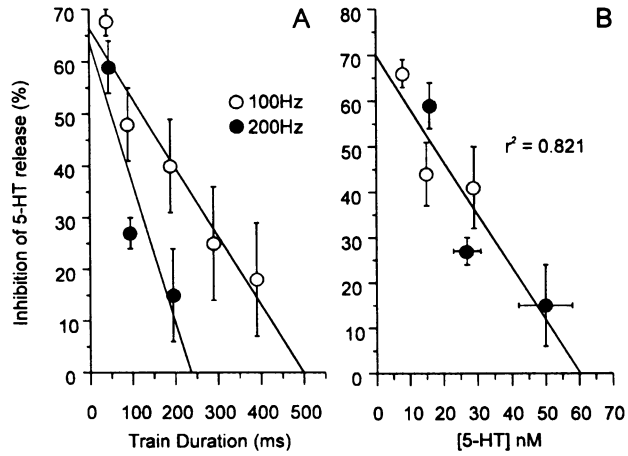
C. Davidson & J.A. Stamford, Anæsthetics Unit (Neurotransmission Laboratory), London Hospital Medical College, Whitechapel, London E1 1BB

In studies of evoked neurotransmitter release, endogenously released transmitter can compete with an exogenously applied agonist for autoreceptors and thereby mask the true effect of the agonist. We were interested to determine how different stimulation parameters might influence the ability of an exogenously applied 5-HT$_{1A}$ agonist to inhibit stimulated 5-hydroxytryptamine (5-HT) release.

Experiments were performed in rat dorsal raphe nucleus (DRN) slices as previously described (O'Connor & Kruk, 1991). Electrical stimulations (5, 10, 20, 30 or 40 pulses at 100 or 200 Hz every 5 min) were applied to the DRN by a bipolar stimulating electrode and 5-HT release was recorded by fast cyclic voltammetry. After four stable control stimulations, the 5-HT$_{1A}$ agonist 8-OH-DPAT (DPAT) was added and its maximum percentage inhibition of 5-HT release calculated.

Figure 1A shows that the effect of DPAT (1.0μM) may depend on the stimulus train duration. DPAT inhibits release of 5-HT from the DRN most on short train durations. By extrapolation of the line, the maximum inhibition of 5-HT release at this concentration of DPAT would be 66%, with the effect of DPAT abolished on trains of 501ms and 236ms duration for 100 Hz and 200Hz stimulation respectively. The effect of DPAT on stimulations of the same train duration depends on how much 5-HT is released. For example the 20pulse/200Hz stimulation releases almost twice as much 5-HT as the 10pulse/100Hz train (27nM and 15nM respectively) while both have almost identical train durations (95 and 90 ms respectively). However, DPAT has a significantly (P<0.05) smaller effect on the 20pulse/200 Hz train.

Figure 1B shows pooled data (n=3/4 for each point ± sem) for both 100 and 200Hz stimulations. The effect of extracellular 5-HT concentration on DPAT inhibition can be seen more



clearly. DPAT has a greater effect when less 5-HT was released. Again, by extrapolation, the maximum inhibition of 5-HT release would be 70% when no 5-HT was released, with the effect of DPAT abolished at an extracellular 5-HT concentration of 60nM.

Although train duration and 5-HT release are interrelated, these data demonstrate that the effect of an exogenously applied agonist is dependent, under these conditions, more on the *concentration* of endogenous agonist released than *the length of time* it has to act on its own autoreceptors,. It is also possible to use this method to calculate the theoretical effect of an agonist in the absence of any endogenous autoreceptor activation.

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J J O'Connor & Z L Kruk (1991) *J Neurosci Meth.* **38**, 25-33

358P THE EFFECT OF 8-OH-DPAT ON ETHANOL INGESTION AND MAINTAINED BEHAVIOUR IN SELF-ADMINISTRATION AND TWO BOTTLE CHOICE STUDIES IN THE RAT

A.W. Wilson, J.C. Neill, & B. Costall, Postgraduate Studies in Pharmacology, School of Pharmacy, University of Bradford, West Yorkshire BD7 1DP.

Previous studies have shown that increased 5-hydroxytryptamine (5HT) neurotransmission leads to a reduction in ethanol consumption, in rodents in free choice tests (Sellers *et al.*, 1992). More specifically the 5HT$_{1A}$ receptor agonist 8-hydroxy 2-(di-n-propylamino) tetralin, (8-OH-DPAT) has been shown to reduce ethanol consumption, (Kostowski *et al.*, 1992). The present studies examined the effects of 8-OH-DPAT on ethanol preference in a two bottle free choice test in the home cage and on ethanol maintained behaviour in a self-administration paradigm. Nine singly housed female Sprague Dawley (CD) rats (350-400 g) were given 16 h access to both 10% ethanol and water and continual access to food. 8-OH-DPAT (30-250 μg/kg, s.c. immediately prior to testing) was without effect on 16 h intake of 10% ethanol and water, and 24 h food intake in the two bottle choice test. For the self-administration experiments, seven female CD rats (300-350 g) were trained to lever press for an oral delivery of 8% ethanol in a 60 min test period on an FR2 schedule of reinforcement, as described in detail elsewhere, (Neill et al., 1994). 8-OH-DPAT (0.05-1.0 mg/kg) was injected s.c. 30 min prior to testing and its effects on ethanol intake (g/kg of body weight), and ethanol maintained behaviour, i.e., the number of lever press responses and dipper deliveries were measured. Locomotor activity (LMA), was measured as line crossings within the drug delivery chamber. Additionally, since low doses of 8-OH-DPAT have been reported to increase ethanol intake in male Wistar rats (Tomkins *et al.*, 1994), the effects of 8-OH-DPAT (0.001-0.01 mg/kg) were examined in the self-administration paradigm (n=6). Data were analysed using one way ANOVA followed by Dunnett's t-test. In the self-administration test, 8-OH-DPAT (0.05-1.0 mg/kg) had a significant effect to reduce the number of lever press responses from control levels of 55.6±10.0 to 2.7±1.0 and 3.6±1.8 at 0.5 and 1.0 mg/kg respectively, ($F(6,24)=4.839$, $p<0.05$); dipper deliveries from control levels of 17.1±3.2 to 6.5±2.97, 0.43±0.43 and 0.14±0.14 at 0.1, 0.5 and 1.0 mg/kg respectively, ($F(6,24)=14.489$, $p<0.001$); and ethanol intake (g/kg) from control levels of 0.342±0.061 to 0.134±0.063, 0.009±0.009 and 0.003±0.003 at 0.1, 0.5 and 1.0 mg/kg respectively, ($F(6,24)=14.229$, $p<0.001$). LMA was significantly reduced from control levels of 245.9±20.2 to 121.9±18.9 and 120.6±15.7 at 0.5 and 1.0 mg/kg respectively, ($F(6,24)=19.402$, $p<0.001$). In contrast 8-OH-DPAT (0.001-0.01 mg/kg) was without effect on ethanol intake and maintained behaviour and LMA. In summary, the present results show that in a self-administration procedure 8-OH-DPAT produced a dose-related reduction in ethanol intake and maintained behaviour, and LMA. At one dose, 8-OH-DPAT (0.1 mg/kg) had a significant effect on ethanol ingestion in the absence of any effect on LMA. A lower dose range of 0.001-0.01 mg/kg was without effect in the self-administration paradigm. In addition, 8-OH-DPAT (30-250 μg/kg) was without effect on 10% ethanol, water or food intake in a two bottle choice test carried out in the home cage. These data show that 8-OH-DPAT had a specific effect to reduce ethanol ingestion in a self-administration paradigm, but had no effect in a two bottle choice test. Therefore the effects of 8-OH-DPAT on alcohol intake may depend on the test paradigm used.

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359P CHARACTERISATION OF 5-HT$_1$-LIKE RECEPTORS MEDIATING VASOCONSTRICTION OF ISOLATED EQUINE DIGITAL VEINS

J. Soydan & J. Elliott Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU

The pathophysiology of the ischaemic disease, equine laminitis (EL), where the sensitive laminae of the horse's foot are starved of nutrient blood flow, is poorly understood. 5-HT is a potent vasoconstrictor of equine isolated digital blood vessels and our previous work suggested that both 5-HT2 and 5-HT1-like receptors were involved in the vasoconstrictor response of equine digital veins to 5-HT (Weller et al 1994). The aim of the present study was to further characterise the responses mediated by 5-HT1-like receptors.

Rings of equine digital vein (EDV) were prepared for isometric tension recording as previously described (Elliott et al., 1994). In all cases, the endothelium was removed mechanically. Vessels were incubated with benextramine (0.1 mM) and pargyline (0.5 mM) for 30 min, washed with drug-free Krebs-Henseleit solution and their contractile responses to depolarising Krebs solution (DKS; 118 mM KCl) measured. Cumulative dose response curves (CRCs) were constructed to sumatriptan (SUM; 1 nM to 30 μM) in vessels in the presence of ketanserin (KET; 30 nM), methiothepin (MET; 10 nM), propranolol (PROP; 1 μM) and yohimbine (YOH; 0.1 μM and 1 μM). Weight-matched adjacent vessel segments obtained from the same horse were examined within the same experiment in the absence of antagonist (controls). The increase in tension obtained was expressed as a percentage of the response obtained to DKS.

The CRC parameters (EC_{50} and maximum response) obtained for SUM in the presence (treated) and absence (control) of the antagonists are presented in the Table. Apparent pK_b values calculated for yohimbine were 6.82 and 6.74 (0.1 and 1 μM respectively).

The high potency of SUM as a vasoconstrictor, its resistance to inhibition by KET and sensitivity to inhibition by MET confirm our previous results that EDV possess 5-HT1-like receptors linked to vasoconstriction (Weller et al, 1994). The lack of effect of PROP tends to rule out an involvement of 5-HT1A and 5-HT1B receptor sub-types. The dose-dependent and competitive inhibition of the responses to SUM obtained in the presence of yohimbine supports the involvement of 5-HT1D receptors. Indeed, the apparent pK_b values for YOH obtained here are in close agreement with those reported for 5-HT1D receptors in blood vessels from other species (Deckert et al., 1994).

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Antagonist	n	EC_{50} (nM)		Max response (% DKS)	
		Treated	Control	Treated	Control
PROP (1 μM)	5	42.6 ± 2.9	41.6 ± 3.0	91.9 ± 1.2	88.5 ± 1.4
KET (30 nM)	4	61.4 ± 1.71	59.7 ± 2.4	70.4 ± 0.4	72.1 ± 0.6
MET (10 nM)	5	481.5 ± 77.6**	66.6 ± 6.2	63.3 ± 2.8**	80.9 ± 1.8
YOH (0.1 μM)	4	121.8 ± 14.5*	73.1 ± 4.5	63.8 ± 1.9	63.6 ± 0.9
YOH (1 μM)	4	480.9 ± 62.6**	73.1 ± 4.5	60.2 ± 1.8	63.6 ± 0.9

(* $p<0.02$, ** $p< 0.001$ vs. control by a Student's t-test)

360P PRESENCE OF 5-HT "1-LIKE" RECEPTORS IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

N.J. Homewood, & S.J. Ireland, Pharmacology 1, Glaxo Research and Development Ltd, Ware, Herts SG12 0DP UK.

The aim of this study was to determine which subtypes of the 5-hydroxytryptamine (5-HT) "1 like" receptor family are expressed on primary cultures of human umbilical vein endothelial cells (HUVEC). The polymerase chain reaction (PCR) has been used to screen cDNA isolated from HUVEC using primers specific for the receptors of interest.

Primary HUVEC were isolated from fresh umbilical cords by collagenase digestion (Jaffe et al., 1973) and grown to confluence over 7 days in 1:1 M 199:RPMI 1640 medium with 2 mM glutamine and 20 % v/v foetal calf serum. Total RNA was isolated from primary HUVEC according to the method of Chomczynski and Sacchi (1987) using RNAzol B (AMS Biotech.) and reverse transcribed to produce cDNA using random hexamers as primers. For PCR, specific primers (21 bases long) were synthesised complementary to regions of the receptor gene sequence least conserved between the subtypes. For each subtype, 2 sense and 2 antisense primers were synthesised. The 4 possible combinations of these primers (1 sense, 1 antisense/tube) synthesise 4 different fragments, each of a specific base length, ranging in size from 269-1101 base pairs long. Control tubes were included in which reverse transcriptase or mRNA were omitted and served to identify DNA contamination of the RNA preparations or PCR reactions. To verify the integrity of RNA preparations, a control reaction was included in which primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used instead of the 5-HT primers. GAPDH was amplified in all RNA samples.

The first PCR experiments used one pair of primers per receptor (5-HT_{1A}, 5-HT_{1F}, 5-HT_{1E}, $5\text{-HT}_{1D\alpha}$, $5\text{-HT}_{1D\beta}$). From this screen only the $5\text{-HT}_{1D\alpha}$ or $5\text{-HT}_{1D\beta}$ products were observed (n=5 single cords). cDNAs were then further analysed using the 4 primer combinations for $5\text{-HT}_{1D\alpha}$ and $5\text{-HT}_{1D\beta}$ subtypes. The results from these experiments provide evidence for the expression of either $5\text{-HT}_{1D\alpha}$ or $5\text{-HT}_{1D\beta}$ (n=3 out of 5) or for apparent coexpression of these receptors in HUVEC (n=2 out of 5).

This PCR-based study shows that primary cultures of HUVEC are likely to express either or both subtypes of the 5-HT_{1D} receptor ($5\text{-HT}_{1D\alpha}$ or $5\text{-HT}_{1D\beta}$) and that expression may differ between individuals. At present, the lack of selective pharmacological probes precludes definitive functional analysis of the 5-HT_{1D} receptor subtypes actually present in a given tissue sample.

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361P CHARACTERIZATION OF 5-HYDROXYTRYPTAMINERGIC RECEPTORS ON THE RETINAL CIRCULATION OF THE RABBIT

M.N. Gaspar[1,2], C. A. Fontes Ribeiro[1], J.G. Cunha-Vaz[2] & T.R.A. Macedo[1], [1]Department of Pharmacology, [2]Department of Ophthalmology, Institute for Research in Light and Image (IBILI), Faculty of Medicine, University of Coimbra, 3000 Coimbra, Portugal.

As far as neurotransmitters and neuromodulators are concerned, there has been a significant advance in ophthalmological pharmacology research (Osborne, 1993). Nevertheless, the characterization of retinal vessel receptors, as well as their specific function, are not completely clarified. This work aimed to study *in vivo* the retinal 5-hydroxytryptaminergic system.

In the present experiments the authors characterized 5-hydroxytryptaminergic receptors on retinal vessels of albino New Zealand rabbits of either sex. Rabbits were anaesthetised with 20 mg/kg ketamine by the intramuscular route. The vascular diameter of retinal arteries and veins was measured at the disc margin on fundus photographs (red free) by two different observers. The vessels were directly visualized by a Topcon's IMAGEnet-H 1024 system (digital angiography) and by video recording. Drugs were injected cumulatively through the pars plana into the posterior vitreous body and also intravenously.

After the intravitreal injection of 0.2 pmol 5-HT, an initial slight vasodilator effect was observed, followed by a dose-dependent arteriolar and venous constriction; the maximal contractile effect was obtained with 1 nmol 5-HT. The maximal vascular diameter reduction (Emax ± s.e.m.) was 29.4 ± 3.5 and 4.0 ± 0.9% at the arteriolar and venous levels, respectively. However, 5-HT administered by the intravenous route induced only a slight arteriolar dilatation.

It has also been observed that sumatriptan, a 5-HT$_{1D}$ receptor agonist, when given by intravitreal injection (0.15 pmol - 1 nmol) has a vasoconstrictor effect at the venous (Emax ± s.e.m. = 8.2 ± 2.4%) and, principally, arteriolar (Emax ± s.e.m. = 44.8 ± 1.0%) level. The maximum effect was obtained with 1 nmol sumatriptan. No significant effect was verified when the same doses of sumatriptan were administered intravenously. The contractile effect induced by 5-HT or sumatriptan was reversed by the intravenous or intravitreal injection of methiothepin (150 pmol), a 5-hydroxytryptaminergic antagonist, and sodium nitroprusside (150 pmol).

The results obtained suggest that there are 5-HT$_{1D}$ receptors on the arteriolar and venous retinal circulation, and that sumatriptan does not cross the blood-retinal barrier.

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362P GR127935 ACTS AS A PARTIAL AGONIST AT RECOMBINANT HUMAN 5-HT$_{1D\alpha}$ AND 5HT$_{1D\beta}$ RECEPTORS

J. Watson, M. Burton, G.W. Price, B.J. Jones, D. Thomas, A. Faruq & D.N. Middlemiss. SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW

Human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors are believed to be coupled via G-proteins to adenylyl cyclase. We have investigated the effects of the potent and selective 5-HT$_{1D}$ receptor antagonist GR127935 (Skingle et al., 1993) on human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors expressed in CHO cells using a GTPγS binding assay to directly measure receptor/G protein coupling (Thomas et al., 1994) and a cAMP accumulation assay as a measure of adenylyl cyclase activity (Watson et al., 1994). 5-HT stimulated basal [^{35}S]GTPγS binding in both the 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ cell lines. This response was shifted to the right by GR127935 and the non-selective 5-HT$_1$ receptor antagonist methiothepin. GR127935 alone partially stimulated basal binding whereas methiothepin alone inhibited basal binding (see table 1). This suggests that methiothepin has negative intrinsic activity at these receptors. 5-HT stimulated basal cAMP levels in 5-HT$_{1D\alpha}$ cells and inhibited forskolin-stimulated cAMP levels in the 5-HT$_{1D\beta}$ cells. Both responses to 5-HT were shifted to the right by GR127935 and methiothepin. In both cell lines, GR127935 produced a partial agonist response, whereas methiothepin increased forskolin stimulated cAMP levels. However, the latter effect may be non-specific as it was also observed in non-transfected cells.

In conclusion, GR127935 is a partial agonist at human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors when expressed in CHO cells.

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Table 1

		[35 S] GTPγS Binding Assay			cAMP Accumulation Assay		
Receptor	Compound	pA2	pEC50	Emax (% basal)	pA2	pEC50	Emax (% control)
5-HT$_{1D\alpha}$	5-HT		8.2 ± 0.2	151 ± 6		7.5 ± 0.1	167 ± 3
	GR127935	8.5 ± 0.2	8.6 ± 0.3	129 ± 5	8.6 ± 0.03	8.6 ± 0.1	136 ± 10
	Methiothepin	8.9 ± 0.3	8.1 ± 0.2	30 ± 7	9.1 ± 0.1	8.9 ± 0.3	148 ± 6
5-HT$_{1D\beta}$	5-HT		7.6 ± 0.03	196 ± 5		7.6 ± 0.1	32 ± 2
	GR127935	9.1 ± 0.2	9.7 ± 0.1	131 ± 10	9.7 ± 0.3	8.9 ± 0.3	83 ± 1
	Methiothepin	8.7 ± 0.2	8.2 ± 0.1	56 ± 2	8.9 ± 0.1	8.3 ± 0.5	124 ± 8

363P [^{3}H]SUMATRIPTAN IS A NOVEL RADIOLIGAND FOR LABELLING 5-HT$_{1D}$ SITES: BINDING IN PIG CAUDATE MEMBRANES

S.C. Cheetham, N.A. Slater, J.A. Viggers, E.C. O'Brien & D.J. Heal, Boots Pharmaceuticals Research Department, Nottingham, NG2 3AA.

At least five 5-HT$_1$ receptor subtypes have been identified using molecular biological and radioligand binding techniques namely 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$ (Martin & Humphrey, 1994). 5-HT$_{1D}$ receptors function as nerve terminal autoreceptors and postsynaptic heteroreceptors modulating transmitter release (Harel-Dupas *et al.*, 1991). Recently sumatriptan, which exhibits high affinity and selectivity for 5-HT$_{1D/1B}$ sites (Peroutka & McCarthy, 1989) has become available as a tritiated radioligand, but there is no methodology in the literature. In this study, we have examined the binding characteristics of [^{3}H]sumatriptan in pig caudate membranes.

Porcine brain was supplied by Advanced Protein Products Limited (Brierley Hill). Membranes (10mg wet weight tissue/tube) were incubated with [^{3}H]sumatriptan at 8 concentrations (0.5-16nM; saturation binding studies) or a single concentration (4nM; drug displacement studies) for 45 min at 25°C. Non-specific binding was defined by 5-HT (10μM).

In preliminary experiments, non-specific and total binding increased linearly with tissue concentration (2.5-15mg wet weight tissue/tube). Subsequent studies were performed at 10mg wet weight tissue/tube as specific binding was maximal (41-76%) and <5% of the radioactivity added was bound to the tissue. Full association was reached within 30 min at 25°C and maintained for at least 150 min. Therefore, an incubation time of 45 min was chosen. Both saturation and competition studies gave data which fitted a single site binding model. [^{3}H]Sumatriptan binding was of high affinity with K_d and B_{max} values of 4.2±0.3nM and 92±2 fmoles/mg protein (n=3), respectively. The rank order of affinity for a range of drugs (Table 1) was in good agreement with those previously reported for 5-HT$_{1D}$ sites in a variety of brain membrane preparations using [^{125}I]GTI or [^{3}H]5-HT as radioligands (Heuring & Peroutka, 1987; Bruinvels *et al.*, 1992). Correlation coefficients were highly significant (r>0.9; P<0.001).

This is the first report of labelling of 5-HT$_{1D}$ sites using the novel radioligand [^{3}H]sumatriptan. This ligand should provide a useful tool for the study of 5-HT$_{1D}$ receptors in the future.

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Table 1. Ki values for inhibition of [^{3}H]sumatriptan binding to pig caudate membranes

	Ki		Ki		Ki		Ki
5-CT	1.2 ± 0.2	Metergoline	14 ± 2	Methiothepin	30 ± 4	Ritanserin	531 ± 28
5-HT	4.0 ± 0.5	RU 24969	20 ± 3	8-OH-DPAT	394 ± 71	SCH 23390	908 ± 271

Ki values (nM) are mean ± s.e.mean (n=3-4). Hill slopes ranged from 0.9-1.2.

364P [^{3}H]SUMATRIPTAN LABELS PUTATIVE 5-HT$_{1F}$ RECEPTOR BINDING SITES IN THE GUINEA-PIG BRAIN

V.L.H. Rhodes*, Y.C. Reilly and A.T. Bruinvels (introduced by M.C.W. Minchin), Department of Molecular Pharmacology, Wyeth Research (UK), Taplow, Maidenhead, Berks. SL6 0PH, UK

Molecular cloning of the 5-HT$_{1F}$ receptor has revealed that the anti-migraine drug sumatriptan has high affinity for this receptor which is insenstive to the 5-HT$_1$ agonist 5-carboxamidotryptamine (5-CT) (Adham *et al.*, 1993). Receptor autoradiography was used to visualize putative 5-HT$_{1F}$ receptor binding sites which show high affinity for [^{3}H]sumatriptan but low affinity for 5-CT in the guinea-pig brain. The experiments were performed as described previously (Bruinvels *et al.* 1994a), but using [^{3}H]sumatriptan (10 nM, 77 Ci/mmol, Amersham, Buckinghamshire, UK) as the radioligand. Non-specific binding was determined in the presence of 10 μM 5-HT. The labelled sections were exposed for 8 weeks on [^{3}H]Hyperfilm.

[^{3}H]Sumatriptan in the presence of 100 nM 5-CT was found to label high densities of receptor binding sites in claustrum, ventrolateral thalamus and deep layers of the frontal, cingular and parietal cortex as well as caudate-putamen. This regional distribution corresponds to earlier studies describing the distribution of the so-called 5-CT insenstive 5-HT$_1$ binding sites, in which high densities of these binding sites were reported in claustrum, olfactory tubercle, lateral septum, amygdala, striatum, hippocampus and cortex of the guinea-pig (Beer *et al.*, 1993, Bruinvels *et al.*, 1994a). Furthermore, the regional distribution of 5-HT$_{1F}$ receptor binding sites is to a certain degree comparable with 5-HT$_{1F}$ receptor mRNA expression in guinea-pig brain (Adham *et al.*, 1993, Bruinvels *et al.*, 1994b).

In conclusion, [^{3}H] sumatriptan labels 5-HT receptor binding sites that are insensitive to 5-CT in guinea-pig brain, possibly corresponding to the recently cloned 5-HT$_{1F}$ receptor. The concentration of these sites in cortex, thalamus and claustrum suggests that 5-HT$_{1F}$ receptor sites have a distinct distribution from 5-HT$_{1D}$ and possibly also 5-HT$_{1E}$ receptors in the guinea-pig brain.

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365P EVIDENCE FOR A ROLE OF BASAL NITRIC OXIDE RELEASE IN THE MODULATION OF SUMATRIPTAN-EVOKED CONTRACTION OF THE RABBIT ISOLATED SAPHENOUS VEIN

D.M.J. Clavell & J.L. Field (introduced by K.F. Rhodes). Department of Molecular Pharmacology, Wyeth Research (UK) Limited, Huntercombe Lane South, Taplow, Maidenhead, Berkshire. SL6 0PH.

The 5-HT_{1D} agonist, sumatriptan, has diverse effects on a variety of blood vessels causing both contraction via receptors located on the smooth muscle and relaxation via receptors located on the endothelium. It had been noticed in our laboratory that endothelium removal potentiated contractile responses to sumatriptan in the rabbit saphenous vein (RSV). Therefore we attempted to determine whether sumatriptan-evoked contraction of the RSV was modulated by nitric oxide (NO) release from the endothelium, by investigating the effects of various amino acid analogues with known effects on nitric oxide synthase activity.

Male New Zealand White rabbits (2-3kg) were killed with pentobarbitone (Sagatal, 60mg/kg; i.v.) and the RSV dissected out. Preparations were either studied intact or mechanically denuded of endothelium by rubbing. Isolated rings (2-3mm in length) of RSV were suspended on parallel tungsten wires under a resting load of 1.0g in Krebs solution at 37°C, gassed with 5% CO_2 in O_2. The presence of an endothelium was determined by contraction with KCl 40mM followed by addition of acetylcholine 1μM. Tissues relaxing > 50% from KCl maximum were considered to be endothelium intact and < 10% endothelium denuded. Tissues were then treated with pargyline (50μM) and phenoxybenzamine (300nM) for a 30min period. Sumatriptan-induced contraction of the RSV was measured isometrically (Martin and MacLennan, 1990), with two concentration-effect curves being constructed in each tissue. Table 1 shows the effects of N^G-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO), incubated for 20 min between first and second concentration-response curves, on the maximum contraction developed to sumatriptan. Also the effects of L-arginine and D-arginine, incubated 15 min before addition of L-NAME are shown. Results are expressed as a percentage of first curve maximum. In a separate experiment, both endothelial intact and denuded tissues were precontracted with the thromboxane A_2-mimetic, U-46619 (30nM) and left for 20 min for tension to stabilise, after which sumatriptan (30nM-100μM) was added.

In intact tissues pre-contracted with a supra-maximal concentration of U-46619 (30nM, n=4), relaxation to sumatriptan (30nM-100μM) could not be shown. In endothelial intact tissues, pretreatment with the nitric oxide synthase inhibitors (NOSi), L-NAME (100μM) and L-NIO (100μM), potentiated contractions to sumatriptan (p=0.001 and 0.03 respectively; Mann-Whitney) but had no effect on pEC_{50} values. Pre-incubation with L-arginine, but not D-arginine, attenuated the effects of L-NAME. Potentiation with the NOSi's was not seen in endothelium denuded preparations. Small contractions (<5% of max) were seen in some tissues with L-NAME (100μM) but equivalent precontraction with 1nM U-46619 did not potentiate responses to sumatriptan. These results suggest a role for NO in sumatriptan's response in the RSV. As relaxation to sumatriptan could not be detected in the precontracted tissues, it appears that endothelium dependent augmented response with a NOSi is due to inhibition of basal NO release from the endothelium, described in other isolated vessels (Trezise et al, 1992).

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Table 1. Analysis of sumatriptan concentration response curve.

	Endothelium Intact			Endothelium denuded		
Treatment (2nd Curve)	pEC_{50} [95% limits]	Max ± s.e.mean %	n	pEC_{50} [95% limits]	Max ± s.e.mean %	n
Time controls (for L-NAME)	6.3 [6.1-6.6]	116.4±12.6	7	6.3 [6.1-6.4]	103.7±4.2	5
+ L-NAME (100μM)	6.5 [6.3-6.7]	250.7±50.2	7	6.4 [6.0-6.8]	101.6±3.3	5
+L-arg (1mM)/ L-NAME (100μM)	6.4 [5.8-7.0]	142.3±15.7	5	6.6 [6.2-7.1]	92.8±8.3	5
+D-arg (1mM)/ L-NAME (100μM)	6.4 [5.8-7.0]	297.3±53.0	7	6.6 [6.2-7.0]	103.2±5.5	5
Time controls (for L-NIO)	5.9 [5.4-6.5]	94.5±24.6	4	6.5 [6.1-6.9]	102.6±1.6	4
+ L-NIO (100μM)	6.3 [5.7-6.8]	207.4±27.5	4	6.3 [5.8-6.8]	114.2±6.5	3

366P INVESTIGATION OF MIANSERIN-INDUCED DOWN-REGULATION OF HUMAN 5-HT_{2A} RECEPTORS EXPRESSED IN A HUMAN NEUROBLASTOMA CELL LINE

R.A. Newton, S.L. Phipps & J.M. Elliott. Oxford University-SmithKline Beecham Centre for Applied Neuropsychobiology, University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE, U.K.

In vivo studies typically show a paradoxical down-regulation of 5-HT_{2A} receptors following chronic exposure to the antagonist mianserin (Blackshear & Sanders-Bush, 1982). We wanted to determine whether this atypical regulation could also occur in an isolated cell culture system. Using the human neuroblastoma cell line SH-SY5Y stably transfected with the human 5-HT_{2A} receptor cDNA we have investigated the effects of mianserin treatment on 5-HT_{2A} receptor density (B_{max}) and 5-HT_{2A} mediated phosphoinositide (PI) hydrolysis.

The cell line displayed a characteristic 5-HT_{2A} receptor binding profile and was functionally coupled to PI hydrolysis (Newton *et al*, 1994). Cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 5% dialysed foetal calf serum (D-FCS) and were treated by replacing the medium 24 h prior to assay with DMEM supplemented with 1% D-FCS and adding mianserin (final concentration 100nM) or vehicle. For 1 h treatments, mianserin was added 23 h later. Membrane preparation for [^{3}H]ketanserin binding involved washing the cells, homogenisation and then washing the membranes three times, with the final wash including a 30 min incubation at 37°C. For PI hydrolysis studies, cells were incubated for 48 h with inositol-free DMEM supplemented with 1% D-FCS and 1μCi/ml [^{3}H]inositol. Treatment was initiated by adding mianserin or vehicle 1 h or 24 h prior to assay. Cells were then washed, LiCl added (10mM, 30min) and 5-HT applied for 15 min. Tritiated inositol phosphates ([^{3}H]IP_x) were separated on Dowex AG1-X8 columns. Data expressed as mean±s.e.mean and statistical analysis performed using the paired Student's t-test.

Exposure of cells expressing the human 5-HT_{2A} receptor to 100nM mianserin for 24 h resulted in a significant decrease in B_{max} from 401±17 to 207±16 fmol/mg protein ($p<0.01$, n=3). There was also a significant increase in the [^{3}H]ketanserin dissociation constant (K_D) from 0.49nM, pK_D=9.31±0.03M to 1.46nM, pK_D=8.86± 0.11M ($p<0.05$, n=3). In order to examine whether the K_D increase was responsible for the decrease in B_{max}, the effect of of 1 h mianserin was examined. Despite a significant increase in K_D from 0.45nM, pK_D=9.35±0.02M, to 2.85nM, pK_D=8.55±0.03M, ($p<0.005$, n=3) there was no change in B_{max} between control and treated cells (352±36 & 379±51 fmol/mg protein, respectively). Therefore the down-regulation seen following 24 h mianserin appears to be a time-dependent phenomenon unrelated to the K_D change.

5-HT stimulated [^{3}H]IP_x production following 100nM mianserin exposure was investigated to assess whether the receptor down-regulation had a functional consequence. A time dependent decrease in maximal [^{3}H]IP_x production was seen in mianserin treated cells which reached significance after 24 h exposure (75.6±5.1% of control, $p<0.05$, n=3). No change in maximal [^{3}H]IP_x production elicited by carbachol (which occurs via endogenous muscarinic receptors) was seen following mianserin treatment.

The use of transfected cells in which 5-HT_{2A} receptors are constitutively expressed suggests that the atypical regulation is unlikely to be accounted for by transynaptic effects or alterations in gene transcription. A direct drug action on the receptor thus seems to be responsible for the 5-HT_{2A} receptor down-regulation and decreased maximal PI hydrolysis.

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367P ADMINISTRATION OF EEDQ ATTENUATES 5-HT$_{2A}$ RECEPTOR-, BUT NOT G-PROTEIN-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN RAT FRONTAL CORTEX

C.J. Kettle, K.F. Martin, S.C. Cheetham & D.J. Heal, Boots Pharmaceuticals Research Department, Nottingham NG2 3AA.

5-HT$_{2A}$ receptors in rat cerebral cortex are linked via G-proteins to the phosphoinositide (PI) signal transduction system (Conn and Sanders-Bush, 1986). Recently, we have shown that administration of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a peptide coupling agent, decreased rat cortical 5-HT$_{2A}$ receptor binding ex vivo (Kettle *et al.*, 1994). Here, we have investigated whether EEDQ treatment modifies 5-HT$_{2A}$ receptor-mediated PI hydrolysis and the protective effects of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI).

Male CD rats (120-140 g; Charles River) received DOI (3 mg/kg) or saline ip. Each group was then divided and given either EEDQ (6 mg/kg) or vehicle ip 30 min later. Rats were killed after 24h and frontal cortices removed. PI hydrolysis was determined as described by Brown *et al.* (1984). Dose-response curves to 5-HT, DOI or NaF were obtained and EC_{20} (concentration to increase PI hydrolysis by 20%) values were calculated. Data were analysed using Dunnett's or multiple t-tests.

5-HT (10^{-8} - 10^{-4} M), DOI (10^{-8} - 10^{-4} M) and NaF ($3x10^{-5}$ - 10^{-1} M) all concentration-dependently increased PI hydrolysis (maximum responses 68 ± 2, 59 ± 2 and 116 ± 13 percent stimulation above basal, respectively). As shown in Table 1, EEDQ administration attenuated the responses to 5-HT and DOI, but not NaF. Although pretreatment with DOI reduced the agonist-stimulated PI hydrolysis, it, nevertheless, also protected against the effects of EEDQ.

Overall, the results show that EEDQ inactivation of 5-HT$_{2A}$ receptors (Kettle *et al.*, 1994) is accompanied by a corresponding reduction in agonist-induced PI hydrolysis. The failure to modify the NaF response argues that this is mediated exclusively by receptor deactivation and, furthermore, that the G-proteins involved are not susceptible to the actions of this peptide-coupling agent.

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Table 1. Effects of EEDQ on EC_{20} values for stimulation of PI hydrolysis by 5-HT, DOI or NaF

Pretreatment	Treatment	5-HT (μM)	DOI (μM)	NaF (mM)
Saline	Vehicle	1.4	7.3	4.5
	EEDQ	16.2**	478***	2.4
DOI	Vehicle	8.0**	17.4	ND
	EEDQ	9.0**†	5.6†	ND

Data are mean values (n = 3 - 5), ** P<0.01, ***P<0.001 versus saline/vehicle, † P<0.05 versus saline/EEDQ. ND = not determined.

368P CLONING, DISTRIBUTION AND CHARACTERIZATION OF A HUMAN 5-HT$_{2B}$ RECEPTOR

D.W. Bonhaus, C. Bach[1], A. DeSouza, H.W. Chan[1], & R.M. Eglen. Institutes of Pharmacology and Bio-Organic Chemistry[1], Syntex Discovery Research, Palo Alto, CA 94304, U.S.A.

The 5-HT$_{2B}$ receptor is one of three 5-HT$_2$ receptor subtypes. In this study the cloning, distribution and pharmacology of the human 5-HT$_{2B}$ receptor is described.

The human 5-HT$_{2B}$ receptor was cloned by PCR using primers based on the third exon of the mouse 5-HT$_{2B}$ receptor (Foguet et al., 1992). A 200 base-pair probe was first isolated from genomic DNA. This probe was then extended and used to isolate full length clones from kidney, liver and pancreas. These clones were identical to those reported by Kursar et al. (1994) or Schmuck et al. (1994).

The distribution of 5-HT$_{2B}$ receptor mRNA was examined by Northern analysis and PCR. Using a 650 base probe, a 2.4 kB band was detected in mRNA isolated from human liver, kidney and pancreas while a 3.2 kB band was detected in liver and kidney mRNA. Using one-step of PCR amplification, 5-HT$_{2B}$ receptor cDNA was found in cDNA from cerebral cortex, liver spleen, pancreas and kidney, but not heart.

The affinities of ligands for the human 5-HT$_{2B}$ receptors were determined using transfected Cos-7 cells (Felgner et al., 1987). For comparison, the affinities of ligands for human 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors (Saltzman et al.,1991) in Cos-7 cells were also determined. The 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors were labelled with [^{3}H]ketanserin, [^{3}H]5-HT and [^{3}H]mesulergine respectively. The affinity of ligands at the human 5-HT$_{2B}$ receptor was similar to that at the rat 5-HT$_{2B}$ receptor (Wainscott et al.,1993). The 5-HT$_{2B}$ receptor was distinguished from 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors on the basis of a lower affinity for ritanserin and a higher affinity for 5-HT and yohimbine.

SB200646A (Forbes et al., 1993) did not discriminate between 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors (Table 1)

These findings therefore, confirm the identity of the human 5-HT$_{2B}$ receptor, suggest that it is expressed in a variety of tissues and further characterize its affinity for 5-HT$_2$ receptor ligands.

Table 1 Affinities of ligands at cloned 5-HT$_2$ receptors

	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$
ritanserin	10.8	8.3	9.6
spiperone	9.0	<6	<6
ketanserin	8.5	6.2	6.7
methysergide	8.4	8.1	8.8
mianserin	8.4	7.7	8.3
mCPP	7.3	7.5	6.6
5-HT	7.2	9.1	6.8
yohimbine	<5.5	6.4	<5
SB200646A	<5	6.2	6.4

Values are the mean pKi of at least three determinations. s.e.mean were less than 10 % of the mean.

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369P EVIDENCE FOR EXPRESSION OF THE 5-HT_{2B} RECEPTOR mRNA IN RAT BRAIN

T.P. Flanigan, A.C. Reavley, J.E. Carey[1] and R.A. Leslie. Oxford University SmithKline Beecham Centre, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE, U.K. and SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow,[1] CM19 5AW, UK.

Recent cloning of the 5-HT_{2B} receptor (Foguet et al., 1992), which mediates serotonin-stimulated contraction of the rat stomach fundus, has resulted in isolation and functional characterisation of its mouse (Loric et al., 1993) and human (Schmuck et al., 1994) homologues. This should facilitate studies on distribution and localisation of this receptor in CNS. Indeed, the presence of 5-HT_{2B} receptor mRNA in mammalian brain is uncertain since it has been suggested that the transcript is present in mouse but not rat brain (Pompeiano, et al., 1994). We have addressed this issue using reverse transcriptase-linked polymerase chain reaction (RT-PCR) analysis of RNA isolated from various regions of rat brain.

Adult male Sprague-Dawley rats were anaesthetised with pentobarbital, perfused transcardially with saline, brains removed and RNA prepared from various regions. RT reactions were then performed using a reverse primer complementary to bases 1631-1653 (inclusive) of the rat 5-HT_{2B} receptor sequence (Foguet et al., 1992). Products were used as templates in a PCR containing a reverse primer complimentary to bases 1568-1589 and forward primer homologous to bases 703-728 of the rat 5-HT_{2B} receptor sequence. PCR products were then analysed by agarose gel electrophoresis at various cycle numbers. Southern blots of these products were probed with a radiolabelled oligonucleotide designed to hybridise with an internal portion of the predicted PCR product (homologous to bases 1255-1279 of the rat 5-HT_{2B} receptor sequence). Positive bands were obtained following 32-38 cycles of PCR in hippocampus cerebellum, cortex, medulla, and mid brain (but not in striatum or hypothalamus), consistent in size with that predicted by the rat 5-HT_{2B} receptor sequence. Their identity was confirmed by Southern blot analysis using the 'internal' oligonucleotide as probe. Control RT reactions in which reverse transcriptase was omitted failed to yield similar results which indicates an RNA source for these signals. Also, the primers used span the intron between exons 2 and 3 of the 5-HT_{2B} receptor gene which negates a genomic DNA source for these signals.

Our data indicate that the 5-HT_{2B} receptor transcript is present in rat brain and supports a role for the protein in 5-HT function in mammalian CNS.

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370P EVIDENCE OF TOLERANCE DEVELOPMENT TO THE HYPERPHAGIC BUT NOT ANXIOLYTIC-LIKE ACTIONS OF SB 200646A, A 5-HT_{2C}/5-HT_{2B} RECEPTOR ANTAGONIST, AFTER CHRONIC TREATMENT

G.A. Kennett, S. Grewal, T.P. Blackburn, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow, Essex CM19 5AW, U.K.

Acutely, SB 200646A, a selective 5-$HT_{2C/2B}$ receptor antagonist, and non-selective 5-$HT_{2C/2B/2A}$ receptor antagonists show anxiolytic-like properties in the rat social interaction test (Kennett et al., 1994). Also, non-selective antagonists of 5-$HT_{2C/2B/2A}$ receptors increase the food intake of freely feeding rats (Kennett, 1993). In the present study, the effects of acute and chronic SB 200646A treatment on rat feeding behaviour and on activity in the social interaction (SI) test were studied.

Male CD rats (200-250 g) were held under a 12 h light cycle (lights on 07.00 h) with free access to food and water. In feeding studies, rats were singly housed on day 1. At 13.00 h on day 2 they were food deprived and given SB 200646A, chlordiazepoxide (CDP) or vehicle (VEH) p.o. 1 h later, food was restored and the amount eaten after 1 and 2 h measured. Other rats were given VEH or SB 200646A (5 or 40 mg/kg) p.o. b.i.d. for 13 days and singly housed on day 13. On day 14, at 13.00 h, they were food deprived and given SB 200646A 5, 40 mg/kg or VEH either as before or for the first time. Food was restored and the amount then eaten measured. Food intakes and body weights were monitored over the 14 day period. In studies of SI, rats were given SB 200646A 5 or 40 mg/kg p.o. b.i.d. x 14 days. and singly housed on day 10. On day 15, SB 200646A pretreated rats were given the same dose of SB 200646A while VEH pretreated rats received either VEH or SB 200646A 5 or 40 mg/kg. 1 h later the SI of weight and treatment matched pairs of rats was scored "blind" under "high light unfamiliar conditions" for 15 min (see Kennett et al., 1994). SB 200646A was given as a suspension (2ml/kg) in saline at pH 4.5. Chronic treatments were given at 8.00 and 20.00 h. All data cited as means ± s.e.m., and analysed by 1 way ANOVA and Dunnett's test.

Acute SB 200646A and CDP modestly increased feeding over 2 and 4 h (VEH 2h, 0.3 ± 0.1 g, SB 200646A 10 mg/kg, 0.9 ± 0.2 g, $p<0.05$, 20 mg/kg 1.2 ± 0.2 g, $p<0.01$, CDP 2 mg/kg, 1.0 ± 0.3 g $p<0.05$, n=10-12). This was not seen in chronic VEH or SB 200646A treated rats. Indeed, a small decrease in weight gain occurred over 14 days. In the SI test, acute SB 200646A increased SI in chronic VEH pretreated rats (chronic VEH + VEH, 73.1 ± 7.5 s, VEH + SB 200646A 5 mg/kg 132.8 ± 11.9 $P<0.01$, 40 mg/kg 133.7 ± 10.4 $p<0.01$). This effect was maintained in chronic SB 200646A treated rats (chronic SB 200646A 5 mg/kg + SB 200646A 5 mg/kg 123.8 ± 11.3 $p<0.05$, chronic SB 200646A 40 mg/kg + SB 200646A 40 mg/kg 128.3 ± 10.7 $p<0.05$, n=10-12). Thus, in these studies, tolerance was observed to the hyperphagic, but not the anxiolytic-like properties of SB 200646A.

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371P DETECTION OF 5-HT$_{2C}$ RECEPTORS IN THE RAT BRAIN USING ANTIBODIES: EFFECT OF 6-HYDROXYDOPAMINE

K.C.F. Fone, A. Sharma & M.S. Duxon, Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham University, Nottingham, NG7 2UH

The 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptor has both a widespread distribution in the CNS and been implicated in anxiety, feeding, locomotor co-ordination and neuroendocrine function. However, the lack of specific, high affinity, 5-HT$_{2C}$ antagonists currently prevents definitive discrimination between the functional role of the three 5-HT$_2$ receptors subtypes so far cloned. The present study further characterises a 5-HT$_{2C}$ receptor specific antibody which has been developed to establish the distribution and function of this protein in the rat CNS.

A polyclonal sheep antiserum was raised against the N-terminal 10 amino acids (MVNLGNAVRS + YC) of the rat 5-HT$_{2C}$ receptor and sephadex purified to yield an immunoglobulin (IgG) fraction. For immunocytochemistry, HEK 293 cells (transfected with human 5-HT$_{2C}$ DNA and untransfected controls) were incubated with 5-HT$_{2C}$ or non-immune IgGs (1:10, 12 h) in the presence and absence of 5-HT$_{2C}$ peptide (10 μM) and a fluorescein-conjugated secondary antiserum (1:20, 60 min) used to visualise the receptor protein by fluorescence microscopy. Western blot analysis of solubilised membrane preparations from selected rat brain regions was performed using SDS-PAGE and immunoreactive proteins were visualised by sequential incubation with 5-HT$_{2C}$ or non-immune IgGs (1:10, 12 h), peroxidase-conjugated secondary antibodies (1:2000, 1 h) and 3,3' diaminobenzidine. In separate experiments, adult male Wistar rats (280-360g, n = 6 each) were pretreated (11 d) with 6-hydroxydopamine (6-OHDA, 2 x 150 μg i.c.v. at a 24h interval, given 30 min after paroxetine, 5 mgkg^{-1} i.p., to prevent uptake into serotonergic neurones) or vehicle (5.68 mM ascorbate in 0154 M saline, 5 μl) prior to analysis of 5-HT$_{2C}$ receptor protein-like immunoreactive (5-HT$_{2C}$-LI) levels by radioimmunoassay on membrane fractions from selected rat brain regions, as described previously (Sharma *et al.*, 1994). 5-HT and noradrenaline levels were determined in extracts from bilateral brain regions by HPLC with electrochemical detection.

The specificity of the 5-HT$_{2C}$ receptor-directed antibody was validated by immunocytochemical visualisation of membrane proteins expressed in HEK 293 cells transfected with human 5-HT$_{2C}$ DNA, which was prevented by co-incubation with synthetic rat 5-HT$_{2C}$ peptide (10 μM). In contrast, untransfected cells failed to show any immunoreactivity with the current 5-HT$_{2C}$ IgGs. In Western blots of membrane preparations from the frontal cortex and hippocampus the 5-HT$_{2C}$ IgGs identified two immunoreactive proteins whose molecular weights (52 and 55 KDa) were similar to that of the 5-HT$_{2C}$ receptor (51.9 KDa, Julius *et al.*, 1988), but a 63 KDa band was identified in the choroid plexus. A further 29 KDa band was identified in all regions which is probably a degradation product. The higher molecular weight bands in these blots may represent regional variation in glycosylation of the receptor, which is currently being investigated. Following 6-OHDA treatment, 5-HT$_{2C}$-LI was significantly decreased ($P<0.02$ Student's *t* test, 42% below control) in the thalamus (from 173.8 ± 21.2 to 101.5 ± 10.3 fmol mg protein^{-1} mean ± s.e.mean.) where there was a concomitant significant ($P<0.05$) 57% depletion of noradrenaline without any significant change in 5-HT. In contrast, no significant change in 5-HT$_{2C}$-LI was observed in the hypothalamus, hippocampus or thoracic dorsal spinal cord (being -1, +25, and -24% different from control, respectively), although 6-OHDA produced significant noradrenaline depletions in these areas (by 60% $P<0.01$, 92% $P<0.001$ and 62% $P<0.02$, respectively).

Immunocytochemical and Western blot analysis confirm that the 5-HT$_{2C}$ IgGs recognise the 5-HT$_{2C}$ receptor both in a transfected cell-line and in rat brain membranes. The current data also suggests that 5-HT$_{2C}$ receptors may be located on catecholaminergic neurones in the rat thalamus.

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372P AGONIST-INDUCED DESENSITISATION OF HUMAN 5-HT$_{2C}$ RECEPTORS EXPRESSED IN A HUMAN NEUROBLASTOMA CELL LINE

S.J. Briddon, R.A. Leslie and J.M. Elliott. Department of Clinical Pharmacology, University of Oxford, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE, U.K.

Desensitisation of several receptors linked to phosphoinositide hydrolysis (PI) has been reported (Wojcikiewicz & Nahorski, 1993). For the 5-HT$_{2C}$ receptor, desensitisation of the electrophysiological response has been shown (Boddeke et al., 1993); here we report desensitisation of the human receptor at the level of PI hydrolysis.

Desensitisation was studied using a previously characterised clone of the human neuroblastoma cell line, SH-SY5Y, which expresses the human 5-HT$_{2C}$ receptor at a level of 110 fmol/mg protein (Newton et al., 1994). PI hydrolysis was measured by incubating cells for 48 hours in inositol free Dulbecco's Modification of Eagle's Medium (DMEM) with 5% dialysed foetal serum (D-FCS) and 1μCi/ml of [^{3}H]inositol. Cells were desensitised by incubation with agonist then washed in DMEM, incubated with 10mM LiCl for 30 min, and stimulated with 1μM 5-HT for 15 min. The reaction was terminated with ice-cold methanol and [^{3}H]inositol phosphates ([^{3}H]IP$_x$)were isolated using Dowex AG1-X8 columns.

Incubation of cells for 2 hours with 5-HT caused a dose-dependent decrease in the resulting [^{3}H]IP$_x$ production induced by 1μM 5-HT, with 50% and maximal desensitisation occuring at 0.2μM and 10μM, respectively. The response of the endogenous muscarinic receptor in this cell to 100μM carbachol was not altered by 5-HT pre-treatment. There was a small rise in the basal levels of [^{3}H]IP$_x$ after desensitisation. Inclusion of 10nM mesulergine in the medium during 5-HT pre-treatment caused an apparent 10-fold increase in the concentration of 5-HT required to produce 50% desensitisation. The degree of desensitisation to 1μM 5-HT was time-dependent, with a rapid initial fall to 34±6% of control response after 20 min, followed by a slower fall to 21±2% of control response at 120 min (mean±s.e.mean; n=4). The pEC$_{50}$ for PI stimulation by 5-HT (7.45±0.08) was not altered after pre-treatment with 1μM 5-HT for 20 min (7.28±0.13) (mean±s.e.mean; n=3). Cells were also incubated with the partial agonists 1-(3-chlorophenyl)-piperazine (m-CPP) (pEC$_{50}$=6.70±0.06; efficacy relative to 1μM 5-HT=0.80±0.07) and quipazine (pEC$_{50}$=5.95±0.26; efficacy relative to 1μM 5-HT=0.61±0.07). Two hour pre-treatment with these compounds also caused a dose-dependent decrease in the resulting response to 1μM 5-HT with 50% desensitisation caused by 4μM m-CPP and 17μM quipazine. Both compounds appeared capable of producing full desensitisation at higher concentrations.

In this system, therefore, the 5-HT$_{2C}$ receptor undergoes a rapid homologous desensitisation to its natural agonist, 5-HT. The concentrations of 5-HT, m-CPP and quipazine causing 50% desensitisation of the response parallel their agonist potency.

S.J. Briddon is a Wellcome Prize Student.

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373P EVIDENCE FOR THE INVOLVEMENT OF 5-HT$_{2B/2C}$ RECEPTORS IN MEDIATING FENFLURAMINE-INDUCED ANOREXIA IN RATS

J.E. Hartley, G. Brown, A. Fletcher and C.T. Dourish, Department of Neuropharmacology, Wyeth Research (UK) Ltd., Huntercombe Lane S., Taplow, Maidenhead, Berks. SL6 0PH

Anorexia induced by fenfluramine may be mediated by 5-HT$_1$ receptors, as metergoline, the non-selective 5-HT$_1$/5-HT$_2$ antagonist, attenuates fenfluramine-induced anorexia in rats (Neill & Cooper, 1989). In an attempt to pinpoint the specific subtype(s) mediating this anorectic effect we have studied the effects of selective 5-HT receptor antagonists on fenfluramine-induced anorexia. Thus, in interaction experiments with fenfluramine, we have used the following receptor antagonists; WAY-100635 (5-HT$_{1A}$) (Fletcher et al., 1994), GR127935 (5-HT$_{1B/1D}$) (Skingle et al., 1993) and SB 200646 (5-HT$_{2B/2C}$) (Kennett et al., 1994). Further studies were undertaken to examine the effect of GR127935 on anorexia induced by the 5-HT$_{1A/1B}$ agonist RU24969 (Kennett et al., 1987).

Male Sprague-Dawley rats (300-400g; n=10) were fasted overnight (16h deprivation) in their home cages and, the following morning, given: SB 200646 (10&50mg/kg p.o.), metergoline (1mg/kg s.c.), GR127935 (0.1&1mg/kg s.c.), WAY-100635 (0.3mg/kg s.c.) or vehicle (by the relevant route). After 30min d/l-fenfluramine (3mg/kg s.c.) or saline was administered. A further 30min later, the rats were allowed access to weighed hoppers of a standard pelleted diet (CRM) and the hoppers were repeatedly weighed such that 1 and 3h food intake were calculated. In a similar experiment, the animals received GR127935 (1mg/kg s.c.) or vehicle, prior to RU24969 (3mg/kg s.c.) or vehicle.

The results of these experiments (see Table 1) show that fenfluramine-induced anorexia is attenuated by metergoline, confirming previous findings, and also by the 5-HT$_{2B/2C}$ receptor antagonist, SB 200646. In contrast, the selective 5-HT$_{1A}$ receptor antagonist, WAY-100635, and the 5-HT$_{1B/1D}$ receptor antagonist, GR127935 had no effect on fenfluramine-induced anorexia. Anorexia induced by RU24969 (RU) (1h intake, expressed as g±s.e.mean, veh+veh: 6.8±0.8g, veh+RU: 1.8±0.7g) was blocked by GR127935 (GR),(GR+veh: 7.0±1.0g, GR+RU: 7.2±1.3g).

These results implicate 5-HT$_{2B/2C}$ receptors in mediating the anorectic effect of fenfluramine. Furthermore, the data suggest that anorexia induced by RU24969 is mediated by 5-HT$_{1B}$ receptors.

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Table 1. The effect of GR127935, SB 200646, metergoline and WAY-100635 on anorexia induced by fenfluramine in the rat.

	GR127935			SB 200646			metergoline		WAY-100635	
mg/kg	0	0.1	1	0	10	50	0	1	0	0.3
vehicle	5.3±1.1	6.2±0.8	5.4±1.1	5.3±0.6	5.9±1.1	5.8±0.9	5.3±1.1	5.8±0.8	4.8±0.7	6.2±0.9
fenfluramine	1.8±0.6*	1.3±0.4*	1.3±0.5*	1.8±0.6*	2.7±0.6	3.9±0.7#	1.8±0.6*	4.0±0.5#	0.2±0.1*	0.1±0.1*

The data are reported as the mean food intake (g) ± s.e.mean., * or # =P<0.05 : * cf. vehicle group, # cf. fenfluramine group

374P EVIDENCE OF A 5-HT$_3$ RECEPTOR-MEDIATED FACILITATION OF THE EMPTYING PHASE OF THE PERISTALTIC REFLEX IN THE ISOLATED GUINEA-PIG ILEUM

B.R. Tuladhar, B. Costall & R.J. Naylor, Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP.

5-HT$_4$ receptor mediated facilitation of the peristaltic reflex in the isolated guinea pig ileum has been previously demonstrated (Costall *et al.*, 1993). In the present study, we present additional evidence of a facilitation of the peristaltic reflex resulting from a stimulation of a 5-HT$_3$ receptor.

Peristalsis was induced by the method described by Costall *et al.*, 1993 with the following modifications: the agonist drugs were added cumulatively to the serosal side at intervals of 5 min and any decrease or increase in the threshold for peristalsis was measured and calculated respectively as the percentage of the minimum or maximum threshold in the two minute period before the addition of the drug. Antagonists were added to the reservoir and equilibrated for at least 45 min before the initiation of peristalsis. A comparison of the effects of agonist and antagonists was carried out using paired preparations.

Peristalsis was facilitated by 0.01 to 0.1 μM of 5-HT. The facilitatory effects were characterised by a decrease in the threshold for peristalsis (maximum decrease at 0.1 μM 50.1±3.1%, n=10) which lasted for 1-2 min after which the threshold returned to nearly the original control values. An increase in threshold was never observed with these concentrations of 5-HT. At higher concentrations, 0.3-1 μM, the facilitatory effect of 5-HT was decreased. At a still higher concentration of 3 μM, 5-HT produced a facilitatory effect qualitatively different from the facilitatory effects observed at lower concentrations of 5-HT (0.01-0.1 μM) with the decrease in threshold lasting for only one stroke (<0.5 min). The decrease in threshold at 3 μM 5-HT was 41.3±5 % (n=10). A significant inhibitory effect on peristalsis characterised by an increase in the threshold (127±16.7%, n=10) and a decrease in the efficiency of propulsion was observed with 10 μM 5-HT.

The relatively longer lasting facilitatory effects observed at 0.01-1 μM 5-HT were not significantly altered by the presence of methysergide (1 μM, n=3) or ondansetron (2 μM, n=4) in the bathing medium. Similarly, the longer lasting facilitatory effects of 5-methoxytryptamine (0.01-1 μM) were not significantly different from that of the control 5-HT response. However, the short lasting facilitatory effect observed at 3 μM 5-HT [not observed with 5-methoxytryptamine (0.01-10 μM, n=6)] was completely abolished in the presence of ondansetron (2 μM, n=4). Methysergide (1 μM, n=3) had no effect on either the longer lasting or the short lasting facilitatory effects of 5-HT.

The relatively longer lasting facilitatory effect of 5-HT is most likely due to the stimulation of the 5-HT$_4$ receptor as described by Costall *et al.*, 1993. However, the short lasting facilitatory effect observed using 3 μM 5-HT is unlikely to be due to stimulation of the 5-HT$_4$ receptor since 5-methoxytryptamine was unable to produce such an effect. The ability of the selective 5-HT$_3$ receptor antagonist ondansetron (Butler *et al.*, 1988) to antagonise this effect indicate a 5-HT$_3$ receptor stimulation.

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375P ABILITY OF TRICHLOROETHANOL TO MODIFY THE 5-HT$_3$ RECEPTOR-MEDIATED DEPOLARIZATION OF THE ISOLATED RAT VAGUS NERVE

KD Johnston, KR Bentley, RL Stowe and NM Barnes. Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT UK.

In addition to the recognition site for 5-HT (5-hydroxytryptamine), it is becoming clear that the 5-HT$_3$ receptor possesses pharmacologically distinct sites which mediate allosteric modulation of the receptor complex. For instance, electrophysiological data demonstrates that both ethanol (Lovinger, 1991), and the active metabolite of chloral hydrate, trichloroethanol, increase the potency with which agonists activate the native 5-HT$_3$ receptor complex (Downie et al., 1993; Lovinger and Zhou, 1993). In the present study we assess the ability of trichloroethanol (TCE) and the structurally related compound, tribromoethanol (TBE) to modulate the function of the 5-HT$_3$ receptor expressed in rat vagus nerve.

Rats were killed by cervical dislocation. From each animal, 2 cervical trunks of vagus nerve (1-1.5 cm), without the nodose ganglia were dissected along the attached carotid artery and placed into ice-cold, gassed (95/5; O_2/CO_2), Krebs buffer (Krebs mM: NaCl 118.0, $NaHCO_3$ 25.0, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4$ 1.2, D-glucose 11.0). After separation from the artery, the nerve was desheathed and transferred to the perfusion platform. The arrangement for extracellular recording used was a modification of the grease-gap method used by Blake et al., (1988). Concentration-response curves to agonists were obtained by including a known concentration of agonist in the perfusing Krebs buffer in the presence or absence of either TCE (5 mM) or TBE (1 mM).

5-HT (10 nM - 30 μM) induced depolarizations of the vagus nerve (maximal magnitude = 0.33 ± 0.77 mV, EC_{50} = 1.02 ± 0.16 μM, (mean ± SEM, n = 4)) that were antagonized by the selective 5HT$_3$ receptor antagonist ondansetron (20 nM; pK_B = 8.9, mean, n=2).

TCE (5 mM) significantly increased the potency and maximal response of 5-HT to depolarize the vagus nerve (EC_{50} = 0.41 ± 0.09 μM, maximal response relative to 5-HT in the absence of TCE = 135 ± 6%, mean ± SEM, n = 4, $p < 0.05$, Student's paired t-test). TCE (2-5 mM) maximally increased the response to a submaximal concentration of 5-HT (1 μM) with lower concentrations having a smaller effect and higher concentrations decreasing the maximal response.

The selective 5-HT$_3$ receptor agonist, phenylbiguanide (PBG) induced depolarizations with near the same potency as 5-HT (EC_{50} = 1.49 ± 0.33 μM, mean ± SEM, n = 3). The PBG-induced depolarization of the rat isolated vagus nerve was also potentiated by TCE (5 mM); EC_{50} = 0.28 ± 0.07 μM, maximal response relative to PBG in the absence of TCE = 124 ± 20% mean ± SEM, n = 3, $p < 0.05$, Students paired t-test.

In a preliminary study, the maximal 5-HT-induced response was also potentiated by TBE (1 mM), the maximal response in the presence of TBE being 121% of that in the absence of TBE. EC_{50} for 5-HT in absence/presence TBE; 0.58 /0.54 μM, (n = 1).

The present study has identified that the active metabolite of chloral hydrate, trichloroethanol, enhances the potency and the maximal response of 5-HT$_3$ receptor agonists to depolarize the isolated rat vagus nerve.

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376P [^{125}I]S(-)ZACOPRIDE LABELS A 5-HT-SENSITIVE NON-5-HT$_3$ RECOGNITION SITE IN RAT ILEUM

J.Ge[1], P.Towers[2], A.C.Patel[3] & N.M.Barnes[1], [1]Department of Pharmacology, The Medical School, University of Birmingham, Birmingham, B15 2TT, UK., [2]Amersham International plc, Forest Farm, Whitchurch, Cardiff, CF4 7YT, UK. and [3]Amersham International plc, White Lion Road, Amersham, Buckinghamshire, HP7 9LL, UK.

It has been demonstrated that both [^{3}H]S(-)zacopride and [^{125}I]S(-)zacopride label the 5-HT$_3$ receptor (Barnes et al., 1990; Laporte et al., 1992; Gehlert et al., 1993). The present study describes an additional 5-HT-sensitive site labelled by the benzamide derivative [^{125}I]S(-)zacopride in rat gut tissue.

Sections (20 μm) of ileum from male Wistar rats (150 - 250 g), killed by cervical dislocation, were preincubated in Tris/Krebs buffer (mM; Tris, 50.0; NaCl. 118.0; KCl, 4.75; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0, glucose 11.0; granisetron 0.01, pargyline 0.01, pH 7.4)) for 60 min at 37°C before incubation in Tris/Krebs buffer plus [^{125}I]]S(-)zacopride (0.1 nM; 1900 Ci/mmol) for 90 min at 25°C in the absence or presence of competing compounds. Tissue sections were subsequently washed and the generated autoradiograms were quantitated using image analysis .

In the presence of granisetron (10 μM), [^{125}I]S(-)zacopride (0.1 nM) labelled a heterogeneous distribution of specific binding sites in rat ileum (defined by 5-HT, 1.0 μM). Highest densities were detected in the mucosa (1.2 ± 0.2 fmol/mg tissue, mean ± S.E.M., n = 3) and represented approximately 80-90 % of the total binding. The inclusion of guanosine triphosphate (GTP; 100 μM) reduced the total binding by 58±5% (mean±SEM, n = 3).

5-HT, 2-methyl-5-HT, N-hexanoyl-5-hydroxytryptophyl-5-hydroxytryptophan amide and N-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide competed for [^{125}I]S(-)zacopride-labelled sites with sub-micromolar or micromolar affinity (pIC_{50}; 6.85±0.11, 6.12±0.05, 6.04±0.07 and 5.58±0.16, respectively, mean±SEM, n = 3). Other selected compounds competed with more modest affinities (pIC_{50}; S(-)zacopride 5.15±0.06, renzapride 4.80±0.07, R(+)zacopride 4.51±0.02 and tropisetron 4.43±0.08, mean±SEM, n = 3).

A variety of other compounds failed to reduce the total binding by more than 9% (1.0 μM; atropine, haloperidol, methysergide, nicotine, ondansetron, paroxetine, phentolamine, pindolol, SCH23390: 10 μM; 5-carboxamidotryptamine, mepyramine, 5-methoxytryptamine, metoclopramide, reserpine: 100 μM; BIMU0001, DAU6215, ranitidine).

Preliminary saturation studies with [^{125}I]S(-)zacopride (0.033-0.47 nM, non-specific binding defined by the inclusion of 5-HT, 10 μM) indicated saturable specific binding in both sections of rat ileum and duodenum (B_{max} =1544 and 2166 fmol g^{-1} grey matter wet weight equivalent; pK_d=10.18 and 10.02; Hill coefficient=1.36 and 1.29; ileum and duodenum, respectively, n=1).

The pharmacology of the [^{125}I]S(-)zacopride labelled non-5-HT$_3$ site most closely resembles the 5-HT$_{1P}$ site, although previous studies (Wade et al., 1991) indicate that benzamides and indoleamines recognise distinct sites on this receptor.

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377P DISTRIBUTION OF THE [3H]-(S)-ZACOPRIDE-LABELLED 5-HT$_3$ RECEPTOR IN PIG FOREBRAIN

PJ McWilliams, S Fletcher, JM Barnes, NM Barnes. Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT UK.

Biochemical investigation of the 5-HT$_3$ receptor has been largely limited to the receptor expressed in neuronal cell lines (for review see Boess and Martin, 1994) although a recent preliminary report demonstrated the purification of the 5-HT$_3$ receptor expressed in bovine brain tissue (Creighton et al., 1994). To facilitate biochemical investigation of the central 5-HT$_3$ receptor, we investigated the distribution of 5-HT$_3$ receptors in the pig forebrain which is conveniently available in large quantities.

Pig brain tissue was obtained from a local slaughter house and was cooled on ice within 30 min of death. Following dissection, brain regions were frozen at -80°C (within 2 hr of death) prior to assay. To prepare the radioligand binding homogenate, brain tissue was defrosted and homogenised in ice-cold Tris/Krebs buffer (mM; Tris, 50; NaCl, 118.0; KCl, 4.75; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; glucose, 11.0; pH 7.4; 100 mg wet weight tissue ml^{-1}) using a Polytron blender (full power; 10 s). The homogenate was washed twice by centrifugation/resuspension in Tris/Krebs buffer and finally resuspended at a concentration of 100 mg wet weight ml^{-1}. For radioligand binding experiments, [^{3}H]-(S)-zacopride (final concentration approximately 1 nM for the distribution study or at a range of concentrations between 0.09 - 6.81 nM for saturation studies) was incubated with brain homogenate in the absence (total binding) and presence of ondansetron (10 µM; non-specific binding) for 60 min at 37°C. Binding was terminated by rapid filtration under vacuum through pre-wet (0.1% v/v polyethyleneimine in Tris/Krebs buffer) Whatman GF/B filters followed by immediate washing with ice-cold Tris/Krebs buffer. Radioactivity remaining on the filters was assayed by liquid scintillation spectroscopy.

Specific [^{3}H]-(S)-zacopride (1 nM) binding was highest in cortical areas of the pig brain (parietal cortex 384±24, temporal cortex 366±61, frontal cortex 365±74, striate cortex 355± 23, entorhinal cortex 298±22 fmol g^{-1} wet weight, mean±SEM, n=3) with lower levels detected in other brain regions (e.g. amygdala 268±19, hippocampus 169±11, nucleus accumbens 155±9, caudate nucleus 144±11, putamen 126±12, thalamus 65±12 fmol g^{-1} wet weight, mean±SEM, n=3). [^{3}H]-(S)-zacopride saturation experiments identified that the binding was saturable and that [^{3}H]-(S)-zacopride labelled an apparently homogenous population of sites with high affinity (Bmax = 1565±160 fmol g^{-1} wet weight, pKd = 8.65±0.07, Hill coefficient = 1.03±0.05, mean±SEM, n=3).

The present study demonstrates that the [^{3}H]-(S)-zacopride-labelled 5-HT$_3$ receptor is distributed throughout the pig brain which therefore provides a suitable tissue preparation to study the biochemical and molecular aspects of the 5-HT$_3$ receptor.

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378P DESENSITISATION OF 5-HT$_3$ RECEPTORS IN THE PRESENCE OF LOW CONCENTRATIONS OF 5-HT$_3$ RECEPTOR AGONISTS

J.T. Bartrup & N.R. Newberry. Oxford University - SmithKline Beecham Centre for Applied Neuropsychobiology, University Department of Clinical Pharmacology, Radcliffe Infirmary, Oxford, OX2 6HE.

As with other ligand-gated ion channels, 5-HT$_3$ receptors desensitise rapidly with the continued or repeated application of agonist. Using N1E-115 cells, Neijt *et al.* (1988) showed that low concentrations of 5-HT, which did not produce a measurable current, could reduce the amplitude of 5-HT$_3$ receptor-mediated inward currents. We have studied this effect in NG108-15 cells.

Undifferentiated NG108-15 cells were recorded at -60mV using whole-cell patch-clamp. The extracellular medium contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1, dextrose 5, N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) 10, $CaCl_2$ 2, (pH 7.4, 24-26°C). Patch electrodes (3-6 MΩ) were filled with an "intracellular" solution of (mM): CsCl 125, HEPES 10, $MgCl_2$ 1, EGTA 1, $CaCl_2$ 0.58 (pH 7.4). 5-HT$_3$ receptor-mediated inward currents were evoked by local pressure application of 5-HT (100µM, 10-15 p.s.i., 0.1-0.5 s) from a drug pipette (tip diameter ~2 µm) situated ~20 µm from the cell. All other drug applications were via the perfusate (2-3 ml/min through a 0.2 ml bath).

Perfusion of low concentrations of three 5-HT$_3$ receptor agonists produced a concentration-dependent reduction of the 5-HT-induced inward current while inducing little or no effect of their own: pIC_{50} values (mean ± s.d., n=6) were 6.2 ± 0.1 (2-Me-5-HT), 7.3 ± 0.1 (5-HT) & 7.7 ± 0.1 (meta-chlorophenylbiguanide, mCPBG). In the presence of agonists, the first activation by pressure applied 5-HT was usually unaffected, even after agonist perfusions for 30 - 150 s, but subsequent activations were gradually reduced. Recovery of the response following washout was rapid after 5-HT, but slower after mCPBG. The agonist effects were seen at +60mV and in nominally divalent cation free extracellular medium and with a high concentration of another calcium chelator (10mM BAPTA) in the "intracellular" solution; so they were probably not modulated by voltage or Ca^{2+} or Mg^{2+}.

The most likely explanation for these observations is that activation of the 5-HT$_3$ receptor switches it to a desensitised state, to which 5-HT is bound with a high affinity, followed by recovery. The continued presence of a low concentration of agonist prevents recovery from the desensitised state. In effect, we may be studying agonist binding to the high affinity desensitised state.

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379P EFFECT OF THE 5-HT$_3$ ANTAGONIST ONDANSETRON ON CHLORDIAZEPOXIDE WITHDRAWAL HYPERTHERMIA

A.J. Goudie, J. Leaviss & M. J. Leathley, Psychology Department, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K.

5-HT$_3$ antagonists alleviate benzodiazepine (BZ) withdrawal-induced anxiety (Costall et al., 1990). However, they do not alleviate BZ withdrawal-induced weight loss (Leathley & Goudie, 1992) and hyperactivity (Mehta & Ticku, 1993). We have assessed the effect of the 5-HT$_3$ antagonist ondansetron on BZ withdrawal-induced hyperthermia. The study involved two phases, chronic treatment and withdrawal. In phase one, 6 groups (see Table 1) of singly housed female rats (ns=9-10) were treated (i.p.) b.i.d. with vehicle (V) or chlordiazepoxide (C) at 20 mg/kg (8 days) and 30 mg/kg (11 days). The subsequent withdrawal phase involved 4 days, during which temperatures were recorded daily (at an ambient temperature of 70^{0}F) 70 mins after the first injection. Controls (group V-V) continued to receive b.i.d. vehicle in this phase. Group C-V was treated with vehicle after C pretreatment and experienced BZ withdrawal. Four groups (the C-O groups) were treated b.i.d. with ondansetron (0.001, 0.01, 0.1 or 1.0 mg/kg) after C pretreatment.

Table 1. Mean group temps ^{0}C ($^+_-$ S.D.) during successive withdrawal days.

Group:-	Day 1	Day 2	Day 3	Day 4.
V-V	37.22 (0.54)	37.03 (0.40)	36.93 (0.47)	36.94 (0.45)
C-V	37.99 (0.55)	38.09 (0.66)	37.85 (0.32)	37.88 (0.48)
C-O 0.001	38.01 (0.48)	37.93 (0.53)	37.88 (0.40)	37.48 (0.52)
C-O 0.01	37.96 (0.44)	37.71 (0.33)	37.58 (0.46)	37.78 (0.47)
C-O 0.1	38.01 (0.38)	37.92 (0.74)	37.99 (0.68)	37.71 (0.47)
C-O 1.0	37.96 (0.30)	37.96 (0.50)	37.95 (0.31)	37.52 (0.31)

Data were analysed with planned t tests. Group C-V differed from controls (V-V) on all days [smallest t=3.15, df=18, p=0.005 on day 1]. Thus withdrawal induced significant, rapid onset, persistent hyperthermia. The effect of ondansetron was assessed by daily comparisons of group C-V with each C-O group. There was no significant alleviation of withdrawal hyperthermia at any dose on any day. At 0.001 mg/kg the largest t was 1.71, df=17, p=0.11 on day 4. At 0.01 mg/kg the largest t was 1.63, df=18, p=0.12 on day 2. At 0.1 mg/kg the largest t was 0.78, df=17, p=0.45 on day 4. At 1 mg/kg the largest t was 2.01, df=18, p=0.06 on day 4. 5-HT$_3$ antagonists appear only to alleviate some BZ withdrawal signs. Thus BZ withdrawal may involve a number of different underlying mechanisms.

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380P RS-39604: A POTENT, SELECTIVE, ORALLY ACTIVE AND LONG-LASTING 5-HT$_4$ RECEPTOR ANTAGONIST

S. S. Hegde, D. W. Bonhaus, E. Leung, E. H. F. Wong, L. G. Johnson, R. D. Clark[1], L. A. Flippin[1] & R. M. Eglen, Institutes of Pharmacology and Organic Chemistry[1], Syntex Discovery Research, 3401 Hillview Avenue, Palo Alto, CA 94304, U.S.A.

Selective antagonism of 5-HT$_4$ receptors may provide therapeutic benefit in certain disorders of the gut, myocardium and lower urinary tract (see Ford & Clarke, 1993 for review). RS-39604 (1-(4-amino-5-chloro-2-(3,5-dimethoxy)benzyloxyphenyl)-3-[1-((2-methylsulfonylamino)ethyl)piperidin-4-yl]-1-propanone) is a novel and selective 5-HT$_4$ receptor antagonist whose pharmacological properties are described below.

In guinea-pig striatal membranes, RS-39604 inhibited specific binding of [^{3}H]-GR 113808 (Grossman et al, 1993) in a concentration-dependent manner (mean pKi ± s.e.mean = 9.1 ± 0.1). RS-39604 displayed a low affinity (pKi < 6.5) for 5-HT$_{1A}$, 5-HT$_{2C}$, 5-HT$_3$, angiotensin (AT$_1$), muscarinic (M$_1$, M$_2$), dopamine (D$_1$, D$_2$) and neurokinin (NK$_1$) receptors. In the rat isolated oesophagus (Baxter et al, 1991), pre-contracted with carbachol (3 μM), RS-39604 antagonized 5-HT-induced relaxation by producing parallel, dextral shifts of the concentration-effect curve to 5-HT without altering the maximum response. Schild analysis of this data yielded a mean pA$_2$ (95% confidence interval, n = 12) and slope ± s.e.mean of 9.3 (9.2 - 9.4) and 1.02 ± 0.12, respectively.

In anaesthetized, vagotomized Yucatan micropigs (Eglen et al, 1994), RS-39604, administered by the intravenous (i.v.) or intraduodenal (i.d.) route, produced dose-dependent inhibition of 5-HT-induced tachycardia (mean ID$_{50}$ (95% confidence interval, n = 4) = 4.7 (3.6 - 6.0) μg·kg^{-1}, i.v., 254.5 (190.6 - 323.6) μg·kg^{-1}, i.d.). At a maximal dose of 30 μg·kg^{-1}, i.v, the inhibitory effect of RS-39604 lasted for greater than 6 h.

In conscious mice (Hegde et al, 1994a), RS-39604, administered by the intraperitoneal (i.p.) or oral (p.o.) route, produced dose-dependent inhibition of 5-hydroxytryptophan (10 mg·kg^{-1}, i.p.)-induced diarrhoea (mean ID$_{50}$ ± s.e.mean, (n = 30) = 81.3 ± 1.9 μg·kg^{-1}, i.p., 1.1 ± 4.2 mg·kg^{-1}, p.o.). In inactin-anaesthetized guinea-pigs (Hegde et al, 1994b), RS-39604 antagonized the contractile effects of 5-HT in the proximal colon by producing parallel, dextral displacement of the dose-response curve to 5-HT. The mean dose-ratios (95% confidence interval, n = 5) at 0.1 mg·kg^{-1}, i.v., 1 mg·kg^{-1}, i.v. and 10 mg·kg^{-1}, i.d. of RS-39604 were 4.6 (1.9 - 10.9), 30.7 (8.5 - 111.3) and 10.8 (2.6 - 44.4), respectively. In a model of visceral pain in conscious rats (Ness & Gebhart, 1988), RS-39604 (0.01 - 1 mg·kg^{-1}, i.v.) did not affect colorectal distension-induced pressor responses (n = 5) whereas morphine (1 mg·kg^{-1}, i.v.) produced significant inhibition of the response implying that 5-HT$_4$ receptors are not involved in nociception in this animal model.

These data suggest that RS-39604 is a potent and selective 5-HT$_4$ receptor antagonist which is orally active and long-lasting *in vivo*.

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381P ABILITY OF 5-HT$_4$ RECEPTOR LIGANDS TO MODIFY RAT STRIATAL DOPAMINE RELEASE *IN VITRO* AND *IN VIVO*

L.J. Steward, J. Ge and N.M. Barnes, Department of Pharmacology, The Medical School, University of Birmingham, Birmingham, B15 2TT, UK.

5-HT$_4$ receptor binding sites have been recently identified in the rat nigrostriatal and mesolimbic dopaminergic systems (Grossman et al. 1993) suggesting that the 5-HT$_4$ receptor may play a role in dopaminergic regulation. In addition, Benloucif et al. (1993) using *in vivo* microdialysis, demonstrated that 5-methoxytryptamine (5-MeOT) produced an increase in striatal dopamine levels. We have previously shown that the 5-HT$_4$ receptor agonists renzapride and (S)-zacopride, increase dopamine release from rat striatal slices (Steward and Barnes, 1994). In the present study we have further characterised the ability of 5-HT$_4$ receptor ligands to modulate the release of dopamine in rat striatal slices and have also investigated the response, using the *in vivo* microdialysis technique.

The methodology for *in vitro* striatal slice studies have been described previously (Steward and Barnes, 1994). For microdialysis studies, female Wistar rats (150-250 g) were anaesthitised with a combination of ketamine (60 mg/Kg, i.p.) and medetonidine (250 µg/Kg, i.p.) and cannulated (A 7.5, V -2.0, L -2.5, relative to Bregma) using standard stereotaxic procedures (Barnes et al., 1992). Approximately 14 days after the cannulation, microdialysis probes were implanted into the striatum (4 mm dialysis probe) and left overnight before being perfused with aCSF (mM: NaCl 126.6, KCl 2.4, KH_2PO_4 0.49, $MgCl_2$ 1.28 $CaCl_2$ 1.1, $NaHCO_3$ 27.4, Na_2HPO_4 0.48, glucose 7.1, pH 7.4) at a rate of 2 µl/min. All drugs were administered via the perfusing aCSF and dopamine levels in the dialysates were quantified by HPLC with electrochemical detection. All data was ANOVA and means comparison t-tested.

In striatal slices, 5-MeOT (10 µM; in the presence of 1 µM pindolol and 1 µM methysergide) and 5-HT (10 µM; in the presence of 10 µM pindolol, 10 µM methysergide and 1 µM ondansetron) enhanced the release of dopamine by 214 ± 25% (mean±S.E.M., n=4, ANOVA P<0.0001, t-test P<0.0001) and 133 ± 5% (mean±S.E.M., n=3, ANOVA P<0.0001, t-test P<0.0001) of basal values (basal dopamine release 0.76 ± 0.19 pmol/min/mgprotein).

The basal extracellular levels of DA from striatum of freely-moving rats was 5.82±1.93 pg/40 µl (mean±S.E.M., n=14). The addition of tetrodotoxin (10 µM) in the aCSF caused a significant reduction in the extracellular levels of DA by some 60±9 % (mean±S.E.M., n=3, ANOVA P<0.05, t test P<0.01). The 5-HT$_4$ receptor agonists S(-)zacopride (100 µM) and renzapride (100 µM) induced marked increases in the dialysate levels of dopamine by some 189±53 % (mean±S.E.M., n=6, ANOVA P<0.00001, t-test P<0.0001) and 175±14 % (mean±S.E.M., n=5, ANOVA P<0.05, t-test P<0.0001) respectively. In contrast, in the continued presence of the 5-HT$_4$ receptor antagonist SDZ 205-557 (100 µM), (S)-zacopride (100 µM), failed to elevate the extracellular DA levels (81 ± 16%, mean ± SEM, n=4). The present results indicate that dopamine release in the rat striatum, is enhanced by the activation of central 5-HT$_4$ receptors.

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382P ML 10302, A POWERFUL AND SELECTIVE NEW 5-HT$_4$ RECEPTOR AGONIST

T. Croci, M. Langlois[1], T. Mennini[2], M. Landi & L. Manara, SANOFI-Midy S.p.A. Research Center, Milan (Italy) [1]BIOCIS-CNRS, Paris (France) and [2]Istituto di Ricerche Farmacologiche Mario Negri, Milan (Italy)

ML 10302 (2-piperidinoethyl 4-amino-5-chloro-2-methoxybenzoate) is identical to a compound recently claimed to be a potent 5-HT$_4$ antagonist (Elz & Keller, 1993). We have characterized ML 10302 *in vitro* in functional and binding assays.

Table 1	GPI[a]		RE[b]	Binding, Ki, nM ± S.E.	
	pA$_2$, 5-HT antagonism	EC$_{50}$ nM	EC$_{50}$ nM	[^{3}H]GR 113808[c]	[^{3}H]BRL 43694[d]
BIMU8	n.e.	43 [86%] (29-63)	n.d.	257 ± 43	0.4±0.1
ML 10302	n.e.	4 [80%] (3.7-4.3)	2.4 [85%] (1.5-3.9)	13±1	314±41
SDZ 205557	~7.8	>1,000	>1,000	25±2	471±45

[a]GPI = guinea-pig ileum myenteric plexus longitudinal muscle, electrically stimulated (Craig & Clarke, 1990); [b]RE = rat esophagus muscularis mucosae, contracted by carbachol (Baxter & Clarke, 1992); [c]guinea-pig striatum membranes (Grossman *et al.*, 1993); [d]rat brain cortex membranes (Nelson & Thomas, 1989); [ ]: maximal effect (intrinsic activity) relative to 5-HT (EC$_{50}$, nM: GPI, 5; RE, 4); ( ): 95% confidence limits; n.e. = non evaluable; n.d. = not determined.

As shown by the concentrations with half-maximal effect (EC$_{50}$, Table 1) ML 10302 had substantial 5-HT$_4$ agonist action (RE and GPI) that was prevented by SDZ 205557 (pA$_2$ ~7.8). The latter behaved as a pure antagonist while ML 10302, like BIMU8, was a partial agonist in view of its lower intrinsic activity than 5-HT. ML 10302, but also 5-HT, shifted to the right the dose-response curve of 5-HT in both GPI and RE, suggesting 5-HT$_4$ desensitization. Thus the antagonist properties of ML 10302 can only be assumed on the grounds of its partial agonist nature. ML 10302 and SDZ 205557 were definitely more selective for the 5-HT$_4$ than the 5-HT$_3$ site judging from their potencies in displacing the binding of the appropriate radioligands (GR 113808 and BRL 43694, Table 1), whereas the opposite applied to BIMU8. In additional binding assays whose results are not presented in detail, ML 10302 up to 10 µM showed slight or no displacement of specific ligands from several sites including 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$, 5-HT$_2$, 5-HT uptake, α_1- α_2- β_1-, β_2-adrenergic, histamine H$_1$, muscarinic, dopamine D$_1$ and D$_2$. This further attests to the selectivity of ML 10302 in a more general context and confirms its value as a better tool for characterization of the 5-HT$_4$ receptors.

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383P OPERATIONAL CHARACTERISTICS OF A 5-HT RECEPTOR MEDIATING DIRECT VASCULAR RELAXATION: IDENTITY WITH THE 5-HT$_7$ RECEPTOR?

G. R. Martin & R. J. Wilson, Receptor Pharmacology Group, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS.

The most prominent haemodynamic response to 5-HT administered to anaesthetised animals by intravenous infusion is vasodepression. In the rat, the operational characteristics of this response suggest involvement of a 5-HT receptor mediating direct vascular smooth muscle relaxation. Cumulated data from various isolated blood vessels indicates close operational and transductional similarities of this receptor with the 5-ht$_7$ recombinant receptor (Martin, 1994). Here we have investigated this possibility further by determining affinity and relative efficacy estimates for a number of 5-HT receptor ligands mediating direct relaxation of rabbit femoral vein.

Vascular rings (4mm) were mounted in Krebs solution at 37°C for measurement of isometric force changes following lethal dose anaesthesia of rabbits by pentobarbitone sodium. Exposure to pargyline (500μM, 30min) blocked metabolism by MAO. In each tissue, two cumulative agonist concentration-relaxation curves were obtained after contracture with U46619 (100nM). For studies with agonists, the first curve was always to 5-carboxamidotryptamine (5-CT) and the second to the test agonist. In the case of antagonist experiments, curve pairs to the same agonist were constructed before and after 60min incubation with antagonist. Agonist affinities and efficacies were estimated using operational model fitting.

5-HT relaxations were resistant to block by ketanserin (1μM), whereas methysergide (0.3μM) and methiothepin (3nM) were potent, unsurmountable antagonists (pA_2=7.8 and 9.5, respectively). Spiperone, however, behaved as a simple competitive antagonist with a pK_B of 7.75±0.05 (16d.f.) and a Schild plot slope parameter of 1.06±0.06. Affinity (pK_A) and efficacy (τ) estimates for agonists, together with apparent pK_Bs estimated for spiperone from a single concentration (0.3μM), were as follows (mean±s.e.mean, n=3-8);

Agonist	pK_A	τ	Relative τ	Spip. pk_B
5-HT	5.70±0.09	3.1±1.1	1.00	7.75±0.05
5-CT	6.69±0.09	6.6±1.3	2.13	7.63±0.06
Di-nPr-5-CT	5.62±0.15	3.2±1.6	1.02	7.41±0.18
5-MeT	5.59±0.05	2.4±0.7	0.77	7.66±0.08
α-Me-5-HT	<4.5	-	-	-
8-OHDPAT	4.53±0.24	1.6±0.6	0.51	7.70±0.22

These results provide a quantitative, operational fingerprint for the 5-HT receptor in rabbit femoral vein. This profile matches that previously described for a smooth muscle relaxant "5-HT$_1$-like" receptor in vascular and gastro-intestinal tissue and closely resembles that described for the 5-ht$_7$ recombinant receptor. Since the vascular receptor stimulates cAMP formation (Trevethick *et al*, 1986) and 5-ht$_7$ mRNA is strongly expressed in human coronary artery (Bard *et al*, 1993), the present data further support the notion that vascular smooth muscle relaxation is mediated by the 5-ht$_7$ receptor.

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384P 5-HT RECEPTOR OF THE LEECH (*HIRUDO MEDICINALIS*) RETZIUS CELLS

M. Koubanakis & L. D. Leake. School of Biological Sciences, University of Portsmouth, King Henry I St, Portsmouth PO1 2DY.

This study aimed at the characterisation of the 5-hydroxytryptamine (5-HT) receptor of the Retzius cells of the leech *Hirudo medicinalis*, using modern agonists and antagonists developed for vertebrate 5-HT receptors. Earlier studies on this receptor were carried out with less selective ligands (Sunderland *et al.*, 1980; 1982). The Retzius cells are giant paired neurones, present in each of the segmental ganglia. and respond to 5-HT by hyperpolarisation (Smith & Walker 1974).

Intracellular recordings were made from Retzius cells in high magnesium leech ringer (NaCl 95 mM, KCl 4 mM, $CaCl_2$ 2mM, $MgCl_2$ 10mM, $MgSO_4$ 10 mM, glucose 10 mM, Tris HCl 10 mM, pH 7.4), using potassium acetate filled glass microelectrodes (R=20-30 MΩ). Measurements of hyperpolarisation, made directly from a Tektronix 5113N oscilloscope and/ or a Brush Gould 2400 pen recorder were used to construct dose response curves. 5-HT agonists were introduced into the 4.5 ml bath of the preparation in 0.5 ml aliquots (providing a ten-fold dilution). The preparations were incubated with antagonists for 5 minutes prior to 5-HT application.

All agonists and antagonists tested were effective. Their potencies are shown in Table 1. It is concluded that the 5-HT receptor on leech Retzius cells does not conform to a single type of vertebrate receptor, but includes elements of 5-HT$_1$,5-HT$_2$ and 5-HT$_3$ receptors. Alternatively, three populations of 5-HT receptors could be present.

Table 1 Potencies of a) agonists expressed as EPMRs (equipotent molar ratios) and b) antagonists expressed as pA_2 values.

a) Agonists			b) Antagonists		
Agonist	Select.	EPMR	Antagonist	Select.	pA_2
5-HT	General	1	Methiothepin mesylate	5-HT$_1$	6.1
5-CT	5-HT$_1$	3.4	Cinanserin	5-HT$_2$	6.5
Buspirone	5-HT$_{1A}$	10000	Ritanserin	5-HT$_2$	7.8
PAPP	5-HT$_{1A/D}$	1000	MDL 72222	5-HT$_3$	8.0
CGS	5-HT$_{1B}$	128	ICS 205930	5-HT$_{3/4}$	6.4
α-Methyl serotonin	5-HT$_2$	20	Ondansetron	5-HT$_3$	6.4
2-Methyl serotonin	5-HT$_3$	10			
5-Meth-oxy trypt-amine	5-HT$_4$	125			

PAPP : 4[2-[4-[3-(trifluoromethyl) phenyl]-1-piperazinyl]ethyl]benzeneamine.
CGS-12066B : 7-Trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline maleate.

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385P THE EFFECT OF 'BROAD SPECTRUM' ANTIEMETIC DRUGS ON NICOTINE-INDUCED EMESIS IN THE *SUNCUS MURINUS*

J.A. Rudd & R.J. Naylor, Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP.

'Broad spectrum' antiemetic drugs such as fentanyl (Barnes *et al.*, 1991), 8-hydroxy-2(di-n-propylamino)-tetralin (8-OH-DPAT, Lucot & Crampton, 1989) and resinferatoxin (Andrews & Bhandari, 1993) can inhibit the emesis induced via diverse stimuli and may therefore be useful to antagonise the emesis induced by unknown mechanisms. In the present study we compare the ability of fentanyl, 8-OH-DPAT and resinferatoxin with ondansetron to antagonise nicotine induced emesis in the *Suncus murinus*.

Suncus murinus weighing 30-90 g (either sex; Bradford University bred) were injected with ondansetron (Ond) or vehicle (V1, distilled water; 2 ml kg^{-1} i.p.) as a 30 min pretreatment, with fentanyl (Fent), 8-OH-DPAT, or vehicle (V2, distilled water; 2 ml kg^{-1} s.c.) as a 15 min pretreatment or resinferatoxin (Res) or vehicle (V3; 10% ethanol:10% Tween 80:80% distilled water; 2 ml kg^{-1} s.c.) as a 3 h pretreatment prior to the administration of nicotine (Nic; 5 mg kg^{-1} s.c.). The latency (Lat) to the first retch/vomit and the total numbers of retches and vomits were recorded over the subsequent 30 min observation period.

Both fentanyl and 8-OH-DPAT caused dose related antagonism of nicotine induced retching and vomiting with a trend to increase the latency; ondansetron was ineffective (Table 1). However, resinferatoxin induced dose dependent retching and vomiting during the pretreatment period (range 3.8±2.6 to 82.5±20.9 retches and 0.0±0.0 to 1.25±0.63 vomits) but subsequently antagonised the retching and vomiting induced by nicotine (Table 1; there was no correlation between the emetic and antiemetic potential of resinferatoxin).

In conclusion, resinferatoxin unexpectedly induced emesis in its own right. However, it also antagonised nicotine induced emesis by unknown mechanisms whereas the antiemetic effects of fentanyl and 8-OH-DPAT indicate a μ-opioid and 5-HT_{1A} receptor involvement. The emetic effects of resinferatoxin have not been reported in the ferret (Andrews & Bhandari, 1993) and may indicate species differences in the organisation of the emetic reflex. 5-HT_3 receptors do not appear to be involved in nicotine induced emesis in the *Suncus murinus*.

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Table 1. The effect of 'broad spectrum' antiemetic agents and ondansetron to modify nicotine induced emesis in the *Suncus murinus*.

Treatment	μg kg^{-1}	Lat (min)	Retches	Vomits	RV/T	Treatment	μg kg^{-1}	Lat (min)	Retches	Vomits	RV/T
Nic+V2		5.4	85.5±11.8	3.3±0.5	4/4	Nic+V3		5.5	71.3±12.3	2.0±0.0	4/4
Nic+Fent	10	8.9	12.5±7.2**	0.6±0.3**	2/4	Nic+Res	1	3.9	92.3±15.5	1.5±0.3*	4/4
	40	-	0.0±0.0**	0.0±0.0**	0/3		10	-	0.0±0.0**	0.0±0.0**	0/4
Nic+8-OH-DPAT	2.5	5.7	31.5±15.8	1.3±0.5**	4/4		100	5.3	32.0±10.9*	0.0±0.0**	4/4
	25	12.5	13.7±6.9**	0.8±0.5**	2/4	Nic+V1		9.2	88.0±5.1	2.0±0.6	4/4
	250	-	0.0±0.0**	0.0±0.0**	0/4	Nic+Ond	1000	8.7	90.8±16.3	3.0±0.8	4/4

The mean Lat is shown for animals developing retching/vomiting; the total numbers of retches and vomits is indicated as mean±S.E.M.; RV/T indicates the no. of animals either retching or vomiting out of the no. of animals tested. Significant differences between respective vehicle control and drug treated animals are indicated as *P<0.05 and **P<0.01 (one-way ANOVA followed by Dunnett's t test).

386P THE EFFECT OF DEXAMETHASONE ON DRUG-INDUCED EMESIS IN THE FERRET

J.A. Rudd, K.T. Bunce[1] & R.J. Naylor. Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP. [1]Pharmacology I, Glaxo Research & Development Ltd., Ware, Herts., SG12 ODP.

Dexamethasone (Dex) is a useful agent to combine with 5-HT_3 receptor antagonists to improve the control of chemotherapy-induced emesis in man (Smith *et al.*, 1990). However, the mechanism of antiemetic action of Dex is unknown and may be related to an activity at central and/or peripheral sites. The present study attempts to investigate the potential of Dex to antagonise the emesis induced by gastric irritation (using intragastric copper sulphate solution), and area postrema activation (using apomorphine and morphine). Cisplatin (CP) was also included in the study as a reference chemotherapeutic agent.

Ferrets (either sex; 0.7-1.4 kg) were injected intraperitoneally with Dex or vehicle (distilled water; 0.5 ml kg^{-1}) as a 60 min pretreatment prior to the administration of $CuSO_4.5H_2O$ (Cu; 100 mg kg^{-1} intragastric), apomorphine (Apo; 0.25 mg kg^{-1} s.c.) or morphine (Mor; 0.5 mg kg^{-1} s.c.). Ferrets receiving CP (10 mg kg^{-1} i.v.) were administered Dex immediately before the injection of CP via a chronic indwelling jugular cannula. The latency to the first retch or vomit and the total numbers of retches and vomits were recorded over the subsequent 30 min (for Cu, Apo and Mor treatment) or 240 min (for CP treatment) observation period.

Dex reduced the retching induced by Cu at the dose of 0.1 mg kg^{-1} and CP at the dose of 5 mg kg^{-1} by 49 and 54 % respectively (table 1). The reductions were not statistically significant (P>0.13). Higher doses of Dex were also ineffective to reduce Cu, Apo, Mor and CP induced retching and vomiting (P>0.05).

In conclusion, the reductions in emesis caused by Dex failed to achieve statistical significance, limiting an interpretation of the data in terms of its relevance to the clinical usefulness of Dex. Future experiments investigating the effect of Dex and ondansetron combinations on drug induced emesis are being performed.

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Table 1. The effect of dexamethasone on copper sulphate, apomorphine, morphine and cisplatin induced emesis in the ferret.

Treatment	mg kg^{-1}	Latency (min)	Retches	Vomits	RV/T	Treatment	mg kg^{-1}	Latency (min)	Retches	Vomits	RV/T
Cu	100.0	3.7	70.0±7.5	8.5±1.0	4/4	Mor	0.5	3.2	48.0±10.6	5.6±1.0	7/7
+Dex	0.05	5.6	52.9±17.4	8.0±2.5	4/4	+Dex	0.1	2.4	25.0±9.1	3.3±1.4	4/4
	0.1	4.8	35.8±5.0	4.3±0.3	4/4		2.5	2.2	42.3±14.6	4.5±1.5	4/4
	5.0	3.8	93.8±28.0	10.0±1.2	4/4		5.0	3.1	45.0±5.1	6.3±0.6	4/4
	10.0	3.4	67.0±15.1	7.8±1.7	4/4		10.0	3.2	37.5±5.6	5.0±1.2	4/4
Apo	0.25	3.0	37.5±6.5	5.3±1.7	4/4	CP	10.0	62.9	70.8±8.9	7.1±1.5	6/6
+Dex	0.1	6.2	39.3±10.6	5.3±1.7	4/4	+Dex	5.0	141.7	33.0±17.4	2.5±2.2	4/4
	2.5	8.7	34.5±17.8	3.5±1.8	4/4		10.0	62.3	92.0±22.2	5.2±2.2	5/5
	5.0	2.6	28.3±5.7	3.3±0.9	4/4		20.0	72.7	65.0±20.5	8.5±2.5	4/4

Results represent the mean±S.E.M. for the numbers of retches and vomits; the mean latency to the first retch or vomit is indicated in min; RV/T indicates the numbers of animals either retching or vomiting out of the numbers of animals tested. There were no significant differences between respective vehicle control (Cu, Apo, Mor and CP) and Dex treated animals (P>0.05, one-way ANOVA followed by Dunnett's t test).

387P EFFECTS IN THE RAT OF SIBUTRAMINE ON FOOD INTAKE AND THE BEHAVIOURAL SATIETY SEQUENCE

J.C.G. Halford, D.J. Heal[1] & J.E. Blundell, BioPsychology Group, Department of Psychology, University of Leeds, Leeds LS2 9JT and [1]Boots Pharmaceuticals Research Department, Nottingham NG2 3AA.

Sibutramine hydrochloride inhibits noradrenaline and 5-hydroxytryptamine (5-HT), but not dopamine, reuptake in vivo (Luscombe *et al.*, 1990; Heal *et al.*, 1992). In rats, it dose-dependently reduces food intake (Fantino *et al.*, 1994). As feeding can be reduced by non-specific interference of behavioural or physiological processes, a sequence of behaviours accompanying feeding ("satiety sequence") has been developed to identify its suppression by natural post-ingestive processes (Antin *et al.*, 1975). This pattern is most accurately monitored by continuous behavioural analysis and is sensitive to drug manipulation (Halford & Blundell, 1993).

Studies were conducted on male Lister hooded rats (250-300g; n=12/group). The ED_{50} dose of sibutramine to reduce food intake (2mg/kg po) was compared with equipotent doses of *d*-amphetamine (1.5mg/kg ip) and *d*-fenfluramine (1mg/kg ip). Following drug dosing, behaviour was continuously assessed for 40 min and classified into 8 categories (eating, drinking, resting, grooming, sniffing, rearing, locomotion and other). The occurrence and duration of each event was recorded using an in-house data collection program ('KEETH').

Table 1 shows the drug effects on food intake and parameters of eating behaviour. Considering the satiety sequence itself, placebo injections produced a classical profile. *d*-Amphetamine markedly increased the duration of sniffing ($p<0.001$) and decreased the duration ($p<0.02$) and frequency ($p<0.002$) of resting, thereby disrupting the satiety sequence. Sibutramine accelerated the decline in feeding by reducing the duration ($p<0.02$) and frequency ($p<0.01$) of eating episodes, and advanced the onset and increased the duration ($p<0.02$) of resting. This enhancement of resting caused a consequent reduction in the duration of grooming, locomotion and rearing. Similar, but not identical, adjustments were brought about by *d*-fenfluramine. This modulation of the behavioural profile is consistent with a 5-hydroxytryptaminergic component in the action of sibutramine and is in accordance with the enhancement of the natural development of satiety.

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Table 1. Effects on food intake (FI), global eating rate (GER), local eating rate (LER), mean eating bout intake (MIE) and mean eating bout length (MBL)

Treatment groups	FI	GER	LER	MIE	MBL
Saline control	6.1g (0.6)	0.15g/min (0.02)	1.13g/min (0.16)	0.40g (0.05)	21.1s (0.06)
Sibutramine 2.0mg/kg po	2.9g*** (0.8)	0.07g/min*** (0.04)	1.09g/min (0.18)	0.31g (0.10)	17.1s (0.08)
d-Amphetamine 1.5mg/kg ip	2.7g*** (0.7)	0.06g/min*** (0.19)	0.97g/min (0.29)	0.17g (0.11)	10.7s (0.15)
d-Fenfluramine 1.0mg/kg ip	3.4g*** (0.6)	0.09g/min*** (0.16)	0.79g/min (0.12)	0.37g (0.16)	27.9s (0.16)

Derived results reflect changes in frequency and duration. ***P<0.005 versus saline (2 x 8 analysis of variance) ( ) = s.e.mean

388P A STUDY IN RATS OF THE EFFECTS OF SIBUTRAMINE ON FOOD INTAKE AND THERMOGENESIS

I.P. Connoley, D.J. Heal[1] & M.J. Stock, Department of Physiology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE and [1]Boots Pharmaceuticals Research Department, Nottingham NG2 3AA

Sibutramine hydrochloride (BTS 54 524) is a novel inhibitor of noradrenaline and 5-hydroxytryptamine reuptake (Buckett *et al.*, 1988) which produces weight loss in patients on a restricted calorie diet (Weintraub *et al.*, 1991). These anti-obesity actions could be due to inhibition of food intake and/or stimulation of metabolic rate; both possibilities have been the subject of the experiments described here.

The effects of 14 days administration of sibutramine (10 mg/kg po) were examined in genetically lean (190 g) and obese (330 g) female Zucker rats (n = 8 per group). Sibutramine produced similar, small weight losses in both genotypes (Lean/Sibutramine = -9 ± 2 g; Obese/Sibutramine = -7 ± 3 g) which differed significantly ($P<0.001$) from the increases in their respective controls (Lean/Con = 9 ± 2 g; Obese/Con = 33 ± 2 g). Although sibutramine significantly ($P<0.05$) reduced food intake in the obese rats during the first week (Obese/Con = 27.2 ± 1.9 g/d; Obese/Sibutramine = 21.8 ± 6 g/d), the average consumption over 14 days did not differ between the sibutramine and control groups. Thus, these data suggest that increases in metabolic rate were involved in the effect of sibutramine on body weight gain.

The effects of sibutramine on oxygen consumption (VO_2) and colonic temperature (Tc) were measured in female Wistar (230 - 240 g) rats (n = 8 or 16 per group) using closed-circuit respirometers at a thermoneutral ambient temperature (29°C). After a 2h baseline measurement (range: VO_2 = 11.02 - 11.75 ml/kg$^{0.75}$/min; Tc = 36.97 - 37.16°C) rats received sibutramine (0.3 - 10 mg/kg po) and VO_2 was measured continuously for a further 2h. Tc was measured immediately before and 2h after sibutramine. Low doses (0.3 mg/kg, 1 mg/kg) had no effect on VO_2 or Tc, 3 mg/kg increased VO_2 by 5 ± 2% ($P<0.05$) without altering Tc and 10 mg/kg markedly enhanced VO_2 by 23 ± 4% ($P<0.001$) and Tc by 0.76 ± 0.13°C ($P<0.001$). At 10 mg/kg sibutramine, the VO_2 reached a plateau 90 - 120 min post-dosing. When sibutramine (10 mg/kg po) was given 1h before VO_2 measurement, the response was found to be sustained ≤6h, with a 31 ± 4% increase ($P<0.01$) in VO_2 and a 1.1 ± 0.1°C increase ($P<0.001$) in Tc at 6h. Injection of sibutramine (10 mg/kg ip) also resulted in a significant ($P<0.01$) increase in VO_2 (peak VO_2 increase = 17 ± 4% at 2h) which was sustained for approximately 3h.

Taken together, these chronic and acute studies with sibutramine indicate a potent thermogenic effect in rats that contributes to its actions to reduce body weight gain.

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389P 5-HT CAUSES ACUTE GASTRIC DAMAGE IN RATS

Apaydin S[1], Gidener S[1], Gelal A[1], Guven H[1], Kupelioglu A[2], Güre A[3]. [1]Department of Pharmacology,[2]Pathology and Physiology. Dokuz Eylul University Medical Faculty 35340 Inciralti, IZMIR, TURKEY

It has been suggested that endogenous 5- hydroxytryptamine (5-HT) plays a role in peptic ulceration (Salim, 1991; Takeuchi and Ohtsuki, 1986). The aim of this study was to investigate the effect of 5-HT on gastric acid secretion and mucosal integrity in rats. Male wistar rats (246± 34 g) were anesthetized with urethane (1.5 g/kg, i.p.) and acid out put measured from an acute gastric fistula. Sistemic administration of 5-HT (3.5 µmole/kg, i.v.) caused a 59.5±11.4 % inhibition in acid out put stimulated by pentagastrin infusion (24 µg/kg/h, i.v.). The increase in acid out put produced by Pentagastrin was 23.85±3.68 µmole/10min. Values are mean±s.e from n=7 experiments. The administration of the $5HT_{1/2}$ antagonists, metergoline (1.1µmole/kg), methiothepine (1.1µmole/kg) and spiperone (2.5 µmole/kg) in to the splenic artery (i.a.) 30 minutes before administration of 5-HT (0.88 µmole/kg, i.a.) significantly ($p<0.05$, one way analysis of variance) reduced (approximately 80% inhibition) the inhibitory effect of 5-HT on Pentagastrin stimulated acid out put. In marked contrast, tropisetrone (0.05µmole/kg), ketanserine (1.2 µmole/kg) and renzapride (0.17 µmole/kg) were without effect (n=3-7). None of the antagonists modified Pentagastrin-induced stimulation of acid out put. The $5HT_1$ agonist, 5-CT (0.88 and 2.6 µmole/kg, i.a.) also inhibited Pentagastrin stimulated acid out put by 28.3±5.1 and 63.8±2.4 % (n=5). In separate studies mucosal integrity was assesed using a histological ulcer index (HUI), (0=normal; 1 =epithelial cell damage; 2=glandular disruption, vasocongestion or oedema in upper mucosa and 3=mucosal disruption,vasocongestion or oedema in midlower mucosa) by light microscopy 4h after treatment with vehicle or 5-HT (20 mg/kg, s.c.) to conscious rats. The HUI in controls and following 5-HT were 0.14±0.1 and 1.57±0.3 respectively (n=7). Atropine (1.5-30 mg/kg, i.p.) and ondansetrone (5 mg/kg, i.p.) had no significant effect on the 5-HT induced increase in the HUI. The results from this study demonstrate that 5-HT causes acute gastric damage and also inhibits gastric acid out put. The inhibitory effect on acid out put appears to be mediated via $5HT_1$ receptors. The increase in HUI was not mediated via activation of muscarinic or $5HT_3$ receptor mechanisms. The mechanism by which 5-HT increases HUI remains to be determined.

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390P EFFECT OF 7 DAYS PAROXETINE ADMINISTRATION ON SOCIAL INTERACTION BEHAVIOUR AND 5-HT2C RECEPTOR FUNCTION IN RATS

S. Lightowler, G. A. Kennett, I. F. Tulloch and T. P. Blackburn Dept. of Psychiatry Research, SmithKline Beecham, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW.

Paroxetine produces anxiolytic-like responses in the rat x-maze (Cadogan et al., 1992) and social interaction (SI)(Lightowler et al., 1992) models of anxiety following 21 days of repeated administration. An effect which may contribute to anxiolysis, and which is also seen in rats following 21 days paroxetine administration, is a reduction in $5\text{-}HT_{2C}$ receptor function (Kennett et al., 1994a). Although these effects are not seen acutely, it is questionable whether 21 days of administration is required. Indeed, anxiolysis has been observed over a shorter time-course clinically (Dunbar et al., 1991). We have therefore investigated the effect of 7 days paroxetine administration in the rat SI test, and on rat $5\text{-}HT_{2C}$ receptor function using the mCPP-induced hypolocomotion test (Kennett et al., 1994a).

In the SI test, male Sprague-Dawley (SD) rats (weighing approx. 300g) were pre-dosed p.o., daily, for 6 days with paroxetine (3 mg/kg) or vehicle (1% methyl cellulose). On day 3 the animals were housed singly and on day 7, 60 min after a final dose p.o., were placed with an unfamiliar, like-treated pair mate into a novel, white perspex test arena under bright light. For each pair, locomotor activity (LA) and active SI (time spent sniffing, grooming, following, mounting, biting, crawling over or under) were scored by an observer for 15 min. For comparison, pairs of rats that had been pre-dosed chronically with vehicle were tested on day 7, 60 min after receiving 4 mg/kg chlordiazepoxide p.o. (CDP). Results, cited as means ± s.e.m., were analysed by 1-way ANOVA and Dunnett's t-type test (n = 17-19 pairs/group).

In the mCPP-induced hypolocomotion test, male SD rats (weighing approx. 300g) were dosed p.o., daily with paroxetine (10 mg/kg) or 1 % methyl cellulose vehicle for 7 days. 24 hr after the last pretreatment, the rats were given mCPP (6 mg/kg i.p.) or vehicle (0.9% saline) 20 min prestest. When tested, the rats were placed singly in automated LA boxes for 10 min under red light and the number of transits recorded. Results, cited as means ± s.e.m., were analysed by 2-way ANOVA and Neuman Keul's test (n=10/group)

Repeated paroxetine significantly ($p<0.01$) increased SI from 156.5 ± 9.7 to 212.4 ± 15.4 s with no effect on LA (1103.4 ± 44.4 and 1057.1 ± 43.4 line crossings for vehicle and paroxetine groups, respectively), indicating an anxiolytic-like effect. The positive control, CDP, also significantly ($p<0.01$) increased SI (to 300.3 ± 14.9 s). with no effect on LA (1130.7 ± 45.4 line crossings).

In animals pre-dosed with vehicle, mCPP significantly ($p<0.05$) reduced LA from 12.1 ± 1.8 to 5.1 ± 2.1 transits. In control animals repeated paroxetine had no effect on LA (15.7 ± 2.0 transits), but attenuated ($p<0.01$) the hypolocomotor effect of mCPP (to 18.5 ± 3.4 transits), indicating a reduction in $5\text{-}HT_{2C}$ receptor function.

These findings demonstrate that 7 days paroxetine administration is sufficient to produce anxiolytic-like activity in the rat SI test. Furthermore, since $5\text{-}HT_{2C}$ receptor antagonism is associated with anxiolysis (Kennett et al., 1994b), the reduction in function of this receptor subtype by the same duration of paroxetine administration may account, at least in part, for its anxiolytic-like activity.

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391P THE ANXIOLYTIC EFFECTS OF SELECTIVE 5-HT REUPTAKE INHIBITORS IN THE ELEVATED PLUS-MAZE ARE NOT EXPLAINED BY REUPTAKE INHIBITION OR AFFINITY FOR 5-$HT_{1A,2A,2C}$ AND $_3$ RECEPTOR SUBTYPES

H.C. Jackson, S.C. Cheetham, G.P. Luscombe, S.E. Mazurkiewicz, J.A. Viggers & D.J. Heal, Boots Pharmaceuticals Research Department, Nottingham, NG2 3AA.

The effects of the selective serotonin reuptake inhibitors (SSRIs) fluoxetine, fluvoxamine, paroxetine and sertraline in the elevated plus-maze (EPM) were compared with their ability to inhibit 5-HT reuptake in vitro and in vivo and binding to 5-HT receptor subtypes.

Male Sprague-Dawley rats (170-200 g; n=6) were dosed p.o. with drug (0.03-30 mg/kg) or vehicle and 60 min later placed in the centre of the EPM. Open and closed arm entries were recorded over the next 10 min. Groups of naive rats (n=5-6) were given the most anxiolytic dose of each SSRI, followed 30 min later by *m*-hydroxybenzylamine (NSD 1015; 100 mg/kg ip) and killed after a further 30 min. 5-HTP (basal level 177±6 ng/g tissue) was measured in whole brain minus cerebellum by HPLC-ECD. Affinities for 5-HT receptor subtypes were determined using tritiated 8-hydroxy-2-(di-n-propylamino)tetralin (5-HT_{1A}), ketanserin (5-HT_{2A}), mesulergine (5-HT_{2C}) and GR 65630 (3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-1*H*-indol-3-yl)-1-propanone; 5-HT_3) (n=2-3). Non-specific binding was defined by 5-HT (10 µM), methysergide (5 µM), 5-HT (10 µM) and metoclopramide (30 µM), respectively.

All SSRIs were active in the EPM. Overall, fluvoxamine was the most potent followed by paroxetine with sertraline and fluoxetine only weakly active. When examined collectively, the anxiolytic effects of the SSRIs cannot be related to 5-HT uptake inhibition in vitro or in vivo, or affinity for any 5-HT receptor subtype which has been implicated in the control of anxiety. In particular, fluvoxamine has no 5-HT subtype affinity and is most active in the EPM at a dose which does not affect 5-HT uptake in vivo. Therefore, none of the potential mechanisms for the SSRIs examined here explains their anxiolytic effect in the EPM which remains to be elucidated.

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Table 1. Effect of SSRIs in the EPM compared with their effects on 5-HT reuptake and receptor subtypes

	Active doses EPM	Most active dose EPM	Open:total arm entries	5-HT uptake Ki (nM)[2]	5-HTP % Inhibition	5-HT_{1A}	5-HT_{2A}	5-HT_{2C}	5-HT_3
						Ki (nM)			
Vehicle (H_2O)	-	-	0.03±0.02[1]	-	-	-	-	-	-
Fluoxetine	30 mg/kg	30 mg/kg	0.13±0.03*	11±1	20±5*	>10,000	1041±60	252±29	5076±247
Fluvoxamine	0.1-10 mg/kg	1 mg/kg	0.25±0.02*	3.6±0.2	-3±3	>10,000	>10,000	>10,000	>10,000
Paroxetine	3, 10 mg/kg	10 mg/kg	0.30±0.02*	0.48±0.01	31±2*	>10,000	>10,000	>10,000	985±49
Sertraline	10 mg/kg	10 mg/kg	0.12±0.01*	2.8±0.3	24±5*	>10,000	9776±890	3618±761	3721±853

Means ± s.e.mean; [1]example of control levels; *P<0.05 vs controls, one-way ANOVA and Dunnett's test; [2]data from Cheetham *et al.* 1993

392P 5-HT-MEDIATED EFFECTS OF NICOTINE ON GABA RELEASE IN GUINEA-PIG CORTICAL BRAIN SLICES

L. Beani, S. Tanganelli, L. Ferraro, M. Morari & C. Bianchi Department of Pharmacology, University of Ferrara, 17/19 Via Fossato di Mortara, 44100 Ferrara, Italy.

The effect of nicotine on spontaneous outflow of endogenous GABA, measured with GCMS method, was studied in guinea pig cerebral cortex slices superfused with normal Krebs solution (Beani et al., 1986). The GABA efflux (255 ± 12 pmol g^{-1} min^{-1}) was steady over two-three hours and was reduced by about 50% in the presence of tetrodotoxin 0.5µ mol l^{-1} or in the absence of calcium. Clearly the basal outflow partly depended on neuronal activity and could be modulated as shown by its inhibition by α_2 and stimulation by α_1 receptor agonists (Ferraro et al., 1993). Nicotine displayed a concentration-dependent, biphasic action: it decreased GABA efflux to 89 ± 2% at 1.86 µmol l^{-1}, to 74 ± 3% at 6.2 µmol l^{-1} and to 66 ± 4% at 18.6 µmol l^{-1}. It was apparently ineffective at 62 µmol l^{-1}. On the other hand, at 186 µmol l^{-1} it incresed GABA efflux to 134 ± 7%, with respect pretreatment values. The inhibition was long lasting and was prevented by mecamylamine 2.5 µmol l^{-1}, while facilitation was short lived and was prevented by tetrodotoxin 0.5 µmol l^{-1}, mecamylamine 2.5 µmol l^{-1} and d-tubocurarine 4.5 µmol l^{-1}. Since, as a rule, the activation of nicotinic receptors causes excitatory responses, the involvement of other transmitters in this complex action on GABA outflow was envisaged. Thus experiments were performed pretreating the slices with α_1, α_2 and 5-HT antagonists. The results obtained support the prevailing role of 5-HT. In fact: i, MDL 72222 1 µmol l^{-1} completely prevented the inhibition caused by nicotine; ii, methysergide 1 µmol l^{-1} reversed the facilitation into inhibition (from 134 ± 7% to 67 ± 3%) and iii, methysergide plus MDL 72222 completely antagonized the effect of high nicotine concentrations. To validate these findings, the effect of exogenous 5-HT on GABA basal efflux was tested. Like nicotine, the amine showed a concentration-dependent, biphasic action: it decreased GABA efflux to 80 ± 3% at 3 µmol l^{-1}, to 69 ± 3% at 10 µmol l^{-1} and was nearly ineffective at 30 µmol l^{-1}. Instead, at 100 µmol l^{-1}, 5-HT increased the GABA outflow to 148 ± 11%. MDL 72222 1 µmol l^{-1} prevented 5-HT inhibition and methysergide 1 µmol l^{-1} reversed the facilitation of GABA efflux into inhibition . It may be concluded that nicotine releases 5-HT by activating high and low affinity receptors located on cortical serotonergic terminals. The amine, in turn, affects the firing rate of GABA interneurones through inhibitory 5-HT_3 and stimulatory 5-HT receptors, depending on its extraneuronal concentrations.

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393P 5-HT RELEASED FROM ISOLATED TRACHEA DERIVES FROM MAST CELLS IN RATS, BUT FROM NEUROEPITHELIAL CELLS IN RABBITS

A. Freitag, I. Wessler* & K. Racké, * Department of Pharmacology, University Mainz; Department of Pharmacology, University-Hospital, J.W. Goethe-University, Theodor-Stern-Kai 7, D-60590 Frankfurt, F.R.G.

Neuroepithelial cells (NECs) are distributed throughout the airwaiy mucosa of vertebrates. Ultrastructurally, NECs have numerous secretory granules, indicating a secretory function. They contain several bioactive peptides and are rich in 5-HT (see Scheuerman *et al.*, 1989). Currently, very little is known about mechanisms affecting secretion from NECs. The present experiments should test, whether 5-HT release from isolated rabbit tracheae reflects secretion from NECs, and look for possible regulatory mechanisms. Since in rats 5-HT is enriched in mast cells (Erspamer, 1966), some comparative experiments were performed on rat tracheae.

Isolated tracheae of adult or newborn rabbits or of adult rats were incubated for up to 12 consecutive 10 min periods in modified Krebs-HEPES medium. The overflow of 5-HT into the incubation medium and 5-HT in tissue extracts was determined by HPLC with electrochemical detection. In some experiments the mucosa was removed mechanically prior to the start of incubation. Values given are means±s.e.m. of at least 4, mostly more than 6, experiments.

Tracheal 5-HT content was 1036±245 and 1189±227 pmol/g in adult and newborn rabbits, respectively, but 18±2.3 μmol/g in adult rats. *Tracheae of adult rats*: spontaneous outflow of 5-HT was 60±13 pmol/g/10 min and addition of 10 μM compound 48/80 evoked the release of 1280±328 pmol/g 5-HT, whereas high potassium (45 mM) evoked a much smaller 5-HT release (18±5 pmol/g). *Tracheae of adult rabbits*: spontaneous outflow of 5-HT was 6.4±0.7 pmol/g/ 10 min. High potassium and A 23187 (10 μM) evoked the release of 24±6 and 17±4 pmol/g, respectively, whereas compound 48/80 did not evoked a significant 5-HT release. *Tracheae of newborn rabbits*: spontaneous outflow of 5-HT was 23±3 pmol/g/10 min. High potassium and A 23187 (10 μM) evoked the release of 47±5 and 147±31 pmol/g, respectively. *DL*-5-Hydroxytryptophan (5-HTP; 2, 20 μM) and 86 μM *L*-5-HTP enhanced 5-HT outflow 2-, 35- and 55fold, respectively. When 20 μM *DL*-5-HPT was present only during the first 20 min of incubation, the increase in 5-HT outflow was transient, returning to almost basal levels within 60 min. However, when a high potassium stimulus was applied 60 min after a 5-HTP pulse, the evoked release of 5-HT was enhanced to 705±105 pmol/g. Under these conditions, high potassium evoked 5-HT release was abolished in calcium-free medium containing 0.5 mM EDTA, and reduced to 38±22 pmol/g in mucosa-denuded preparations. Neither, nicotine (up to 100 μM), oxotremorine (10 μM) nor substance P (1 μM) significantly affected the spontaneous outflow of 5-HT from 5-HTP-conditioned tracheae.

In conclusion, NECs and mast cells are the source of 5-HT released from rabbit and rat tracheae, respectively. Since NECs are of particular importance in newborns, studying 5-HT release from tracheae of newborn rabbits may be a useful model to illuminate secretory control mechanisms in NECs. 5-HT release from NECs appears to be triggered by an intracellular rise in calcium, and calcium influx through voltage-regulated channels may be one activating pathway.

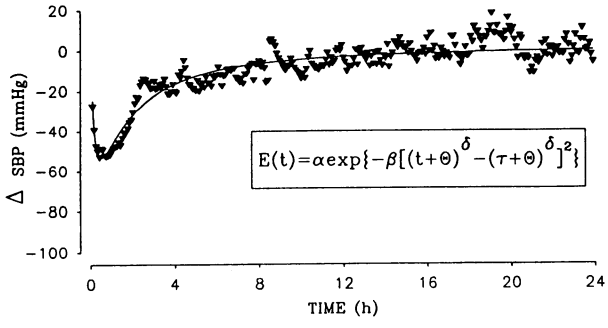
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394P A MATHEMATICAL MODEL FOR THE ANALYSIS OF HAEMODYNAMIC PROFILES OBTAINED BY THE TELEMETRY SYSTEM IN THE RAT

E. Bonizzoni, A. Forlani, C. Casati & A. Monopoli, Research Labs, Schering-Plough S.p.A., 20060 Comazzo, Milan, Italy

The newly developed radio-telemetry system offers a number of advantages for the measurement of blood pressure and heart rate in laboratory animals (Brockway et al., 1991). However, there are no statistical methods available which permit a valuable utilization of the many data gathered using this continuous recording of hemodynamic parameters. With this study we propose a family of mechanistic nonlinear functions suitable for the measurement of the effects of drugs on blood pressure and heart rate in spontaneously hypertensive rats.



The mathematical function (Fig. 1) contains the constants α = peak value, τ = time to peak, ß = parameter related to amplitude, Θ = parameter related to asymmetry and δ = Box-Cox transformation. The response E(t) corresponds to the hemodynamic value recorded at time t after drug administration, minus the baseline value. Least squares estimates of the constants are obtained by nonlinear curve fitting (Draper and Smith, 1981). This procedure allows a definition of a grid of initial estimates for the constants among which the best combination is selected as starting point for the iterative computation. These models not only apply to drugs showing a single peak of activity (Fig. 1), but may be also generalized to the case of drugs with double-peak effects. For further details see Bonizzoni et al., 1995.

Fig 1. Example of fitting of systolic blood pressure (SBP) 24 h profiles obtained by telemetry in conscious rats administered with the A2a adenosine agonist, 2-hexynyl-5'-N-ethylcarboxamidoadenosine (2-Hexynyl-NECA) (0.03 mg/kg i.p.). There is a maximum effect (α) of -53.2 ±5 mmHg (baseline=181 ±2 mmHg) at 0.49 ±0.05 h (τ) after drug administration. Continuous line represents the mean constant curve of 7 spontaneously hypertensive rats and dots are the mean of residuals obtained as difference between observed and predicted values of each individual hemodynamic profile.

Using this mathematical model, estimates of peak effect and tmax are based on the whole response profile (Ludbrook, 1994). Other parameters such as persistence of the effects (e.g. t½ or t¼) or amount of the effects (e.g. Area Under Curve) are also easily evaluated. With this approach the large amount of information provided by the telemetry system is adequately examined.

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395P SYNERGY BETWEEN TRANSFECTED ADENOSINE A_1-RECEPTORS AND CONSTITUTIVE P_2-PURINOCEPTORS IN CHO-K1 CELLS

Megson, A.C., Townsend-Nicholson, A.* & Hill, S.J. Department of Physiology & Pharmacology, Queen's Medical Centre, Nottingham NG7 2UH and *Garvan Insitute of Medical Research, St Vincent's Hospital, Sidney, Australia.

We have previously provided evidence for the coupling of both transfected human adenosine A_1-receptors and endogenous nucleotide receptors to mobilisation of intracellular calcium in CHO-K1 cells (Iredale & Hill, 1993; Iredale *et al.*, 1994). In the present study we have investigated the effect of these two receptor systems on inositol phospholipid hydrolysis.

CHO-K1 cells transfected with either the human adenosine A_1-receptor (CHO-A1) or the bovine histamine H_1-receptor (CHO-H1) were grown as described previously (Townsend-Nicholson & Shine, 1992; Iredale *et al.*, 1994; Iredale *et al.*, 1993). Monolayer cell cultures were loaded for 24 hr with 3H-myo-inositol (37 KBq/well) in 24 well cluster dishes in inositol-free DMEM containing 2 mM glutamine. Cells were then washed and incubated in Hanks/HEPES buffer (290 µl/well), pH 7.4. Agonists were added in 10 µl medium and the incubation continued for 40 min. Incubations were terminated with ice-cold methanol/0.12 HCl (1:1 v/v) and total ^{3}H-inositol phosphates (3H-IP) isolated as described previously (White *et al.*, 1993).

Data expressed as mean ± s.e. mean.

N^6-cyclopentyladenosine (CPA) (10µM) produced a 2.8 ± 0.2 fold increase in ^{3}H-IP in CHO-A1 cells (EC_{50} 14.0 ± 0.8 nM; n = 5) which was completely sensitive to inhibition by pertussis toxin (100 ng/ml; 24h). The response to CPA was antagonised by the A_1-selective antagonist 1,3-dipropylcyclopentylxanthine (K_D 1.2 ± 0.3 nM; n = 3). ATP (1mM) also stimulated ^{3}H-IP accumulation in these cells (2.4 ± 0.2 fold; EC_{50} 2.1 ± 0.1 µM; n = 3), but this effect was only partially attenuated in pertussis-toxin treated cells (19 ± 7% inhibition; n = 3). A combination of CPA (100 nM) and ATP (100µM) produced a greater than additive accumulation of ^{3}H-IP (447 ± 13 % of the response to 100µM ATP compared to the expected additive response of 206 ± 11 %; n=3). A similar synergy was observed with 10nM CPA and 0.1 mM ATP (324 ± 6 % of the ATP response compared to an additive effect of 148 ± 5%). In contrast, the ^{3}H-IP response to a combination of histamine (100 nM; pertussis toxin-insensitive) and ATP (100µM) in CHO-H1 cells (400 ± 8% of the response to ATP alone) was very similar to the predicted additive value (396 ±8 %; n = 4). A similar additivity was observed between the responses to 10 nM histamine and 100µM ATP (n = 4).

These data indicate that both transfected adenosine A_1-receptors and constitutive P_2-purinoceptors can stimulate inositol phospholipid hydrolysis in CHO-K1 cells and provide evidence for a synergistic interaction between the two receptor systems in these cells.

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396P STIMULATION OF NITRIC OXIDE SYNTHASE BY ENDOTHELIAL ATP RECEPTORS DOES NOT CORRELATE WITH INOSITOL TRISPHOSPHATE LEVELS

C.A.Brown, G.F.Wilkinson and M.R.Boarder, Department of Cell Physiology and Pharmacology, University Road, Leicester LE1 9HN.

Bovine aortic endothelial cells contain two co-existing receptors for extracellular ATP, the P_{2Y}-purinoceptors and P_{2U}-purinoceptor. Previous work in our laboratory has shown that the P_{2Y}-purinoceptor mediated relaxation of collateral artery rings was sensitive to the cyclooxygenase inhibitor indomethacin, whereas the relaxation caused by nucleotide stimulation was unaffected (Wilkinson *et al* 1994). The aim of this study was to determine whether the two receptors were differentially linked to nitric oxide synthase (NOS).

Endothelial cells were prepared from fresh bovine aortae and were used between passage 1-10 on 24 well plates. Cells were labelled for 10 min with [^{3}H]arginine (3uCi/ml) in balanced salt solution prior to stimulation with agonists, after which [^{3}H]arginine and [^{3}H]citrulline in the cell extracts were separated on Dowex 50 columns. The stimulation of [^{3}H]citrulline accumulation was taken as an index of NOS activity. For inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) formation cells were stimulated for 5s, and $InsP_3$ levels present in cell extracts measured with a protein binding assay. When protein kinase C was stimulated with tetradecanoyl phorbol acetate (TPA) or inhibited with Ro 31 8220 the drugs were present 10 min before, and during, the stimulation with agonist. UTP was used as a selective P_{2U} agonist, and 2MeSATP as a selective P_{2Y} agonist in these cells.

Addition of either 30uM 2MeSATP or 300uM UTP to [^{3}H]arginine labelled cells produced a rapid increase in accumulation of intracellular [^{3}H]citrulline which was sustained in a linear fashion over a period of several minutes.Concentration response studies showed that 2MeSATP was more potent than UTP (pA_{50} 5.96 ± 0.22 (n=3), and 4.89 ± 0.22 (n=3) respectively), and that the maximal response observed with UTP was 99.9 ± 5 % of the maximal response to 2MeSATP. The NOS inhibitor N^G - nitro-L- arginine methylester (L-NAME) inhibited [^{3}H]citrulline accumulation induced by 30µM 2MeSATP or 300uM UTP in a dose dependent manner (pA_{50} 5.93 ± 0.06 and 6.55 ± 0.16 respectively). Accumulation of [^{3}H]citrulline induced by 30µM 2MeSATP or 300µM UTP was not modulated in the presence of the phorbol ester TPA (100nM), (97 ± 20%, and 88 ± 14% of those in the absence of TPA respectively). However, in the presence of the PKC inhibitor Ro 31-8220 (10µM) NOS stimulation by both agonists was significantly attenuated (53.5 ± 4.7%, and 50.8 ± 3.9% of the responses in the absence of Ro 31-8220 for 2MeSATP and UTP respectively). In contrast to effects on NOS, TPA (100nM) caused a 60% reduction, and Ro 31 8220 (10 µM) caused a 100% increase, in the 2MeSATP (30µM) stimulation of $Ins(1,4,5)P_3$ levels.

These data show that, contrary to the expectations generated by vasorelaxation studies, both P_{2Y}- and P_{2U}-purinoceptors contribute in a similar manner to the regulation of synthesis of NO in cultured endothelial cells. Furthermore, in the presence of stimulators and inhibitors of protein kinase C, there is a dissociation between the agonist stimulation of phospholipase C and stimulation of NOS.

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397P CYCLIC AMP GENERATION MEDIATED BY AN ENDOGENOUS ADENOSINE RECEPTOR IN HUMAN EMBRYONIC KIDNEY (HEK 293) CELLS

Jacqui Cooper, [1]Stephen J.Hill & [1]Stephen P.H. Alexander, Division of Therapeutics, Department of Medicine, and [1]Department of Physiology & Pharmacology, University of Nottingham Medical School, Nottingham, NG7 2UH, UNITED KINGDOM.

The present classification of adenosine receptors (Fredholm *et al.*, 1994) allows their division into subtypes which are coupled to inhibition (A_1 and A_3) or stimulation of adenylyl cyclase activity (A_{2a} and A_{2b}). We have recently characterized the A_2 adenosine receptor mediating a stimulation of cAMP accumulation in rat astrocytes as being of the A_{2b} subtype on the basis of agonist potency and antagonist affinity (Peakman & Hill 1994). In the present report, we provide evidence that the endogenous adenosine receptor which couples to stimulation of cAMP accumulation in intact human embryonic kidney (HEK 293) cells is also of the A_{2b} subtype.

[^{3}H]-cAMP accumulation was monitored in [^{3}H]-adenine-pre-labelled HEK 293 cells (Peakman & Hill, 1994) at 37°C for 15 minutes in the presence of the phosphodiesterase inhibitor rolipram (100 μM). Antagonist affinities were calculated using increasing antagonist concentrations in the presence of a fixed concentration of 5'-N-ethylcarboxamidoadenosine (NECA, 10 μM, Peakman & Hill, 1994). Data (means ± SEM) presented were derived from experiments conducted on at least three separate cell preparations and were initially expressed as [^{3}H]-cAMP production as a percentage conversion from total [^{3}H]-adenine nucleotides.

Basal accumulation of [^{3}H]-cAMP was 0.36 ± 0.06 % conversion. The maximal response to NECA generated a stimulation of [^{3}H]-cAMP to 4.08 ± 0.30 % conversion. Adenosine analogues evoked a concentration-dependent stimulation of [^{3}H]-cAMP accumulation with the rank order of potency: NECA (7 ± 1 μM) > 2-chloroadenosine (45 ± 12 μM) ≥ adenosine (69 ± 13 μM). CGS 21680 failed to generate a significant response at concentrations up to 10 μM. Adenosine antagonists antagonised NECA-evoked cAMP generation with the rank order of affinity: xanthine amine congener (19 ± 5 nM) > 8-cyclopentyl-1,3-dipropylxanthine (112 ± 25 nM) = PD 115,199 (121 ± 17 nM).

These data are very similar to our recent observations on A_{2b} adenosine receptor-stimulated cAMP generation in intact rat astrocytes. Our results, therefore implicate the presence of endogenous A_{2b} adenosine receptors on HEK 293 cells. Since these cells are commonly used as recipients for transfected receptors, the presence of an endogenous receptor positively-coupled to cAMP generation will allow comparisons to be made with other positively-coupled receptors, or inhibitions evoked by negatively-coupled receptors to be revealed.

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398P INVESTIGATION INTO THE INTERACTION BETWEEN ADENOSINE RECEPTORS AND ADRENOCEPTORS IN GUINEA-PIG AORTIC SMOOTH MUSCLE

H.L.Maddock & K.J. Broadley, Department of Pharmacology, Welsh School of Pharmacy, University of Wales, Cardiff, CF1 3XF

The vasodilator responses of 5'-N-ethylcarboxamidoadenosine (NECA), thought to be mediated by A_{2b} adenosine receptors (Martin 1992), were compared in noradrenaline-(NA), phenylephrine-(PE), adrenaline-(ADR) and methoxamine-(ME) precontracted guinea-pig thoracic aorta.

From each guinea-pig, four 5mm rings of endothelium-denuded thoracic aorta, were mounted under a resting tension of 1g, in Krebs bicarbonate solution at 37°C gassed with O_2:CO_2 (95:5). Cumulative concentration-response curves (CCRC's) were constructed for NA, PE, ADR and ME to determine a submaximal concentration suitable for preconstriction. Each agonist was either in the presence or absence of idazoxan 10^{-6}M, prazosin 10^{-6}M or propanolol 10^{-6}M. Aortic rings were precontracted with NA, PE, ADR or ME and when the tension had plateaued, CCRC's were constructed to NECA. Antagonists were added 20 min before agonist addition. Statistical significance ($P < 0.05$) was tested using Student's t-test of paired data.

All the adrenergic agonists caused concentration related increases in the aortic tension. The concentrations of NA, PE, ADR and ME selected for precontracting the aortic rings were 10^{-5}, $3x10^{-6}$, $3x10^{-6}$ and 10^{-5}M respectively. NECA relaxed the aortic rings in a concentration-dependent manner, the maximum relaxation being dependent on the contractile agonist used. The maxima for NECA in PE, ADR and ME precontracted aortic rings (61.8±9.5%, 44.4±4.7%, 59.8±5.4 (n=4), respectively) were significantly greater ($P<0.05$) than with NA (10.9±3.8%, n=4). There was no significant difference ($P<0.05$, n=4) between the contractions to NA, PE, ADR and ME (0.78±0.22, 0.60±0.16, 0.76±0.28, 0.93±0.26g, respectively).

Further experiments were conducted to determine whether these differences in magnitude of relaxation by NECA were due to variable involvement of β-, α_1- and α_2-adrenoceptors. Propanolol had no significant effect ($P<0.05$, n=4) on either the maximal response (control 24.6±3.9%, test 18.3±4.4%) or the EC_{50} value (control 1.3(1.1-1.4)$x10^{-6}$M, test 7.8(5.9-10.2)$x10^{-7}$M) of NECA in NA precontracted aortic rings. Prazosin displaced the CCRC's for NA, PE, and ADR to the right. The concentrations used for precontracting the aortic rings in its presence were therefore raised to 10^{-3}, 10^{-4} and $3x10^{-4}$M, respectively. Idazoxan failed to displace the CCRC's for NA, PE, and ADR and concentrations used for precontracting in its presence were $3x10^{-5}$, $3x10^{-6}$, $3x10^{-6}$M, respectively. The maximal inhibition by NECA of NA contractions in the presence of idazoxan (12.9±7.1%) or prazosin (16.2±4.9%) did not alter significantly ($P<0.05$, n=4) compared to the control (10.7±2.7). The presence of idazoxan (50.1±6.0%) or prazosin (82.5±10.1%) had no significant ($P<0.05$, n=4) effect on the maximal inhibitory response of NECA in PE precontracted aortic rings compared with the control (62.9±9.6%). The maximum inhibition in the presence of idazoxan (39.6±4.9%) or prazosin (38.4±11.5%) when contracted with ADR was not significantly different ($P<0.05$, n=4) compared with the control (44.3±4.6%). The NECA inhibitory response therefore appears most effective when the aorta is contracted by PE rather than NA. The differences in magnitude of relaxation by NECA when precontacted with NA, PE, ADR or ME cannot be explained by differential stimulation of α_1, α_2 or β receptor classes by these agonists.

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399P HYPERTENSION INDUCED BY 1,3-DIPROPYL-8-SULFO-PHENYLXANTHINE (DPSPX): EFFECTS ON SYMPATHETIC NEUROTRANSMISSION IN THE RAT TAIL ARTERY

Karoon, P., Rubino, A. & Burnstock, G. Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E, 6BT.

Chronic administration of the adenosine antagonist 1,3-dipropyl-8-sulfophenylxanthine (DPSPX) induces increase of the systemic blood pressure together with hyperplasia and hypertrophy of vascular smooth muscle (Matias *et al.*, 1991). The structural changes of blood vessels in DPSPX-treated animals resembles morphological changes observed in models of genetic hypertension where enhanced sympathetic nerve activity also occurs (Head, 1989). Here we examine whether sympathetic neurotransmission is altered in DPSPX-induced hypertension.

Male Wistar rats (250-300 g) were infused with DPSPX (30 μg/Kg/h) or saline for 7 days using Alzet osmotic minipumps and were killed by an overdose of sodium pentobarbitone. Two proximal tail arteries were dissected, one used for electrochemical detection of noradrenaline (NA) using high-performance liquid chromatography and the other cut to 3-4 mm ring segments for organ bath pharmacology (Brizzolara *et al.*, 1992). Statistical analysis was performed by using umpaired Student's *t* test.

Constant infusion of DPSPX caused significant elevation in systolic blood pressure of treated rats compared with saline control rats (164.0±2.9, n=5 versus 140.0±2.8, n=7 mm Hg). Neurogenic contraction evoked by transmural nerve stimulation (TNS; 65 V, 0.1 msec, 4-64 Hz, for 1 sec) of arterial rings was sensitive to antagonism by prazosin and desensitisation by α,β-methylene ATP, thus indicating a dual noradrenegic and purinergic component of perivascular sympathetic neurotransmission. Vascular responses to TNS were significantly greater in DPSPX- compared to saline-treated rats. At 64 Hz contractile responses were 2.41±0.15 (n=5) and 1.18 ±0.22 (n=5) in DPSPX- and saline-treated rings, respectively. Long-term DPSPX treatment had no effect on the concentration-response curves to both neurotransmitters NA (0.1-300 μM) and ATP (0.01-3 mM) applied exogenously. PD_2 values for NA and ATP were 5.14±0.18 (n=5) and 3.24±0.05 (n=5) in saline-treated and 5.40±0.38 (n=5) and 3.18±0.03 (n=4) in DPSPX-treated rats, respectively. The NA content of the tail artery was significantly increased from 3.44±0.30 (n=5) in saline-treated rats to 4.50±0.26 ($P<0.05$; n=4) ng/cm tissue in DPSPX-treated animals.

In conclusion, DPSPX-induced hypertension, enhanced perivascular sympathetic neurotransmission in the rat tail artery, via a prejunctional mechanism. These changes, accompanied by the morphological alterations reported by others (Matias *et al.*, 1991), closely resemble those described for genetic hypertension (Head, 1989). These results offer indirect evidence that adenosine may play a role in hypertension by changing the perivascular sympathetic neurotransmission.

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400P SUPPRESSION OF HEPATOCYTE GROWTH FACTOR-INDUCED ANGIOGENESIS BY SURAMIN

D. E. Hu & T.-P. D. Fan, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

Hepatocyte growth factor /scatter factor (HGF/SF) is a heparin-binding glycoprotein which stimulates vascular endothelial cell migration, proliferation, and organization into capillary-like tubes *in vitro* and induces angiogenesis *in vivo* (Grant *et al.*, 1993). Suramin is an effective analogue of heparin/heparan sulphate which inhibits the activities of several heparin-binding growth factors such as transforming growth factor-beta, fibroblast growth factors (FGFs) and platelet-derived growth factor (Myers *et al.*, 1990). Here we use a rat sponge model to examine the angiogenic activity of HGF/SF and its blockade by suramin.

Sterile polyether sponges with attached cannulae were implanted subcutaneously in male Wistar rats (180-200 g) and neovascularisation was assessed by using ^{133}Xe clearance to measure blood flow through the implants every two days over a period of 14 days. Test substances (50 μl) were injected to the sponges through the cannulae daily. To exclude acute vascular effects of these peptides, they were applied 24 h before ^{133}Xe measurement.

Daily doses of 150 ng of recombinant human hepatocyte growth factor (rhHGF) caused an intense neovascularisation. The 6 min ^{133}Xe clearance value (all are mean ± s.e.m.) of the control group on Day 6, 8, 10 were 16.1±1.8, 20.9±1.5, 25.9±1.9 % (n = 6) while rhHGF increased it to 23.0±1.2, 30.3±0.8, 34.3±1.2 %, respectively (n = 6, $P < 0.05$). Morphometric analysis of 8-day-old sponges showed that rhHGF increased the total fibrovascular growth area from 20.4 ± 6.4 % to 48.6 ± 6.8 % (n = 12, $P < 0.05$). The rhHGF-induced neovascular response was inhibited by co-injection of 3 mg suramin or a polyclonal anti-rhHGF antibody (rhHGFAB; 10 μg). The ^{133}Xe clearance values on Day 6, 8, 10 for the co-administration groups were 16.0±0.5, 21.8±1.6, 26.3±1.3 % (n = 6, $P < 0.01$, vs rhHGF group) and 17.2±1.2, 21.7±0.6, 24.7±0.7 % (n = 6, $P < 0.05$, vs rhHGF group), respectively. Histological and morphometric studies of sponge sections showed that both the cellular infiltration and neovascularisation in the rhHGF-treated sponges were profoundly suppressed by suramin and rhHGFAB. The total fibrovascular growth area decreased from 48.6 ± 6.8 % to 23.4 ± 6.7 % and 25.1 ± 5.9 %, respectively (n = 12, $P < 0.05$). Lower doses (15 ng day^{-1}) of rhHGF produced no apparent effect on sponge-induced neovascularisation.

We have previously shown that suramin inhibits the angiogenic activities of heparin-binding growth factors such as basic FGF and vascular endothelial growth factor, but not non-heparin binding growth factors like platelet-derived endothelial cell growth factor (Hu & Fan, 1992 & 1994). Taken together, our data suggest that suramin is an effective antagonist of the *in vivo* action of heparin-binding angiogenic factors such as HGF. Further studies are necessary to delineate the exact role of HGF in vascular development and to explore the therapeutic potential of its antagonists and suramin analogues.

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401P THE EFFECTS OF SURAMIN ON ELECTRICAL RESPONSES IN THE RAT ISOLATED TAIL ARTERY

G.J. McLaren, C. Kennedy & P. Sneddon, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW.

Suramin has been shown to antagonise P_{2X}-purinoceptors in a number of smooth muscle preparations and to inhibit excitatory junction potentials (e.j.ps) in the guinea-pig vas deferens (Sneddon, 1992), which are mediated by ATP acting at P_{2X}-purinoceptors. We have now investigated the effects of suramin on electrical responses to sympathetic nerve stimulation in the rat isolated tail artery, where it has been proposed that ATP acts as a cotransmitter with noradrenaline.

The ventral tail artery was pinned out in a 2 ml organ bath and perfused with Krebs' solution at 35°C, bubbled with 95% O_2, 5% CO_2. Intracellular microelectrodes (30-50 MΩ) were used to record electrical responses to field stimulation of the sympathetic nerves, evoked by trains of 10 pulses at 0.5, 1 and 2 Hz (pulse width 0.3 ms, submaximal voltage). In all experiments suramin and phentolamine were allowed to equilibrate for 45 min. All data presented below refer to the mean ± s.e.mean and are compared using Student's t-test for unpaired data.

Stimulation of the sympathetic nerves produced an electrical response with two distinct phases. Each stimulus evoked a rapid transient e.j.p. and, as a train of pulses progressed, a slow depolarisation developed. The e.j.ps showed neither facilitation nor depression during the train of 10 pulses. The amplitude of the slow depolarisation increased with frequency of stimulation.

The mean resting membrane potential of smooth muscle cells in the rat tail artery was -68.3±1.1 mV (n=29). Neither suramin (100 μM) nor phentolamine (2 μM) had any effect on the resting membrane potential of cells. The magnitude of e.j.ps in control cells was 10.5±0.8 mV (n=29). Suramin (100 μM) produced a gradual decline in e.j.p. magnitude and e.j.ps were completely abolished after 30-40 min. In the presence of suramin the amplitude of the slow depolarisation was significantly increased from control values of 1.5±0.2 mV (n=11), 2.0±0.2 mV (n=24) and 2.5±0.3 mV (n=11) to 5.1±0.9 mV (n=11), 4.2±0.2 mV (n=24) and 3.7±0.4 mV (n=11) at 0.5, 1 and 2 Hz respectively. These actions of suramin did not reverse even after 2 h washout. Phentolamine (2 μM) abolished the slow depolarisation at all stimulation frequencies. The magnitude of e.j.ps was unchanged in the presence of phentolamine, 10.8±1.1 mV (n=13), compared to control, 10.5±0.8 mV (n=29). In the presence of both suramin and phentolamine the electrical response was abolished.

The ability of suramin to inhibit e.j.ps in rat tail artery is consistent with the proposal that it is a P_{2X}-purinoceptor antagonist and supports a role for ATP as an excitatory cotransmitter in the sympathetic nerves innervating this tissue. Suramin is also able to increase the α-adrenoceptor-mediated slow depolarisation by a mechanism which is as yet undetermined.

Sneddon (1992) *Br. J. Pharmacol.*, 107, 101-103.

402P ACTIONS OF ANTAGONISTS AGAINST INWARD CURRENTS INDUCED BY ATP IN RAT NODOSE NEURONES

B.S. Khakh[1], P.P.A. Humphrey[1] & A. Surprenant[2]. [1]Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge UK and [2]Glaxo Institute for Molecular Biology, Geneve, Suisse.

It has been shown that ATP activates inward currents mediated by P_{2X} purinoceptors in single nodose neurones (Khakh *et al.*, 1994). We have now attempted to study the mechanism by which suramin, PPADS and pyridoxal-5'-phosphate (P5P) act as antagonists.

The methods used for dissociation of rat nodose neurones, whole cell patch clamp recording and drug application have already been described (Khakh *et al.*, 1994). One concentration-effect (c-e) curve was determined to ATP in each cell, either in the absence or presence of a single concentration of antagonist, which was present at least 8 min prior to the determination of a c-e curve. Responses were expressed as a % of the initial response to 100μ M ATP. All data are mean±s.e.m values obtained from between 3-5 cells.

ATP (0.1-100μM) activated inward currents in a concentration-dependent manner with an EC_{50} of 6.8±2.8μM, Hill slope of 0.89±0.03 and a maximal response at 100μM of 89±6% of the initial response to 100μM ATP (2351.9 ±172.3pA n=41). Suramin produced a complex profile of antagonism, such that responses to 100μM ATP in the presence of 10, 30 and 100μM suramin were 45.3±10.6, 33.9±5.9 and 76.9±23.4%. PPADS and P5P produced a relatively concentration-dependent antagonism such that responses to 100μ M ATP were 40.9±6.8, 23.2±6.4 & 4.3±1.9% and 29.7±3.0, 35.3±6.0 & 15.9±5.8% for PPADS (1,3 &10μM) and P5P (10, 30 & 100μM), respectively. Due to the complex nature of the c-e curves no attempt was made to analyse data in terms of affinity estimates. Determination of current voltage relations using ramp protocols from -130 to + 60mV, in the absence and presence of fixed concentrations of antagonist (suramin 30μM; PPADS 3μM; P5P 30μM), during the application of 100μM ATP showed that all three compounds produced a voltage independent antagonism

Suramin (30μM) caused an approximately 10 fold increase in rise time for the ATP (100μM) activated inward current (from 31.7±8.5msec to 354.5± 101.5msec). In contrast PPADS and P5P produced not much more than a two fold increase in rise times (from 75.7±24.1 and 30.6±9.4msec to 177.6± 68.5 and 37.5±6.9msec, for 3μM PPADS and 30μM P5P respectively). In these experiments antagonists all produced a similar reduction of response to ATP (suramin 30μM 60.3±4.6%; PPADS 3μM 76.8±6.3%; P5P 30μM 64.8±6.8% reduction). Time-dependent control cells produced no change in rise time over the course of experimentation (from 34.8±3.7msec initially to 29.2±6.5msec at the end of the experiment). Antagonist equilibration studies showed that suramin could produce a maximal effect within 2 minutes of contact with cells, reducing the response to a near EC_{50} concentration of ATP (3μM) from 82.7±4.5% just prior to antagonist addition to 15.9±0.9% within 2 minutes (expressed as % initial response to 3μM ATP). In contrast PPADS (3μM) and P5P (30μM) required 8min of contact time to produce a maximal effect reducing responses from 93.3±2.0 and 85.6±4.4% just prior to addition of antagonist to 11.2±2.7 and 6.6± 1.0%. Control cells whereby no antagonist was added showed that successive applications of 3μM ATP every 2mins produced little desensitisation. Selectivity of antagonists was determined against a near maximal concentration of 5HT (30μM) and changed responses from 96.1± 10.1% in control cells to 122.2±11.4, 105.4±14.1 and 116.0±17.2 % in 100 μM suramin, 10μM PPADS and 100μM P5P, respectively.

We conclude that suramin, PPADS and P5P all act as antagonists of the ATP induced inward currents by blocking the P_{2X} purinoceptor and not the intrinsic cation channel. However, the kinetic profile of antagonism seen with suramin differs from that using the other two antagonists and the mechanisms involved are under further investigation.

KHAKH, B.S., HUMPHREY, P.P.A. & SURPRENANT A. (1994). *J.Physiol.*, in press.

403P ADRENERGIC MODULATION OF SODIUM FLUORESCIN TRANSPORT ACROSS BOVINE BRAIN MICROVESSEL ENDOTHELIAL CELLS

N. Borges, I. Azevedo, F. Shi* & K.L. Audus*, Institute of Pharmacology and Therapeutics, Medical Faculty, 4200 Porto, Portugal and *Department of Pharmaceutical Chemistry, The University of Kansas, School of Pharmacy, Lawrence, Kansas 66045-2504, USA

The ability of adrenergic drugs to modulate blood-brain barrier transport has been previously described using *in vivo* models (Sarmento *et al.* 1991; 1994). Therefore, it seemed of interest to use an *in vitro* model to better characterize the role of both α- and β-adrenoceptors in modulating the transport across this particular capillary endothelium. Transport of sodium fluorescein (SF) across primary, polarized cultures of bovine brain microvessel endothelial cells was studied using side-by-side chambers. SF was added to the apical side and its concentration in the opposite chamber was assayed at 2, 10, 20, 30, 40, 55 and 70 min. Drugs were added after 30 min to the chamber facing the basal side of the cells. Percent differences between the slope obtained with the SF concentrations before and after the addition of the drug were determined and are presented in Table 1.

Drugs having mainly an α-adrenoceptor agonist effect (lower concentrations of noradrenaline (NA) and adrenaline (AD) and phenylephrine (PHE)) increased SF transport whereas the β_2-adrenoceptor agonist clenbuterol (CLE) and the highest AD concentration slightly reduced the monolayer permeability. Blockade of α-adrenoceptors by prazosin (PRAZ) converted the 0.1 μM NA-induced increase in transport to a decrease.

Table 1. Percent changes in sodium fluorescein transport induced by adrenergic drugs (Student's t test for paired observations).

Drugs	Dose (μM)	variation in transport (%)	n	P
NA	0.1	+47±14	4	<0.05
NA	1	+81±13	7	<0.001
NA	10	-7±5	5	not sig.
AD	0.1	+34±9	5	<0.025
AD	1	-14±1	5	<0.001
CLE	0.1	-23±4	4	<0.01
PHE	0.1	+95±28	5	<0.05
NA+PRAZ	both 0.1	-23±4	4	<0.02

The inhibition of pinocytosis by vincristine or by glucose withdrawal abolished the 1μM NA and CLE mediated effects (data not shown). We conclude that α-adrenoceptor stimulation increases and β-adrenoceptor stimulation slightly decreases the monolayer permeability to sodium fluorescein. Both effects most probably involve alterations in transcytotic transport across the endothelial cells.

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404P REDUCED β-ADRENOCEPTOR-MEDIATED BEHAVIOUR WITHOUT A REDUCTION OF CORTICAL β-ADRENOCEPTOR BINDING

P.R. Gard, J.P. Fawcett[1] & M.R. Nunn[1] Department of Pharmacy, University of Brighton, Moulsecoomb, Brighton BN2 4GJ and [1]School of Pharmacy, University of Otago, PO Box 913, Dunedin, New Zealand.

Chronic administration of antidepressants causes down-regulation of cortical β-adrenoceptors (β-ADRs) in the rat. Binding studies indicate that the receptors affected are of the β_1-subtype, with no effect on β_2-ADRs, and that repeated treatment for approximately 8 days is required (Beer *et al.*, 1987). Behavioural studies, however, show that there is a decreased β_2-ADR response after administration of antidepressants and that this occurs within 21h of a single dose (Gard *et al.*, 1991). This study investigated brain β-ADR binding in rats exhibiting behavioural evidence of rapid central β_2-ADR down-regulation.

Male Lewis rats received either desipramine (10mg kg^{-1}), salbutamol (2mg kg^{-1}), clenbuterol (2mg kg^{-1}) or saline (1ml kg^{-1}) i.p. either as a one-off dose or twice daily for 4 days. Salbutamol-induced drinking was determined 21h after the final dose by the method described by Gard *et al.* (1991). At the conclusion of the behavioural test (3 h.), cerebral cortices were rapidly removed and stored at -30°C. For determination of total β-ADR binding, cortical membranes were incubated for 20 min at 37°C with [^{125}I]-iodopindolol (30-800pM), GTP (200μM) and phentolamine (150μM); non-specific binding was determined in the presence of isoprenaline (200μM). β_2-ADR binding was determined as above but with the addition of CGP 20712A (100μM).

The results of the binding studies (Table 1) indicate that, with the exception of desipramine which caused a significant reduction in β_2-ADR number, the single treatments had no effect on β-ADRs. Repeated treatment with salbutamol or clenbuterol resulted in down-regulation of β_2-ADRs, although at this time point there was no significant effect of desipramine. The results of salbutamol-induced drinking have been reported previously: all of the treatments caused a significant reduction of this β_2-ADR-mediated behaviour (Gard *et al.*, 1991).

These results support and extend the findings of Beer *et al.*, (1987) in that repeated treatment with β_2-ADR agonists caused down regulation of cortical β_2-ADRs. However they also demonstrate that the previously reported effects of these treatments on salbutamol-induced drinking are not indicative of, nor correlated with, a change in cortical β_2-ADR status.

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Gard, P.R., Fawcett, J.P., Kerr, K.P. (1991) *Eur. J. Pharmacol.* 197, 217-219

Table 1. The effects of repeated and single treatment with desipramine, salbutamol and clenbuterol on β-adrenoceptor binding in the rat cortex (mean ± s.e.mean; *:P<0.05; **:P<0.01); ANOVA followed by Student's t-test.

	Single treatment						Repeated treatment					
	Total β-adrenoceptors			β_2-adrenoceptors			Total β-adrenoceptors			β_2-adrenoceptors		
	n	Kd (pM)	Bmax (fmol/mg)	n	Kd (pM)	Bmax (fmol/mg)	n	Kd (pM)	Bmax (fmol/mg)	n	Kd (pM)	Bmax (fmol/mg)
Saline	5	332±39	31.2±1.9	8	202±24	14.3±1.6	9	305±19	36.6±2.0	6	191±20	14.3±1.2
Desipramine	7	314±22	36.3±1.3	7	166±14	12.0±0.6*	8	260±15	34.3±2.9	6	210±21	15.4±1.1
Salbutamol	8	275±24	36.8±2.8	5	247±35	16.0±1.2	9	283±18	36.5±3.7	7	239±30	11.2±0.8*
Clenbuterol	3	305±40	34.4±1.4	6	259±41	13.2±1.5	7	409±28**	34.8±4.2	5	308±28**	10.4±0.8*

405P RAPID DOWN-REGULATION OF B-ADRENOCEPTORS BY DESIPRAMINE BUT NOT OTHER ANTIDEPRESSANTS: 5-HT_{2A} RECEPTORS ARE UNAFFECTED

Newman-Tancredi A., Verrièle L. and Millan M.J. (Introduced by M. Spedding), Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy-sur-Seine (Paris, France).

Chronic treatment with a variety of antidepressants (ADPs) down-regulates [^{3}H]dihydroalprenolol-labelled β adrenergic (β-AR) and [^{3}H]ketanserin-labelled serotonin (5-HT)$_{2A}$ receptors with a time-course similar to that of their clinical activity (Racagni & Brunello, 1984). However, Riva & Creese (1989), using the more β-AR-selective radioligand [^{3}H]CGP-12177, showed that desipramine (DMI) down-regulated β-AR even on *acute* treatment. To test whether acute down-regulation of [^{3}H]CGP-12177 (0.015 to 2.0 nM) binding is a general feature of ADPs, we studied the effect of desipramine (15 mg/kg^{-1}, single dose or 14 once-daily doses), and of single doses of amitryptiline (10 mg/kg^{-1}), clorgyline (1 mg/kg^{-1}), and fluoxetine (10 mg/kg^{-1}) on β-ARs in frontal cortex. Data were compared with those for 5-HT_{2A} receptors (labelled with [^{3}H]ketanserin, 0.25 to 8 nM) in frontal cortex. Male Wistar rats (250 g), injected subcutaneously with drug or vehicle (water, 4 ml/kg), were killed 24 hours later. Saturation binding isotherms (8 points) produced linear Scatchard plots. Non-specific binding was defined by 10 μM alprenolol (β-AR) or 10 μM spiperone (5-HT_{2A}). Results are expressed as a percentage of control values (mean ± s.e.m, n). Control Bmax (fmol mg^{-1} tissue) and Kd (nM) were 6.25 ± 0.27 and 0.13 ± 0.01 (β-AR, n=6) and 29.2 ± 1.3 and 1.08 ± 0.07 (5-HT_{2A}, n=6). Drug treatment did not significantly affect the Kds of the radioligands ($p > 0.05$, results not shown). DMI significantly reduced the number of binding sites for [^{3}H]CGP-12177 both on chronic (-29 %) and acute (-15 %) treatment. The other ADPs tested did not have a significant effect following acute administration. None of the drugs produced 5-HT_{2A} down-regulation acutely, but a 20% decrease in 5-HT_{2A} sites was seen following chronic DMI treatment. These results confirm the ability of DMI to down-regulate [^{3}H]CGP-12177-labelled cortical β-ARs acutely but indicate that this change may *not* be a general property of ADPs. Further, acute administration of DMI and others ADPs does not appear to affect 5-HT_{2A} receptors in the cortex suggesting that 5-HT_{2A} down-regulation may be a function of chronic treatment.

Racagni G. and Brunello N. (1984), *TIPS* 5, 527-531.
Riva M.A. and Creese I. (1989), *Mol. Pharm.* 36, 211-218.

Table 1: Influence of ADPs upon β-adrenoceptor and 5-HT_{2A} receptor density.

		DMI (14 days)	DMI (1 day)	Amitryptiline (1 day)	Clorgyline (1 day)	Fluoxetine (1 day)
β-AR	Bmax	71.4 ± 2.3 *** (5)	84.7 ± 2.2 ** (12)	97.5 ± 2.7 (6)	101.8 ± 3.0 (6)	102.0 ± 3.6 (6)
5-HT_{2A}	Bmax	79.9 ± 3.7 * (5)	105.6 ± 3.7 (12)	102.3 ± 4.6 (6)	101.4 ± 9.7 (6)	118.5 ± 8.2 (6)

Data expressed as a percentage of control values (= 100 %). Statistics based on absolute values (fmol mg^{-1} tissue). **p<0.01 in Dunnett's test; *p<0.05, ***p<0.001 in Student's two-tailed t-test.

406P EQUIPOTENT MODULATION OF NORADRENALINE REUPTAKE IN THE RAT CEREBRAL CORTEX BY DESMETHYLIMIPRAMINE (DMI) AND THE SELECTIVE α_2-ADRENOCEPTOR AGONIST MEDETOMIDINE

J.W. Dalley & S.C. Stanford, Department of Pharmacology, University College London, London WC1E 6BT.

The α_2-adrenoceptor agonist, medetomidine (MED), induces biphasic changes in levels of noradrenaline (NA) in dialysates of the rat frontal cortex (Dalley & Stanford, 1994). The inhibitory effect of 0.5 μM MED on NA efflux appears to be mediated *via* activation of α_2-autoreceptors but the neural mechanism underlying its stimulatory effect at 5-50 μM is unknown. The aims of the present study were to assess (1) whether MED augments extraneuronal NA by inhibiting NA uptake (like DMI) and (2) whether the effects of the α_2-adrenoceptor agonists clonidine and UK 14,304 resemble those of MED.

Dialysis probes were implanted vertically in the frontal cortex of halothane-anaesthetised male SD rats (250-300 g) and perfused with Ringers solution (NaCl 150 mM, KCl 4 mM, $CaCl_2$ 1.2 mM, pH 6.1) at 1.0 μl min^{-1}. Drugs were added to the perfusate in increasing concentrations (0.5, 5.0 and 50 μM) for 1 h following three 20 min basal samples collected 2.5 h after surgery. Dialysate NA was analysed using HPLC-ECD. Aliquots of crude cortical synaptosomes were incubated in Tris-Krebs buffer (pH 7.4) containing (mM): NaCl 136, KCl 5, $MgCl_2$ 12, $CaCl_2$ 2.5, *d*-glucose 10, *l*-ascorbate 1, Tris base 20, pargyline 0.25. The assay was initiated by addition of [^{3}H]-NA (50 nM) and terminated after 3 min by filtration. Duplicate samples were incubated at 37°C and 4°C, in both the presence and absence of test drugs, to estimate specific [^{3}H]-NA uptake. The results were analysed statistically by one and two-way ANOVA.

Spontaneous NA efflux in cortical dialysates was approximately 20 fmol 20 μl^{-1} and remained constant over 5 h ($F=0.89$; df 14,56; $P=0.578$). Clonidine and UK 14,304, (5 μM), both reduced NA efflux by 40-50 % compared with basal levels ($F=362.6$; df 1,3; $P<0.001$ and $F=27.19$; df 1,3; $P<0.02$ respectively) with no further reduction at 50 μM. MED and DMI caused a concentration-dependent increase in NA efflux (50 μM *cf* basal: $F=29.68$; df 1,4; $P=0.006$ and $F=13.57$; df 1,4; $P=0.021$ respectively). Specific synaptosomal uptake of [^{3}H]-NA was 1.02 ± 0.10 pmoles mg^{-1} protein ($n=9$); this was significantly inhibited by MED and DMI with estimated IC_{50} values of 7 μM ($F=11.15$; df 3,35; $P<0.001$) and 8 μM ($F=6.52$; df 3,42; $P<0.001$), respectively. Neither clonidine ($F=0.11$; df 2,11; $P=0.896$) nor UK 14,304 ($F=0.04$; df 2,11; $P=0.954$) affected [^{3}H]-NA uptake.

These results indicate that low micromolar concentrations of the α_2-adrenoceptor agonist MED inhibit NA uptake in the cerebral cortex with a potency similar to DMI. Since this effect was not shared by either clonidine or UK 14,304 it is concluded that α_2-adrenoceptors are not directly involved in this response.

We are indebted to Orion-Farmos, Pfizer and Boehringer Ingelheim for their generous gift of test drugs.

Dalley, J.W. & Stanford, S.C. (1994) *Br. J. Pharmacol.* 112, 154P.

407P COMBINED EFFECTS OF STORED Ca^{2+} AND EXTRACELLULAR Ca^{2+} IN STIMULATING RELEASE OF [^{3}H]-NORADRENALINE FROM SH-SY5Y CELLS

J.R. Purkiss and G.B. Willars (introduced by M.R. Boarder), Department of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, Medical Sciences Building, Leicester, LE1 9HN.

We have recently reported that bradykinin and muscarinic receptor-mediated stimulation of [^{3}H]-noradrenaline ([^{3}H]NA) release from SH-SY5Y human neuroblastoma cells is partially dependent on mobilisation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) sensitive Ca^{2+} stores, (Purkiss et al., 1994). Here we describe data suggesting a greater than additive effect of Ca^{2+} mobilisation and Ca^{2+} influx on [^{3}H]NA release.

SH-SY5Y cells, passage 80-90, on 24 well plates were loaded with [^{3}H]NA and release measured according to the method of Murphy et al. (1992). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined in adherent cells loaded with fura-2-AM, (Grynkiewicz et al., 1985). Statistical analysis was by Student's t-test, where appropriate, preceeded by ANOVA to determine the standard error of the difference between groups.

Emptying of $Ins(1,4,5)P_3$ sensitive Ca^{2+} stores by treatment with thapsigargin (2μM) in low Ca^{2+} (100nM) caused $[Ca^{2+}]_i$ to transiently rise (rise of 48 ± 0.3nM, n = 3). Re-instating Ca^{2+} (1.3mM) to the extracellular medium caused $[Ca^{2+}]_i$ to rapidly rise again and fall to a plateau due to capacitative entry (150.6 ± 10.7 and 72.0 ± 4.3nM above basal, peak and plateau respectively). Thapsigargin treatment followed by addition of Ca^{2+} did not stimulate [^{3}H]NA release (basal, 4.58 ± 0.2%, 1.3mM Ca^{2+}, 4.27 ± 0.15%, n = 3, release expressed as a percentage of total [^{3}H]NA content). Stimulation of muscarinic receptors with the specific agonist methacholine (1mM) and both nicotinic and muscarinic receptors with the non-specific agonist carbachol (1mM) resulted in biphasic $[Ca^{2+}]_i$ rises (carbachol: 669.0 ± 85.7 and 122.3 ± 11.1, methacholine: 904.3 ± 152.6 and 144.3 ± 2.2nM, peak and plateau respectively, n = 3). Both agonists stimulated release of [^{3}H]NA (carbachol, 8.50 ±0.1%, methacholine, 4.48 ± 0.1%, $p<0.05$,n = 3). Stimulation of the nicotinic receptors alone (30μM nicotine) however, only resulted in a [^{3}H]NA release of 1.3 ± 0.38% (n =3). The product of addition of the methacholine and nicotine-stimulated releases was found to be significantly smaller than the carbachol-stimulated release ($p<0.05$). The Ca^{2+} ionophore, ionomycin (5μM) stimulated release of [^{3}H]NA (13.65 ± 0.73%, n = 3) but this was significantly reduced in cells pre-treated with thapsigargin (8.29 ± 0.86%, $p< 0.05$, n = 3). When cells were stimulated with ionomycin in low Ca^{2+}, to prevent influx, release was reduced to 1.89 ± 0.44% (n = 3).The product of the addition of releases from thapsigargin pre-treated cells and from cells in low Ca^{2+} was significantly smaller than the ionomycin-induced release from cells with intact Ca^{2+} stores in 1.3mM Ca^{2+} ($p<0.05$). Stimulation in 100nM Ca^{2+} following thapsigargin pre-treatment virtually abolished ionomycin stimulated release (0.06 ± 0.01%, n = 3).

These data show that ionomycin stimulated release of [^{3}H]NA is facilitated by efflux of Ca^{2+} from intracellular stores as well as influx from the extracellular medium. Influx through capacitative refilling alone is insufficient to stimulate release but influx mediated by ionophore or via nicotinic acetylcholine receptor channels combines with Ca^{2+} mobilised from intracellular stores to promote [^{3}H]NA release in a greater than additive manner.

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408P 2-(2-BENZO-FURANYL)-2-IMIDAZOLINE-INDUCED ALTERATIONS IN LOCAL CEREBRAL GLUCOSE UTILISATION

N. French, D.J. Nutt & J.A. Pratt[1], Psychopharmacology Unit, University of Bristol, Bristol BS8 1TD and [1]Department of Physiology & Pharmacology, University of Strathclyde, Glasgow G1 1XW.

In a previous [^{14}C]2-deoxyglucose study, the α_2-adrenoceptor antagonist, idazoxan, was shown to decrease local cerebral glucose use (LCGU) in a number of rat brain regions (French *et al.*, 1992). Although idazoxan binds with equally high affinity to imidazoline$_2$ (I_2) sites, its effects on LCGU are probably mediated through α_2-adrenoceptors as they are similar to those evoked by RX811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline), an α_2-adrenoceptor antagonist which does not bind to I_2 sites (French *et al.*, 1993). In order to explore this further, the present study examined the effect of 2-(2-benzofuranyl)-2-imidazoline (2-BFI) on LCGU. 2-BFI is approximately 1500 times more selective for I_2 sites than α_2-adrenoceptors in rat brain (Hudson *et al.*, 1994).

Male Long Evans rats (347 ± 11 g) were given an i.v. injection of vehicle (0.9% saline) or 2-BFI (1, 3 or 10 mg kg^{-1}) and 5 min later, an i.v. bolus of [^{14}C]2-deoxyglucose. Autoradiograms from 20μm brain sections were analysed using a densitometer and LCGU was calculated according to Sokoloff *et al.*, (1977). Data were analysed using one-way ANOVA followed by Newman-Keuls multiple range test. The statistical method of Ford *et al.* (1985) was used to compare the effect of 2-BFI with that of idazoxan and RX811059.

2-BFI altered LCGU in 10 out of 77 structures (Table 1). Correlation analysis revealed that the pattern of changes evoked by 2-BFI was not similar to that produced by either idazoxan ($r = 0.16$) or RX811059 ($r = -0.11$). In contrast, there was a significant correlation between the effect of idazoxan and RX811059 ($r = 0.37$; $P<0.01$).

These data suggest that 2-BFI produces a pattern of change in LCGU distinct from that of idazoxan, which may underlie differences in the psychopharmacological profiles of the drugs. The idazoxan-induced alterations in LCGU appear to be mediated through α_2-adrenoceptors.

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Table 1. Control LCGU and changes in LCGU (μmol/100g/min ± s.e.mean) induced by 2-BFI in various regions of rat brain (n = 5/group). *P<0.05.

Structure	Control	2-BFI (mg kg^{-1} i.v.)		
		1	3	10
Inferior colliculus	126±8	126±11	159±6*	131±6
Superior olive	93±4	99±4	111±6*	90±3
CA2 moleculare	82±3	76±3	77±4	68±1*
Subiculum	76±3	75±3	77±4	96±3*
Fornix	60±2	61±4	62±2	80±4*
Somatosensory cortex	103±3	96±4	93±2*	89±2*
Motor cortex	101±3	95±4	94±3	89±1*
Mediodorsal thalamus	72±2	64±2	69±3	81±3*
Ventromedial thalamus	88±2	92±4	92±3	103±2*
Arcuate nucleus	46±2	59±2*	55±1*	-

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409P EVIDENCE THAT OPIOIDS ARE NOT INVOLVED IN THE DOWN-REGULATION OF CENTRAL β_1- AND α_2-ADRENOCEPTORS BY SIMULATED ELECTROCONVULSIVE THERAPY

J. Ogilvie, S.C. Cheetham, J. Gosden, S.A. Butler, & D.J. Heal, Boots Pharmaceuticals Research Department, Nottingham NG2 3AA

Simulated electroconvulsive therapy (ECS) down-regulates central β_1- and presynaptic and postsynaptic α_2-adrenoceptors (Heal *et al.*, 1989, 1991). Jackson & Nutt (1990) suggested that some ECS effects are mediated via changes in opioids. In view of this, we have now investigated whether endogenous opioids are involved in the ECS-induced down-regulation of β_1- and α_2-adrenoceptors in rat brain.

Male CD rats (70-120g) were injected ip with naloxone (3mg/kg) or saline (2ml/kg). These groups were subdivided and 15 min later given either ECS (200V, 2s; once or 5 times over 10 days) under halothane anaesthesia, or halothane alone. All experiments were performed 24h after the final treatment. β_1-Adrenoceptors were quantitated in frontal cortex using [^{3}H]CGP 12177 ((-)-4-)3-t-butylamino-2-hydroxypropoxy)-benzimidazol-2-one) at 8 concentrations (0.02-1.2nM). Non-specific binding was defined by 500nM CGP 20712A (1-2-[(3-carbamyl-4-hydroxy)phenoxy]ethylamino-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol). Clonidine (0.2mg/kg ip) hypoactivity and clonidine (0.03mg/kg ip) mydriasis were used to assess pre- and postsynaptic α_2-adrenoceptor function, respectively, as described by Heal *et al.* (1981, 1994).

Although acute ECS had no effect (data not shown), repeated ECS down-regulated central β_1-adrenoceptors and α_2-adrenoceptor function (Table 1). Acute or repeated naloxone pretreatment was not only without effect itself, but it also failed to influence the action of ECS on these adrenoceptors (Table 1). Thus, if changes in β_1- and α_2-adrenoceptors underpin the therapeutic effect of electroconvulsive therapy, these data indicate that such efficacy is independent of central opioids.

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Table 1. Effect of naloxone pretreatment on the ECS-induced down-regulation of central β_1-adrenoceptors and α_2-adrenoceptor function

	β_1-Adrenoceptor (Bmax)	Hypoactivity score	Mydriasis (mm)
Saline/halothane	94.5 ± 1.6	23.6 ± 1.9	0.26 ± 0.02
Naloxone/halothane	95.6 ± 1.7	23.8 ± 1.7	0.24 ± 0.04
Saline/ECS	85.9 ± 1.6**	15.6 ± 1.4**	0.09 ± 0.02**
Naloxone/ECS	84.5 ± 2.4**	18.4 ± 1.1*	0.11 ± 0.02**

Values mean ± s.e. mean (n = 9-10). Bmax = fmol/mg protein. *P<0.05, **P<0.01 versus appropriate halothane group (one-way analysis of variance followed by Newman-Keuls multiple comparison procedure. No differences between saline/ECS and naloxone/ECS groups.

410P INFLUENCE OF α_2-ADRENOCEPTOR DENSITY ON THE INHIBITORY EFFECT OF LITHIUM ON cAMP LEVELS IN THE RAT CEREBRAL CORTEX

M.L. Cuffí, L. Carbonell, F. Mármol & J. Forn (A.C. Dolphin). Department of Pharmacology, School of Medicine, University of Barcelona. Spain.

It has been reported that low lithium concentration ($0.3x10^{-3}$M) has a significant inhibitory effect on cAMP content induced by isoprenaline in brain tissue pretreated with the α-adrenoceptor blocker phenoxybenzamine (Mármol *et al.*, 1992).
The aim of the present study is to investigate if α_2-adrenoceptors recovery, after previous administration with the irreversible antagonist EEDQ, modifies the inhibitory effect of lithium on the cAMP levels induced by stimulation of ß-adrenoceptors with isoprenaline in rat cerebral cortex. The density of α_2-adrenoceptors was measured by binding experiments on membranes from cortical tissue, using [^{3}H]-yohimbine (0.5-12 nM) as a ligand; non-specific binding was evaluated in the presence of phentolamine ($1x10^{-5}$M). The cAMP content was determined by an isotopic displacement method using [^{3}H]cAMP and a binding protein in cortex slices. The density of α_2-adrenoceptors measured with [^{3}H]-yohimbine after treatment with EEDQ (4 mg/kg, ip) was reduced more than 90% at 6 h (Bmax for [^{3}H]-yohimbine 7.21 ± 0.5% of control). By 14 days the α_2-adrenoceptors density had recovered to 68% of control. The binding affinity of [^{3}H]-yohimbine for the α_2-adrenoceptors was unaltered during recovery from EEDQ-induced receptor inactivation. The process of α_2-adrenoceptor repopulation yielded estimates for *r* (appearance constant) of 15.02 ± 1.36 fmol/mg prot/day, *k* (disappearance constant) of 0.074 ± 0.0067 day^{-1} and a $t_{1/2}$ of 9.51 ± 0.80 days. The inhibitory effect of lithium ($0.3x10^{-3}$M) on the cAMP levels were significantly different from the control group (isoprenaline alone) only at 6 and 24 h after EEDQ treatment.
In summary, the administration *in vitro* of lithium in rat cerebral cortex has only an inhibitory effect on cAMP levels when the population of α_2-adrenoceptors is less than 25% of control. These results agree with previous experiments in brain slices in which the inhibitory effect of lithium on isoprenaline induced cyclic AMP levels was increased by blockade of α-adrenoceptors.

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Table 1. Recovery of α_2-adrenoceptors and cAMP levels at various time intervals after EEDQ treatment in rat cerebral cortex. cAMP and Bmax values are shown as means ± S.E.M. (n=3). Statistical comparisons with Student's t-test ($^aP<0.05$,$^bP<0.01$).

Time (days)	0.25	1	2	4	7	10	14
cAMP (pmol/mg prot)	86.7±2.7^a	89.5±2.3^b	102.8±3.3	104.1±4.2	110.1±1.4	117.8±4.0	102.8±3.2
Bmax (fmol/mg prot)	14.6±1.0	31.9±4.6	52.5±3.2	87.0±5.5	104.1±2.4	114.9±2.9	137.

411P AFFINITY AND SELECTIVITY OF 2-(-2-BENZO-FURANYL)-2-IMIDAZOLINE FOR MAMMALIAN BRAIN NON-ADRENOCEPTOR IDAZOXAN BINDING SITES (I_2-SITES)

A.L. Hudson, N.J. Mallard[1], D.J. Nutt & C.B. Chapleo[2], Psychopharmacology Unit, School of Medical Sciences, University of Bristol, BS8 1TD, [1]Cardiff Clinical Trials Ltd, University of Wales College of Medicine, Cardiff, CF4 4XN, [2]Reckitt & Colman Products, Dansom Lane, Hull, HU8 7DS.

The α_2-adrenoceptor antagonist idazoxan (IDX), also binds to non-adrenoceptors. These have been termed I_2-sites (Ernsberger, 1992). Although widely distributed in mammalian brain and periphery, the function of I_2-sites is unclear. It has been postulated that I_2-sites are linked to monoamine oxidase as they are downregulated *in vivo* by the MAO inhibitor clorgyline (Olmos *et al.*, 1993). In addition, microdialysis studies show a semi-selective I_2-site ligand, 2-(1,3-benzodioxanyl)-2-imidazoline (RX821029) decreases monoamine turnover in rat striatum (Lalies & Nutt, 1993). In a search for a more selective I_2-site ligand we have examined another derivative of IDX, 2-(2-benzofuranyl)-2-imidazoline (2-BFI), for affinity at I_2-sites versus α_2-adrenoceptors in rat, rabbit and guinea pig whole brain membranes.

Brain membranes were prepared from male Wistar rats (300g), New-Zealand rabbits (3.5-5.0kg, both sexes) and male guinea pigs (300-500g). [^{3}H]RX821002 (1-2nM) and [^{3}H]IDX (2-3nM, in the presence of 5μM rauwolscine) were used to label α_2-adrenoceptors and I_2-sites respectively. Membranes with [^{3}H]-ligand and displacing drug were incubated (50mM Tris-HCl buffer, pH 7.4) in triplicate to equilibrium (30min, 22°C). Bound and free radioactivity were separated by filtration. Specific binding of [^{3}H]RX821002 and [^{3}H]IDX was defined in the presence of 10μM rauwolscine and 10μM 6-fluoro-idazoxan respectively. The inhibition constants (K_i) are shown in Table 1 (mean±s.e.mean; n = 4-6 experiments).

2-BFI displayed high affinity for I_2-sites in rat, rabbit and guinea pig brain membranes, and was comparable or of higher affinity than idazoxan (Hudson *et al.*, 1992) or its isomer RX821029 (Table 1). Hill coefficients for 2-BFI were close to 1. In contrast, 2-BFI demonstrated lower affinity for α_2-adrenoceptors than RX821029, giving 2-BFI a selectivity ranging from 840 up to 2,800 in favour of I_2-sites. These results show that 2-BFI is more selective for I_2-sites than RX821029 and may therefore be of greater use for *in vivo* studies. Furthermore, radiolabelled 2-BFI should prove useful for binding studies and evaluating its selectivity with respect to other receptors (Lione *et al.*, this meeting).

Table 1	Ki (nM)		
RAT	I2-sites	α2-receptors	$\alpha 2/I_2$-site
RX821029	1.8 ± 0.3	244.1 ± 18.0	136
2-BFI	1.2 ± 0.2	1860.0 ± 244.0	1550
RABBIT			
RX821029	2.7 ± 0.5	436.2 ± 26.0	161
2-BFI	1.3 ± 0.4	3736.0 ± 147.0	2874
GUINEA PIG			
RX821029	3.7 ± 0.5	171.5 ± 6.9	46
2-BFI	1.9 ± 0.3	1600.0 ± 45.3	842

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412P [^{3}H]2-(-2-BENZO-FURANYL)-2-IMIDAZOLINE: A NEW RADIOLIGAND FOR THE STUDY OF RABBIT BRAIN NON-ADRENOCEPTOR IDAZOXAN BINDING SITES (I_2-SITES)

L.A. Lione, D.J. Nutt, J. Lewis, J. Hunter[1], P. Towers[2], & A.L. Hudson, Psychopharmacology Unit, School of Medical Sciences, University of Bristol, BS8 1TD, [1]SmithKline Beecham Pharmaceuticals, Harlow , Essex, CM19 5AD, [2]Amersham International plc, R & D Ligands Group, Cardiff, CF4 7YT.

For several years [^{3}H]Idazoxan (IDX) has been used as a radioligand for the study of α_2-adrenoceptors. [^{3}H]IDX also binds with high affinity to imidazoline$_2$-sites (I_2-sites; Ernsberger, 1992), although the function of these sites remains unclear. Recently, 2-(-2-benzofuranyl)-2-imidazoline (2-BFI) has been identified as a selective ligand for I_2-sites (Hudson *et al.*, 1994). We now report the binding characteristics of [^{3}H]2-BFI to rabbit whole brain membranes.

Whole brain membranes were prepared from New-Zealand rabbits (3.5-5.0kg, both sexes) and incubated in triplicate (0.25-0.45mg protein, 50mM Tris-HCl buffer, 1 mM Mg^{2+}, pH 7.4, 22°C) with [^{3}H]-2-BFI or [^{3}H]IDX (in the presence of 5μM rauwolscine to preclude α_2-adrenoceptor binding). Bound ligand was separated by filtration, with the specific component of binding to I_2-sites determined with 10μM 2-(4,5-dihydroimidaz-2-yl)-quinoline (BU224) or 6-fluoro-IDX. Data were analysed using Prism software (GraphPad Software, 1994).

Saturation binding studies revealed that both ligands bound with high affinity, [^{3}H]2-BFI bound with higher affinity (K_D= 0.27 ± 0.08nM) than [^{3}H]IDX (K_D= 1.75 ± 0.35nM) but to a similar number of sites (B_{max} values, 111.2 ± 17.4 and 82.4 ± 12.4 fmol mg^{-1} protein respectively). Both [^{3}H]2-BFI and [^{3}H]IDX also bound to an apparent second population of sites with lower affinity (8.9 and 33.8 nM respectively). Subsequent competition assays were performed using 1nM [^{3}H]2-BFI, a concentration predicted to label mainly the high affinity component of binding. Specific binding accounted for greater than 90% of total bound. Several imidazolines known to have high affinity for I_2-sites displaced [^{3}H]2-BFI in a concentration-dependent manner, notably IDX, 6-fluoro-IDX, 2-BFI, BU224, and cirazoline (Table 1). In contrast, other drugs known to have low affinity for I_2-sites, were weak at displacing [^{3}H]2-BFI; in particular the α_2-adrenoceptor agonist clonidine, the α_2-adrenoceptor antagonists efaroxan and rauwolscine and the I_1 site-selective drug moxonidine (Table 1; mean inhibition constants(Ki)±s.e.mean, 1-site fit, n = 3 or 4). A range of compounds having affinity for other receptors and ion channels were also of low affinity for the site labelled by [^{3}H]2-BFI.

Table 1	Ki (nM)		Ki (nM)
2-BFI	0.77 ± 0.15	Clonidine	5,590 ± 550
Idazoxan	4.40 ± 0.63	Amiloride	66.8 ± 16.3
Fluoro-IDX	5.07 ± 0.39	Efaroxan	36,000 ± 460
BU224	0.57 ± 0.11	Rauwolscine	>100μM
Cirazoline	2.36 ± 0.56	Moxonidine	>100μM

These data indicate [^{3}H]2-BFI is a useful new radioligand for the study of I_2-sites in rabbit whole brain membranes.

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Hudson, A.L. *et al.*, (1994) This meeting.

413P THE EFFECT OF A SELECTIVE I_2-SITE LIGAND, 2-(-2-BENZOFURANYL)-2-IMIDAZOLINE, ON *IN VIVO* NORADRENALINE RELEASE IN RAT BRAIN

M.D. Lalies and D.J. Nutt, Psychopharmacology Unit, School of Medical Sciences, University of Bristol, BS8 1TD.

Non adrenoceptor idazoxan binding sites (now termed imidazoline$_2$ (I_2) sites, see Ernsberger, 1992), which are widely distributed in mammalian brain and periphery, are believed to have a functional interaction with monoamine oxidase (MAO). It has been demonstrated that I_2 sites in rat cortex are downregulated by chronic treatment with the MAO inhibitor clorgyline (Olmos *et al.*, 1993) and more recently, a number of I_2 site ligands have been shown to reduce MAO activity in rat adipose and liver tissue (Carpéné *et al.*, 1994). Using the technique of *in vivo* microdialysis we have examined the modulation of noradrenaline (NA) release in rat frontal cortex (FC) and hippocampus (HIPP) by a highly selective I_2 site ligand, 2-(2-benzofuranyl)-2-imidazoline (2-BFI), which has a 1500 fold selectivity for I_2 sites over α_2-adrenoceptors in rat brain membranes (Hudson *et al.*, 1994).

Under chloral hydrate anaesthesia concentric dialysis probes, perfused with artificial CSF at 3 µl min^{-1}, were implanted into the FC (AP +3.2; L ±2.8; V (bregma) -3.7 mm) and HIPP (AP -5.3; L ±5.0; V (bregma) -6.5 mm) of each rat (male Wistar, 270-310g). Dialysates were collected every 15 min and assayed by HPLC-ECD for NA content. When a stable baseline was established rats were injected (i.p.) with either saline or 2-BFI (2.5, 5, 10 or 20 mg kg^{-1}), n=3 per group, and sampling continued for a further 120 min.

Whilst saline injection had no effect on NA levels in either brain region, 2-BFI caused transient increases in both, in a dose dependent manner. 2-Way ANOVA revealed significant effects ($p \leq 0.01$) in FC at doses 5 - 20 mg kg^{-1} whereas all 4 doses had a significant effect ($p \leq 0.01$) in HIPP. The peak effect of 2-BFI was at 30 min (see Table 1), after which NA levels gradually returned to basal. At 120 min (see Table 1) NA was back to pre-drug levels in all except the highest dose group.

In conclusion, 2-BFI, a highly selective I_2 site ligand, can increase extracellular NA levels in rat brain. This may reflect inhibition of MAO as has previously been found with 2-BFI in peripheral tissues (Carpéné *et al.*, 1994).

Table 1. Noradrenaline levels (pg per sample, mean±s.e.mean) in frontal cortex and hippocampus before (basal) and at two time points (30 & 120 min) after saline or 2-BFI (2.5 - 20 mg kg^{-1} i.p.) administration.

	FRONTAL CORTEX				HIPPOCAMPUS		
	Basal	30min	120min		Basal	30min	120min
Saline	2.5±0.1	2.4±0.1	2.3±0.2	Saline	1.9±0.3	1.7±0.2	1.6±0.2
2.5	1.9±0.3	1.8±0.4	1.8±0.3	2.5	2.3±0.4	2.6±0.4	1.6±0.2
5.0	2.9±0.1	4.2±0.3	2.7±0.2	5.0	1.9±0.1	4.1±0.7	2.0±0.2
10.0	2.4±0.2	5.1±1.1	2.6±0.3	10.0	1.8±0.1	4.8±0.1	2.0±0.1
20.0	2.4±0.2	10.8±1.3	3.9±0.6	20.0	2.3±0.2	9.0±0.6	2.8±0.5

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414P CNS α_2-ADRENOCEPTORS AND BLOOD PRESSURE IN THE RAT

J.P. Nunes, Instituto de Farmacologia e Terapêutica, Faculdade de Medicina, 4200 Porto, Portugal

The role of alpha$_2$-adrenoceptors in the central regulation of blood pressure has been questioned since drugs such as clonidine stimulate both alpha$_2$-adrenoceptors and imidazoline-preferring receptors. Alpha$_2$-adrenoceptors encoded by the RG20 and RG10 genes (Lanier *et al.*, 1991) have been shown to be present in the rat CNS (Zeng and Lynch, 1991). The present work was undertaken to study the influence of alpha$_{2D}$-adrenoceptors, encoded by the RG20 gene, on blood pressure in the rat.

Male wistar rats were used. Oligodeoxynucleotides (phosphodiester) were stereotactically injected into the right lateral cerebral ventricle, with a single injection per rat (a volume of 20 µl, corresponding to 4 nmol, was injected), under general anaesthesia induced by pentobarbital sodium (50 mg Kg^{-1}). The following products were injected in different series of rats: an antisense oligodeoxynucleotide directed at nucleotides 4-21 of the RG20 gene (RG20ASP, n=9; 5'ATCC GGCTGCAGGGAGCC 3'); a sense oligodeoxynucleotide corresponding to nucleotides 4-21 of the RG20 gene (RG20SP, n=6); a missense oligodeoxynucleotide similar to RG20ASP but with a mismatch in 3 nucleotides (RG20MSP, n=5; 5'ATCCAGCGGCTGGGAGCC 3'; mismatches underlined); an antisense oligodeoxynucleotide directed at nucleotides 65-82 of the RG10 gene (RG10ASP, n=6); distilled water (n=9). The blood pressure was measured by means of the tail-cuff method; five measurements of blood pressure were obtained, and the arithmetic means calculated. Blood pressure measurements were recorded for 4 days before the intracerebroventricular injections, and for 5 days after.

Pre-injection blood pressure values of the 5 groups of rats were not statistically different (127±2/ 73±4 mm Hg for rats injected with RG20ASP and 128±3/ 82±4 mm Hg for rats injected with distilled water). The injection of RG20ASP caused a significant ($P<0.05$) increase in systolic blood pressure, both at 1 and 2 days after the injection (147±3/ 79±4 and 147±4/ 76±4 mm Hg, respectively), when compared to the rats injected with distilled water (133±2/ 74±2 and 124±2/ 77±4 mm Hg, respectively). The rats injected with RG20SP, RG20MSP and RG10ASP had blood pressure values similar to the controls injected with distilled water. In days 3-5 after the injections, the different groups of rats showed similar values of blood pressure.

We conclude that alpha$_2$-adrenoceptors encoded by the RG20 gene may play a role in blood pressure regulation in the rat.

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415P MONOAMINERGIC SYSTEMS ARE INVOLVED IN THE ANTICONVULSANT ACTION OF SODIUM VALPROATE AGAINST PILOCARPINE-INDUCED SEIZURES

C.S. Biggs, B.R. Pearce and P.S. Whitton, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX

Seizures induced in rodents by the cholinomimetic pilocarpine are considered to be a model of intractable epilepsy, for which sodium valproate (VPA) is an effective treatment. Previous investigations have produced conflicting data regarding an involvement of central monoaminergic systems in the anticonvulsant action of VPA (Horton *et al.*, 1977; Lazarova *et al.*, 1983), although VPA is known to alter brain extracellular monoamines *in vivo* (Biggs *et al.*, 1992). Additional research has revealed that the balance between stimulation of 5-HT and DA receptor subtypes implicated in the central regulation of seizure thresholds may be of primary importance. It follows that the use of more specific pharmacological probes might uncover a more subtle interaction of VPA with these systems. We have attempted to investigate the relative contributions of central 5-HT and DA receptors to the anticonvulsant action of VPA, using the pilocarpine rodent seizure model in conjunction with D_1, D_2, 5-HT_{1A} and 5-HT_2 selective drugs.

Male Wistar rats (250-300g) were individually housed in test arenas and treated with scopolamine methyl bromide (1mg/kg) at t= -30 min. At this time, animals in pretreatment groups received the relevant dose of drug or saline. At t=0 min, all animals received a convulsant dose of pilocarpine nitrate (400 mg/kg) and half the animals, an anticonvulsant dose of VPA (300 mg/kg). During the subsequent 180 min period, rats were observed and assessed for seizure activity using a point score system (Janusz and Kleinrok, 1989).

All rats challenged with pilocarpine alone convulsed tonically with a mean seizure score of 2.8±0.1 (n=18). In contrast, rats co-treated with pilocarpine and VPA were partially protected from seizures (seizure score 1.4±0.2; n=8). Pretreatment with the 5-HT_{1A} antagonist NAN-190 (5mg/kg) resulted in a marked reduction in the efficacy of VPA (2.1±0.4; $p<0.05$, n=5), as did the D_2 antagonist raclopride (1 mg/kg. 2.2±0.2; $p<0.05$, n=5) Conversely, the potent D_1 antagonist SCH 23390 (0.25 mg/kg) greatly enhanced the action of VPA against pilocarpine seizures, animals only displaying pre-seizure facial myoclonus (nominal score 1.0, n=5). In striking contrast, the D_1 agonist SKF 38393 (15 mg/kg) completely reversed the anticonvulsant effect of VPA, to the extent that the mean seizure score recorded was greater than that for pilocarpine treatment alone (3.05±0.3; $p<0.05$, n=5). These data strongly support a role for central DA receptor tone in determining the efficacy of VPA in this seizure model, with evidence for a weaker involvement of 5-HT_{1A} receptors.

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416P RENAL NERVE STIMULATION-INDUCED NORADRENALINE AND ATP RELEASE IN NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS

L.C. Rump, I. von Kügelgen* & C. Bohmann, Department of Internal Medicine IV, University of Freiburg, Hugstetter Str. 55 and Institute of Pharmacology*, Hermann-Herder-Str. 5, D-79106 Freiburg, FRG

Noradrenaline (NA) and adenosine 5'-triphosphate (ATP) are cotransmitters in postganglionic sympathetic neurons of various tissues and species (Burnstock 1990) and there is also evidence for α-adrenoceptor blockade-resistant pressor responses in the rat isolated kidney which are due to the cotransmitter ATP (Rump et al. 1990). The aim of the present study was to investigate a possible role of ATP as a cotransmitter in SHR and WKY isolated kidneys (age: 12-14 weeks). This was done by determining pressor responses to renal nerve stimulation (RNS) as well as the RNS induced outflow of endogenous NA and ATP. Kidneys were isolated, perfused with Krebs-Henseleit solution and the renal nerves were stimulated electrically. The RNS induced outflow of NA and ATP was measured by high pressure liquid chromatography with electrochemical detection (Bohmann et al. 1995) and the luciferin-luciferase-technique (von Kügelgen & Starke 1991), respectively. RNS for 30 s induced a greater outflow of ATP at 4 Hz (SHR: 12.7 ± 3.3 pmol/g kidney, n=12; WKY: 16.7 ± 2.1 pmol/g kidney, n=13) than at 1 Hz (SHR: 1.6 ± 1.7 pmol/g kidney, n=12; WKY: 3.9 ± 2.9 pmol/g kidney, n=11). At 4 Hz the RNS induced outflow of NA and ATP as well as pressor responses to RNS were abolished by tetrodotoxin (1 μM). The α_1-adrenoceptor antagonist prazosin (0.03 μM) did not alter the RNS induced outflow of NA but markedly reduced pressor responses to RNS by 90% and 80% and inhibited the RNS induced outflow of ATP by 70% and 60% in SHR and WKY kidneys, respectively. The α_2-adrenoceptor antagonist rauwolscine (0.1 μM) given in addition to prazosin (0.03 μM) increased the RNS induced outflow of NA and ATP and markedly enhanced the prazosin-resistant pressor responses to RNS. These pressor responses were then abolished by the P_2-purinoceptor antagonist suramin (300 μM). The present results demonstrate that in SHR and WKY kidneys pressor responses to RNS are mainly noradrenergic, but to a small extent also purinergic in origin. RNS induces the release of NA and ATP. As observed in other isolated tissues, a major part of the released ATP is derived from non-neuronal cells due to activation of postjunctional α_1-adrenoceptors. Furthermore, the results indicate that the release of both cotransmitters is modulated via α_2-adrenoceptor mediated autoinhibition in SHR and WKY kidneys.

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417P STUDY OF THE CONTRACTILE RESPONSE ELICITED BY EXTRACELLULAR CALCIUM AFTER DEPLETION OF NORADRENALINE-SENSITIVE INTERNAL CALCIUM STORES IN RAT AORTA

M.P. D'Ocon, M.D. Ivorra, S. Chuliá & M.A. Noguera, Departament de Farmacologia, Facultat de Farmacia, C/ Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia , Spain.

We have recently shown that depletion of internal Ca^{2+} stores sensitive to noradrenaline (NA) elicits a contractile response dependent on extracellular Ca^{2+} in rat aorta. (Noguera & D'Ocon, 1993 a, b). We analyzed the mechanism involved in the genesis of this spontaneous contraction by provoking it in the presence of different agents.

After depletion of intracellular Ca^{2+}-pools by repetitive NA 1 μM addition in Ca^{2+}-free medium, subsequent loading in Ca^{2+}-containing solution (in the absence of NA) induces a considerable increase in the resting tone (IRT), which represents 60.1 ± 6.1% (n=7) of the contraction elicited by NA in Ca^{2+}-containing solution. Similar results were obtained when methoxamine (100 μM; n=4) was used as agonist but not when clonidine (1 μM; n=7) was used. This IRT was inhibited in a concentration-dependent manner by α-adrenoceptors antagonists (prazosin, phentolamine, yohimbine), by Ca^{2+}-entry blockers (nifedipine and verapamil) and by non-specific relaxing compounds (papaverine and caffeine) (Noguera & D'Ocon, 1993 a,b). Benoxathian (0.1-0.0001 μM), a selective α_{1A}-adrenoceptor antagonist inhibited the IRT in a concentration-dependent manner (IC_{50}=3.2 nM; n=3) and the highest concentration completely inhibit it. The α_{1B}-adrenoceptor antagonist cloroethylclonidine (CEC 100 μM) did not affect the IRT (control response = 51.5±5.5%, n=5; CEC = 51.8±2.3%, n=5). These results suggests that the α_{1A}-adrenoceptors mediate this response, which is also related to voltage-dependent Ca^{2+} channels. Cromakalim (1-0.0001 μM) inhibited the IRT (IC_{50}=0.74 nM, n=4) and its inhibition was antagonized by glibenclamide (10 μM, n=6). Therefore, K^+-channels seems also to be involved in this process. However ryanodine, a plant alkaloid that depletes intracellular Ca^{2+} stores, and thapsigargine, a putative selective Ca^{2+}-ATPase inhibitor, did not modify the IRT but altered the refilling of intracellular stores sensitive to NA, as does cloroethylclonidine (Table 1)

Table 1. Contractile responses to noradrenaline (1 μM) in Ca^{2+}-free medium after depletion of internal Ca^{2+} stores and a refilling period in the presence of Ca^{2+} and testing agents.

REFILLING PERIOD		NORADRENALINE (%)		
AGENT	(μM)	*phasic*	*tonic*	*n*
CONTROL		23.5±2.4	9.3±1.9	9
benoxathian	0.1	13.6±2.3*	2.7±0.4*	3
CEC	100	0	0	5
cromakalim	0.1	23.1±2.8	8.4±1.6	4
ryanodine	10	0	7.6±1.5	7
thapsigargine	1	10.1±0.9*	6.0±0.7*	4

Contractions are expressed as a percentage of the noradrenaline-induced contraction in Ca^{2+}-containing solution. All values represent mean±s.e.mean, n=number of experiments. *p<0.05

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418P AGONIST AND ANTAGONIST POTENCIES AT THE ADRENOCEPTOR MEDIATING SUPPRESSION OF I_{Ca} IN RAT SYMPATHETIC NEURONES

S.M. Duffy (Introduced by D.A. Brown), Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

Inhibition of the calcium current (I_{Ca}) by noradrenaline in rat sympathetic neurons has been suggested to be mediated via an α_2-receptor, based on observations that the action of noradrenaline is mimicked by the α_2-agonist clonidine, and antagonised by the α_2-antagonists yohimbine and idazoxan (Song *et al.*, 1989; Schofield, 1990). The purpose of this investigation was to obtain a further, quantitative, characterization of this adrenoceptor.

I_{Ca} was recorded from isolated s.c.g. neurones using the whole-cell patch-clamp technique, using CsCl filled electrodes. Noradrenaline reduced I_{Ca} with an EC_{50} of 86.5nM (95% confidence limits; 71.9-104, n=16) with a maximal suppression of 51.2 ± 2.4% (mean ± s.e.m.). I_{Ca} was also reduced by the α_2-adrenergic agonists, UK14304 (n=9), clonidine (n=4) and oxymetazoline (n=5), with EC_{50} values of 37.3nM (22.9-60.6), 57.1nM (25.5-127) and 26.9nM (14.7-49.0) respectively, and maximal suppression values of 40.5±3.7, 21.5±3.4 and 22.1±3.1 respectively. In contrast, I_{Ca} was not suppressed by low concentrations of the α_1-agonist, phenylephrine (1μM, n=3), or the β agonist, isoprenaline (10μM, n=3).

Dose-response curves were obtained for noradrenaline in the presence of antagonists. Because of I_{Ca} run-down during long experiments, dose-response curves were constructed in the presence of a single concentration of antagonist, using 3 or 4 cells. The concentration of antagonist was then altered and dose-response curves obtained in previously unused cells. Three concentrations of antagonist were used, and Schild plots constructed from the data. Phentolamine (10nM - 1μM) shifted the agonist dose-response curve to the right. Constraining the slope of the Schild regression to unity yielded an apparent pK_B value of 7.97 (95% confidence limits, 7.80-8.13). Yohimbine (10nM-1μM) also shifted the agonist dose-response curve to the right with an apparent pK_B value of 7.46 (7.24-7.68). Prazosin was ineffective at concentrations below 1μM. At higher concenrations (1-30μM), prazosin antagonised noradrenaline with an apparent pK_B of 5.36 (5.16-5.57).

The results obtained accord with inhibition via an α_2-receptor, at which clonidine and oxymetazoline act as partial agonists (unlike frog sympathetic neurones where clonidine is inactive: Lipscombe *et al.*, 1989).

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419P SL 89.0591: A NOVEL α_1-ADRENOCEPTOR ANTAGONIST WHICH SHOWS PREFERENTIAL EFFECTS ON THE LOWER URINARY TRACT

F. Lefèvre-Borg, P. George, S.E. O'Connor, J. Lechaire, A.M. Galzin, S. Arbilla, C. Pimoule, D. Graham & S.Z. Langer, Departments of Biology and Chemistry, Synthelabo Recherche, 31 Avenue P.V. Couturier, 92225 Bagneux, France

Benign prostatic hypertrophy (BPH) causes chronic micturition disturbances as a result of obstruction of urine outflow. Sympathetically-mediated contraction of smooth muscle in the prostate, bladder and urethra makes an important contribution to this outflow obstruction. Hence, α_1-receptor antagonists, such as alfuzosin (Lefèvre-Borg *et al.*, 1993; Jardin *et al.*, 1991), which inhibit sympathetic tone to the lower urinary tract, offer effective treatment for BPH. Since α_1-antagonists also produce hypotension, agents of this class which show reduced vascular effects would be of therapeutic interest. Here we describe the pharmacological properties of SL 89.0591 (2-[[3-[4-(5-chloro-2-methoxyphenyl)piperazin-1-yl]propyl]amino] pyrimidine-4-carboxamide), a novel α_1-antagonist.

The affinities of α_1-adrenoceptor subtypes for SL 89.0591 were determined by inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptor cDNA stably transfected cell lines (α_{1b}-hamster smooth muscle cells, α_{1c}-bovine brain, α_{1d}-rat cerebral cortex). SL 89.0591 was highly potent and showed a modest selectivity for the α_{1c}-subtype (pK_i values α_{1b} 8.31±0.024, α_{1c} 9.18±0.041, α_{1d} 8.51±0.025). By contrast, SL 89.0591 shows no affinity for α_2 receptors (rat cerebral cortex [^{3}H]-clonidine binding pIC_{50} < 5.0). SL 89.0591 was a competitive antagonist of phenylephrine-induced contractions in the rabbit isolated urethra (pA_2 8.2±0.2, n=9). In anaesthetised cats, SL 89.0591 potently antagonised increases in urethral pressure (UP) produced by electrical stimulation of hypogastric sympathetic nerves after i.v. (ID_{50} 0.025 mg kg^{-1}) or intraduodenal (ID_{50} 0.16 mg kg^{-1} 95% c.l. 0.11-0.23) administration. The potency order for a series of α_1-antagonists in this model (ID_{50} values mg kg^{-1} i.v., 95% c.l., n=3-9) was: prazosin (0.018, 0.015-0.021) = bunazosin (0.019, 0.010-0.028) ≥ SL 89.0591 (0.025, 0.017-0.037) > terazosin (0.054, 0.039-0.074) = phentolamine (0.058, 0.044-0.077) > alfuzosin (0.076, 0.063-0.092). In a similar rat model SL 89.0591 also inhibited increases in UP (ID_{50} 0.005 mg kg^{-1} i.v., n=3). SL 89.0591 was weakly antihypertensive in conscious spontaneously hypertensive rats producing a 20% fall in systolic blood pressure (ID_{20}) at 1.2 mg kg^{-1} i.a. and 15.0 mg kg^{-1} p.o. (95% c.l. 11.6-20.7). The order of antihypertensive potencies (ID_{20} values mg kg^{-1} i.a., 95% c.l., n=4-10) was: prazosin (0.015, 0.013-0.022) > bunazosin (0.045, 0.009-0.083) ≥ terazosin (0.054, 0.029-0.080) > alfuzosin (0.18, 0.013-0.23) > phentolamine (0.38, 0.29-0.48) > SL 89.0591 (1.2, 0.82-1.92).

Thus, SL 89.0591, a potent α_1-selective antagonist, is very active in vivo at inhibiting sympathetic tone to the lower urinary tract but lowers blood pressure only at very high doses. The reference compounds did not possess the same degree of functional uroselectivity. SL 89.0591 is currently in clinical development for BPH. In this context, its modest α_{1c}-selectivity is of interest since the α_{1c}-subtype predominates in the human prostate (Faure *et al.*, 1994).

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420P PHARMACOLOGICAL CHARACTERIZATION OF α_1-ADRENERGIC SUBTYPES MEDIATING CONTRACTIONS OF RABBIT URETHRA, TRIGONE AND MESENTERIC ARTERY

V. Deplanne & A.M. Galzin, Department of Preclinical Research, Synthélabo Recherche, 31 Avenue P.V. Couturier, 92225 Bagneux, France

It has been proposed that α_1-adrenoceptors could be divided into α_{1L} and α_{1H} subtypes, based on affinity for prazosin, while the cloned $\alpha_{1a/1c}$, α_{1b} and α_{1d} subtypes could be members of the α_{1H} family (Ford *et al.*, 1994). α_1-adrenoceptor antagonists are currently being developed for the treatment of benign prostatic hypertrophy, one of the major issues remaining to separate antihypertensive from uroselective compounds (Lefèvre-Borg *et al.*, this meeting). The aim of the present experiments was therefore to characterize the α_1-adrenoceptor subtypes involved in contractile response of urogenital tract vs vascular bed in the rabbit.

Segments of male adult rabbit urethra, trigone and endothelium-denuded mesenteric artery were set up under 1 g tension in a Krebs solution at 37°C. Cumulative concentration-response curves (CRC) to oxymetazoline were obtained in the absence and presence of WB4101 (1-3 nM), phentolamine (10-100 nM), prazosin (0.1-1 µM) or chloroethylclonidine (CEC, 50 µM for 30 min, 30 min washout), alone or in combination. Experiments were performed in the presence of rauwolscine (1 µM) and propranolol (1 µM).

Oxymetazoline showed a higher potency for α_1-adrenoceptors in mesenteric artery (pD_2 7.39±0.04) than in both urethra and trigone (pD_2 6.92±0.06 and 6.94±0.08, respectively). In urethra, CEC reduced maximum responses to oxymetazoline by 30%, while significantly shifting to the right the CRC (pD_2 6.26±0.10). In trigone, CEC did not affect maximum responses, but shifted the CRC to the right (pD_2 6.18±0.12). In contrast, CEC reduced maximum responses in mesenteric artery by only 20%, without modifying the pD_2 of oxymetazoline (7.25±0.07). In both urethra and trigone, neither WB4101 (1-3 nM) nor phentolamine (10 nM) alone or in combination with CEC significantly modified the CRC to oxymetazoline. At 100 nM, phentolamine shifted the response to oxymetazoline in urethra (pA_2 7.55±0.20), while the CRC in trigone was only slightly modified at lowest oxymetazoline concentrations. In mesenteric artery, WB 4101 or phentolamine produced rightwards shift of the CRC to oxymetazoline (pA_2 8.49±0.30 and 8.59±0.20 for WB 4101 3 nM, pA_2 8.15±0.18 and 7.84±0.39 for phentolamine 10 nM, in the absence and presence of CEC respectively). Prazosin (0.1-1 µM) in the presence of CEC significantly shifted to the right the CRC to oxymetazoline in both urethra and trigone (respective pA_2 in urethra 6.66±0.62 and 6.75±0.35, in trigone 7.38±0.36 and 7.37±0.22). There is no explanation for these unexpectedly low values. In mesenteric artery, prazosin (0.1 µM) induced a more pronounced shift than in other tissues (pA_2 8.13±0.18).

The results obtained with CEC suggest that α_{1B}-adrenoceptor subtype is functional in urethra and mesenteric artery, but not in trigone. A functional $\alpha_{1A/1C}$-adrenoceptor is not detectable in urethra and trigone (lack of effect of WB4101 and phentolamine at low concentrations), but is present in mesenteric artery. α_{1D}-adrenoceptors may be present in urethra and mesenteric artery, but not in trigone (effect of 100 nM phentolamine).

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421P CONTRACTILE RESPONSES TO PHENYLEPHRINE OF THE CORPUS CAVERNOSUM PENIS OF THE RABBIT ARE MEDIATED VIA α_{1B}-ADRENOCEPTORS

R. Chess-Williams[1], K. Furukawa,[2] & T. Uchiyama[2] Departments of [1]Biomedical Science, University of Sheffield, Sheffield, UK and [2]Pharmacology, Toho University School of Medicine, Tokyo, Japan.

Stimulation of the α_1-adrenoceptors of the corpus cavernosum penis causes contraction of the smooth muscle, and the intracavernosal injection of α-adrenoceptor antagonists has been used for the treatment of impotence (Brindley, 1983). The present study characterizes the α_1-adrenoceptor mediating contraction of the corpus cavernosum penis of the rabbit.

Strips of the corpus cavernosum penis from New Zealand White rabbits were isolated and set up under 2g resting tension in gassed Krebs-bicarbonate solution at 37°C. Cumulative concentration-response curves to phenylephrine were constructed in the absence and presence (after a 30 min antagonist equilibration period) of WB4101 (30-300nM), 5-methylurapidil (0.1-1.0μM) and chloroethylclonidine (25μM and 100μM). All experiments were performed in the presence of cocaine (10μM), corticosterone (10μM) and propranolol (1μM). Control experiments were performed without the addition of antagonists.

WB4101 and 5-methylurapidil caused rightward shifts of concentration-response curves to phenylephrine without altering maximum responses. Mean maximal responses (±SEM) to phenylephrine were 4.5 ± 1.1g and 4.2 ± 1.0g in the absence and presence of WB4101 (300nM) whilst for 5-methylurapidil (1μM) these values were 3.8 ± 1.0g and 3.4 ± 0.5g respectively.

Mean pA_2 values of 8.1 ± 0.1 and 7.6 ± 0.1 were obtained for WB4101 and 5-methylurapidil respectively. The slopes of Schild plots were not different to unity (WB4101 = 1.0 ± 0.2, 5-methylurapidil = 0.85 ± 0.15).

Incubation with chloroethylclonidine did not significantly alter maximum responses to phenylephrine (control = 4.8 ± 1.3g, 100μM chloroethylclonidine = 4.2 ± 1.1g) but did produce concentration dependent rightward shifts of concentration-response curves, increasing geometric mean EC_{50} values from 6.9(2.0-24.0)μM to 39.3(20.2-76.4)μM at 25μM (n=7, $P<0.5$) and to 220.9(116.6-418.3)μM at a concentration of 100μM (n=7, $P<0.01$).

These results indicate that the predominant α_1-adrenoceptor mediating contractions of the rabbit corpus cavernosum penis to phenylephrine has a low affinity for WB4101 and 5-methylurapidil and is sensitive to inactivation by chloroethylclonidine. The receptor therefore has the pharmacological properties of the α_{1B}-adrenoceptor subtype. The lack of effect of chloroethylclonidine on maximum responses suggests that a substantial receptor reserve exists for responses to phenylephrine in this tissue.

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422P THE α-ADRENOCEPTOR MEDIATED ACTIONS OF CHLOROETHYLCLONIDINE IN RAT AORTA

Mary O'Rourke & J.R. Docherty, Department of Physiology, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland.

The alkylating agent chloroethylclonidine (CEC) is reported to selectively inactivate α_{1B}- but not α_{1A}-adrenoceptors, but is reported to have unusual actions in rat aorta: actions unlike those of classical irreversible antagonists (see Aboud et al., 1993). Contractions to NA in rat aorta following pretreatment with CEC have also been shown to be resistant to α-adrenoceptor antagonists (Oriowo & Bevan, 1990). We have investigated further the actions of CEC in rat aorta.

Rings of rat aorta (endothelium removed) were set up under 1 g tension in organ baths, and two concentration-response curves were carried out to NA: control and following vehicle or test agents. Responses in the second concentration-response curve were expressed as a percentage of the maximum response obtained in the first (control) curve.

Unlike irreversible alkylating agents such as phenoxybenzamine (10 μM for 10 min, 1 hr washout), which reduced the maximum response to NA to 7.8±7.0% of control (n=7), CEC (100 μM for 30 min, 1 hr washout) produced a large 100 fold shift in the NA concentration-response curve without reducing the maximum response (NA maximum response: after vehicle, 117.4±8.5%, n=21; after CEC, 115.5±12.7%, n=15). The shift was not parallel in that the response to low concentrations of NA was shifted less. Following exposure to CEC, a component of the response to NA was resistant to the α_1-adrenoceptor antagonist prazosin (10 μM) or to PBA (10 μM for 10 min) (following CEC and subsequent PBA, maximum response to NA was 91.4±6.4% of control, n=4). In receptor protection experiments, in which prazosin, yohimbine or NA (all 10 μM) were given before and during exposure to CEC, all three agents prevented the antagonist actions of CEC against low concentrations of NA. However, yohimbine or NA, but not prazosin, prevented the occurrence of a prazosin-resistant response to NA following CEC.

It is concluded that there are two components to the actions of CEC against NA in rat aorta. The first is irreversible α_1-adrenoceptor antagonism, demonstrated against low concentrations of NA. The second component is a contractile interaction with high concentrations of NA which could be prevented by prior, but not subsequent, exposure to yohimbine, suggesting an irreversible agonism at α_2-adrenoceptors.
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423P INHIBITORY ACTIONS OF NIFLUMIC ACID AND NIFEDIPINE ON NORADRENALINE-INDUCED CONTRACTION OF THE ISOLATED RAT AORTA

D.N. Criddle, R. Soares de Moura, I.A. Greenwood[1], R.M. Helliwell[1] & W.A. Large[1]. Departamento de Farmacologia, IB-CB Universidade do Estado do Rio de Janeiro, Brasil & [1]Department of Pharmacology, St Georges Hospital Medical School, London.

Niflumic acid (NFA) is a potent inhibitor of Ca-activated chloride (Cl^-) currents in rat and rabbit portal vein (Pacaud et al., 1989; Hogg et al., 1994) and may thus be useful as a pharmacological tool to assess the involvement of Cl^--channels in agonist-induced contraction of smooth muscle. Previous studies indicate that noradrenaline (NA) can activate a Cl^- conductance in rabbit portal vein (Byrne & Large 1988; Hogg et al., 1994). Therefore it is possible that the depolarisation induced via such a mechanism may lead to an opening of voltage-sensitive Ca-channels and thus contribute to vascular contraction. In order to assess whether such an involvement might be present in NA-induced contraction of quiescent vascular smooth muscle, we have compared the effects of NFA and nifedipine on NA- and KCl-induced contractions of the isolated rat aorta.

Aortae from male Wistar rats (250-300g) were mounted as rings for the recording of isometric tension using conventional techniques and bathed in Krebs-Henseleit solution (37°C) gassed with 95% O_2 5% CO_2. NA was applied either cumulatively or transiently, to elicit phasic contractions (using a single, maximal concentration of 1μM), in the presence and absence of NFA or nifedipine. In addition, in order to assess whether the effects of NFA were specific to NA-induced changes, the actions of both drugs on 60mM KCl-induced contraction were examined.

NA (10^{-9}-10^{-6}M), applied cumulatively, induced sustained contraction of the aortae with a maximal response of 1.4±0.1g (n=11). NFA (3 & 10μM) did not alter basal tone, however, the concentration-effect curve for NA in the presence of NFA was displaced to the right concentration-dependently and the maximal response depressed by 24.4±5.6% and 38.2±4.8%, respectively (n=7). Similarly, nifedipine (10^{-7} & 10^{-6}M) inhibited the responses to NA with reductions of the maximal response of 34.3±4.1% and 39.3±3.8%, respectively (n=4). Transient applications of NA (1μM) produced phasic contractions (0.8±0.1g, n=11) that were inhibited reversibly by NFA (3 & 10μM) with a reduction of 54.6±5.0% at the higher concentration (n=11). Nifedipine (1μM) elicited a 62.2±5.3% reduction of NA-induced contractions (n=3). In aortae precontracted with 60mM KCl, NFA (1, 3 & 10μM) produced no significant relaxation, whereas nifedipine (10^{-7}M), applied post-NFA, elicited complete inhibition of the induced tone (n=8).

Our results show that NFA reversibly inhibits NA- but not KCl-induced contraction of the rat aorta. The inhibitory effects of NFA on NA-induced responses were comparable with those of nifedipine. Our data thus support the view that activation of Ca-activated Cl^--channels may play an important role in the vasoconstrictor action of NA.

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424P THE EFFECTS OF α-ADRENOCEPTOR ANTAGONISTS ON COLD-INDUCED VASOCONSTRICTION IN THE AUTOPERFUSED HINDLIMB OF THE RAT

Paula J.W. Smith[1,2], David J. Webb[1] & Daniel S. McQueen[2] The University of Edinburgh, [1]Department of Medicine, Western General Hospital, Edinburgh EH4 2XU & [2]Department of Pharmacology, 1 George Square, Edinburgh EH8 9JZ.

Raynaud's Disease (RD) is characterised by prolonged cold-induced vasospasm, the pathogenesis of which remains unresolved. Patients with RD have enhanced α-adrenoceptor responsiveness (Freedman *et al.*, 1989), and *in vitro* studies using human hand veins find cooling augments α2-adrenoceptor-mediated vasoconstriction (Bodelson *et al.*, 1990). The autoperfused rat hindlimb preparation has been used to examine the role of α-adrenoceptors in cold-induced vasoconstriction *in vivo*, which may also be responsible for the vasospasm found in RD.

Male Wistar rats (382±17g) were anaesthetised with sodium pentobarbitone (60mg/kg i.p.) and the right jugular vein and carotid artery were cannulated for drug administration and measurement of mean arterial pressure (MAP) respectively. The rats were heparinised (1000U/kg) prior to cannulation of the right and left femoral arteries. The right hindlimb was perfused with blood from the left femoral artery using a peristaltic pump which was set at a constant flow rate so that hindlimb perfusion pressure (HLPP), measured by a pressure transducer situated between the pump and right hindlimb, matched the MAP. Cooling was achieved by passing the blood through a rapid heat-exchange element before it entered the right hindlimb. Temperature-HLPP curves were obtained by cooling the blood from 37 to 21°C in 4C° steps held for 2 minutes, before and after i.a. bolus administration of the following adrenoceptor antagonists: phentolamine (α1/α2); prazosin (α1); yohimbine (α2); prazosin followed by yohimbine. Doses used have been shown to be effective against agonists. Results are expressed as the -log slope of temperature-HLPP curves (mean ± s.e.mean, n=4-6).

Stepped-cooling of the blood from 37 to 21°C resulted in a rise in HLPP, implying cold-induced vasoconstriction. The effects of the antagonists are shown in Table 1 (* p<0.05, ** p<0.01, with respect to control; paired t-test).

The effect of phentolamine suggests a role for α-adrenoceptors in mediating the cold-induced vasoconstriction in the rat hindlimb. However, lack of antagonism by the selective α1- and α2-antagonists, alone or in combination, indicates that the action of phentolamine does not involve α-adrenoceptors. Phentolamine has also been reported to have effects on ATP-sensitive K+ channels (Schwietert *et al.*, 1992), and further studies will be required to establish the mechanism(s) responsible for its action against cold-induced vasoconstriction in the rat hindlimb.

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Table 1: -Log slope of temperature-HLPP curves before (control) and after antagonist, and basal HLPP (mmHg) at 37°C

	control	phentolamine (10μg/kg)	control	prazosin (100μg/kg)	control	yohimbine (300μg/kg)	control	prazosin and yohimbine
- log slope	1.42±0.15	1.12±0.24 *	1.48±0.12	1.50±0.11	1.44±0.12	1.37±0.03	1.53±0.18	1.26±0.08
basal HLPP	92±2	83±8	120±6	103±5**	110±2	93±4**	119±10	98±7

425P COMPLEX ANTAGONISM OF α_1-ADRENOCEPTOR-MEDIATED CONTRACTION OF THE RAT SMALL MESENTERIC ARTERY

P.H. van der Graaf, N.P. Shankley & J.W. Black, Analytical Pharmacology, King's College School of Medicine & Dentistry, 123 Coldharbour Lane, London SE5 9NU.

At least three different subtypes of α_1-adrenoceptors have been proposed in vascular tissues (Muramatsu *et al.*, 1990). However, the α_1-adrenoceptors in resistance vessels have not yet been adequately characterised. Here we report the results of the interaction between noradrenaline (NA) and a series of α_1-adrenoceptor antagonists in rat mesenteric arteries.

Small mesenteric arteries (s.m.a., internal diameter 150-350 μm) from male Wistar rats (225-300 g) were mounted as 2 mm ring segments in a myograph (37°C, gassed with 95%O_2/5%CO_2) as described by Mulvany & Halpern (1977). The endothelium was removed and tissues were calibrated with a single concentration of 10 μM NA. After a 15 min washout period, tissues were incubated for 90 min with 30 μM cocaine, 6 μM timolol and antagonist or the appropriate vehicle. Single NA concentration-effect (E/[A]) curves were obtained by cumulative dosing.

All of the antagonists investigated produced concentration-dependent, parallel, rightward shifts of the NA E/[A] curves. However, Schild analysis yielded slope parameters (b) significantly greater than unity for tamsulosin, phentolamine, prazosin, WB-4101 and spiperone (Table 1). In contrast, the Schild plot slope parameters for 5-methylurapidil and HV723 (Muramatsu *et al.*, 1990) were not significantly different from unity, characteristic of simple competitive antagonism. Previously, we showed with prazosin that the steep Schild plots cannot be explained by the usual interpretative models, namely insufficient antagonist equilibration time, antagonist removal from the biophase or nonspecific antagonism (Van der Graaf *et al.*, 1993). Consequently, we have considered the hypothesis that NA can also exhibit a receptor-mediated inhibitory action in the s.m.a. A model of this hypothesis has shown that the data can be adequately fitted when there is a steep agonist curve plus high efficacy at α_1-adrenoceptors. In this case, the model predicts that the estimated K_B values have not been distorted by the steep Schild plot and Schild plot slopes of unity will occur when the antagonists are non-selective for the two receptors.

Table 1

	pK_B (pA_2) ± s.e.	b ± s.e.	d.f.
tamsulosin	(9.8 ± 0.2)	1.35 ± 0.09*	23
phentolamine	(7.7 ± 0.1)	1.40 ± 0.09*	27
prazosin	(8.5 ± 0.1)	1.63 ± 0.13*	43
WB-4101	(8.4 ± 0.1)	1.52 ± 0.19*	22
spiperone	(7.5 ± 0.2)	1.37 ± 0.12*	25
5-methylurapidil	8.38 ± 0.11	1.04 ± 0.07	29
HV723	8.96 ± 0.08	0.98 ± 0.06	52

*b significantly greater than unity ($P<0.05$)

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426P CHRONIC INHIBITION OF ADENOSINE RECEPTORS LEADS TO HYPERTENSION AND ENHANCES β-ADRENOCEPTOR-MEDIATED FACILITATION OF NORADRENALINE RELEASE

S.Guimarães, A.Albino-Teixeira & M.Q.Paiva, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200-Porto, Portugal

Recently it was shown that long-term treatment of rats with DPSPX (1,3-dipropyl-8-sulphophenylxanthine)- a non-selective antagonist of adenosine receptors - leads to a hypertensive state (Matias *et al.*1991) and alters the prejunctional effects of the selective α-adrenoceptor agonist UK-14,304 (Guimarães *et al.*1994).The present study was undertaken to compare the role of β-adrenoceptor-mediated facilitation of noradrenaline release in the tail artery of normotensive rats and of rats rendered hypertensive by chronic administration of DPSPX.

The prejunctional effect of the β-adrenoceptor agonist isoprenaline (3-100 nM) was determined by the change (increase) in tritium overflow elicited by electrical stimulation of arterial rings preloaded with ^{3}-H-noradrenaline (Guimarães *et al.*1978). Four stimulations (S_1-S_4) (1Hz, 2 ms, 100 V) were applied with 50-min intervals, S_1 being taken as control. The agonist was added to the perifusion fluid 20 min before S_2, S_3 and S_4 in concentrations increasing by a factor of 5. To eliminate the influence of prejunctional α_2-autoreceptors the tissues were pre-exposed to 1 μM phenoxybenzamine for 45 min.

Isoprenaline caused a concentration-dependent increase of tritium overflow elicited by electrical stimulation and was more effective on arterial rings from hypertensive than on those from normal rats; 60 nM isoprenaline, which increased by 30% tritium overflow evoked by electrical stimulation in vessels from control rats were as effective as 6.6 nM isoprenaline in vessels from DPSPX-treated animals. Furthermore, the maximal effect of isoprenaline - an increase in tritium overflow by 31.6±2.7% (mean±s.e.) (n=6) (in control rats) - increased to 46.6±5.3% (in hypertensive rats) (n=6; $p<0.05$; unpaired rings).

These results show that the sympathetic nerve endings of the rat tail artery are endowed with prejunctional β-adrenoceptors which mediate a clear facilitation of the noradrenaline release elicited by electrical stimulation. This facilitation is enhanced in hypertensive rats both in terms of sensitivity and maximal effect. These changes are compatible with an upregulation of the prejunctional β-adrenoceptors or with some change at the level of G_S proteins. Finally, these results show that adenosine and β-adrenoceptors interact at prejunctional level and suggest that this "crosstalk" interferes in the regulation of blood pressure. Impairment of this interaction may lead to the development of a hypertensive state.

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427P THE INFLUENCE OF HYPERTHYROIDISM ON β-ADRENOCEPTOR-MEDIATED RELAXATION OF ISOLATED SMALL ARTERIES

J. Zwaveling, E.A. Winkler Prins, M. Pfaffendorf & P.A. van Zwieten, Department of Pharmacotherapy, Academic Medical Centre, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

It may be expected that hyperthyroidism will induce significant changes in the pharmacodynamic behaviour of the cardiovascular system (Klein, 1990), but this matter has not been studied systematically.

We investigated vasodilator responses to ß-adrenergic compounds in isolated small arteries of chronic hyperthyroid rats. Hyperthyroidism was induced by feeding male Wistar rats of approximately 250 gram with 5 mg/kg L-thyroxine (T4)-containing chow during four weeks. T4 treatment caused significant increases in serum T4 levels (treated vs. control: 266.3 ± 13.4 and 55.9 ± 2.1; means ± SEM,n=8; $P<0.05$) and decreases in serum TSH levels (0.33 ± 0.03 vs. 0.87 ± 0.13, n=8; $P<0.05$). Mesenteric small arteries were prepared and normalized according to Mulvany and Halpern (1977) to their individual optimal lumen diameter (± 310 μm for both groups) in an isometric wire-myograph. Concentration-effect curves (CEC's) for vascular relaxation were constructed after precontraction with methoxamine (10 μM) which resulted in a stable wall tension of 3.32 mN/mm ± 0.09 (means ± SEM). CEC's were constructed for the vasodilator effects of salbutamol, forskolin and isoproterenol. The responses to isoproterenol were also studied in the presence of the $ß_2$-adrenoceptor antagonist ICI 118,551 (3 μM) or the K^+_{ATP} channel-blocker glibenclamide (1 μM).

Hyperthyroidism of the donor rats induced leftward shifts of the CEC for isoproterenol (pD_2 treated vs. control: 6.52 ± 0.08 and 6.16 ± 0.09 respectively, n=7; $P<0.05$; means ± SEM., Student's unpaired t-test). Pretreatment of the donor animals with L-thyroxine neither influenced the salbutamol-induced relaxation nor that caused by forskolin. ICI 118,551 caused a rightward shift of the CEC for isoproterenol. The shift was more pronounced (pD_2 6.52 ± 0.08 to 5.86 ± 0.12, n=6) in vessels from hyperthyroid rats than in preparations from euthyroid rats (pD_2 6.16 ± 0.09 to 5.86 ± 0.12, n=6). Accordingly, the same final pD_2-values were achieved in both types of preparations. Glibenclamide induced a comparable leftward shift in both types of preparations: pD_2 6.52 ± 0.08 to 6.96 ± 0.06 (n=6) in vessels from hyperthyroid rats and pD_2 6.16 ± 0.09 to 6.46 ± 0.12 in those from euthyroid rats.

In conclusion, the increased sensitivity of the resistance vessels to ß-receptor agonists, associated with hyperthyroidism is probably located at the ß-receptor level since the responses to forskolin were not influenced by the thyroid state of the donor animals. ATP-sensitive potassium channels may be involved in the ß-receptor mediated relaxation in both types of preparations (from hyper-and euthyroid donor animals), as indicated by the influence of glibenclamide. The vascular sensitization caused by hyperthyroidism may simultaneously involve both $ß_1$- and $ß_2$-adrenoceptors in a rather complex manner.

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428P THE INFLUENCE OF TONE ON β-ADRENOCEPTOR-MEDIATED RELAXATION OF THE ARTERIAL VESSEL WALL, *IN VITRO*

R. Begonha, D. Moura & S. Guimarães, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200-Porto, Portugal

Besides varying from tissue to tissue, ß-adrenoceptor distribution can vary with species, age, vessel size in one vascular bed (Bevan *et al.*, 1980; Guimarães *et al.*, 1994) and location along the length of a single vessel (Guimarães *et al.*, 1993). The present investigation was undertaken to study the influence of the tone on β-adrenoceptor-mediated responses to isoprenaline on rings from coronary, mesenteric, splenic and pulmonary arteries removed from dogs under pentobarbital anaesthesia (Guimarães *et al.*1993). In the coronary artery, adrenaline and noradrenaline were also used as relaxation-inducing agonists. Five different levels of tone were produced (of about 35%, 50%, 65%, 80% and 95% of the maximum) by adding phenylephrine (0.6, 1.0, 2.0, 4.0, and 10 μM, respectively) to the bath.

In the coronary artery at spontaneous tone, 1-3 nM of noradrenaline or adrenaline caused either relaxation or contraction, while after induced tone, both noradrenaline and adrenaline caused concentration-dependent relaxations, noradrenaline being more potent (EC_{50} of 0.16 (0.13-0.20) μM (geometric means and 95% confidence limits) and 0.38 (0.22-0.67) μM) , respectively; n=6; $P<0.05$). Only in the coronary artery did isoprenaline relax the tissue irrespective of the previous level of tone. In all the other arteries, isoprenaline was able to cause concentration-dependent relaxations only if the previous tone was submaximal. At 80% of the maximum, isoprenaline caused relaxation in the mesenteric and pulmonary arteries but in the splenic one it caused relaxation only when the tone was of about 65% of the maximum or less. While in the coronary artery, atenolol and ICI-118,551 were equipotent in antagonizing isoprenaline, in all the other arteries ICI-118,551 was much more potent than atenolol (Table 1).

Table 1. Antagonism (pA_2 ±S.E.M.) exerted by atenolol and ICI-118,551 against the relaxant effect of isoprenaline

Artery	Atenolol	n	ICI-118,551	n
Mesenteric	6.59 ± 0.09^a	5	9.31 ± 0.13^b	5
Splenic	7.13 ± 0.22^c	4	8.90 ± 0.27^d	5
Pulmonary	6.89 ± 0.18^e	7	9.50 ± 0.24^f	6
Coronary	8.03 ± 0.18^g	4	7.53 ± 0.33^h	4

Significant ($P < 0.05$) were the differences between [a] and [b]; [a] and [c]; [c] and [d]; [e] and [f]; [e] and [g]; [a] and [g]; [f] and [h].

It is concluded that: 1) the relaxant effect of isoprenaline is critically dependent on the tone; 2) $β_2$-adrenoceptors predominate in the mesenteric, pulmonary and splenic arteries, while $β_1$-ones predominate in the coronary artery.

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429P LOSS OF EFFICACY AND RECEPTOR RESERVE FOR ISOPRENALINE AT THE β_2-ADRENOCEPTORS OF THE PORTAL VEINS OF HYPERTENSIVE RATS

Sheila A. Doggrell & Ailsa J. Surman, Department of Pharmacology, School of Medicine, University of Auckland, Private Bag 92019, Auckland, New Zealand

β_2-Adrenoceptor vascular responsiveness is reduced in established human and rat hypertension (Brodde & Michel, 1992). It is possible that this β_2-adrenoceptor hypo-responsiveness is due to pressure induced vascular changes. We have compared the attenuating effects of isoprenaline and verapamil on the contractility of portal veins, which are not subject to systemic pressure, from Wistar Kyoto (WKY) rats and Spontaneously Hypertensive rats (SHRs). Secondly, we have studied the effects of bromoacetylalprenolol-menthane (BAAM), an irreversible β-adrenoceptor antagonist, on the isoprenaline responses. These studies used methods described previously (Doggrell, 1990). In weight-matched SHR and WKY rats, the SHR portal veins were not significantly heavier than the WKY rat portal veins.

We observed a selective hypo-responsiveness of the portal veins to isoprenaline in hypertension. The pD_2 values were 8.30 $\pm$ 0.06 (8) and 7.88 $\pm$ 0.06 (7), $p < 0.05$ unpaired t-test, and the maximum attenuation values were 0.90 $\pm$ 0.03 (8) and 0.53 $\pm$ 0.06 (7), $p < 0.01$, from the portal veins of WKY rats and SHRs, respectively. On the WKY rat portal vein the pD_2 value for verapamil was 6.45 $\pm$ 0.13 (8) and the maximum attenuation value was 1, and these values were not significantly different from the values on the SHR portal vein. On the WKY portal vein, BAAM treatment at 3 x 10^{-7} or 10^{-6}M for 30 min caused nonparallel shifts of the isoprenaline response curves and a reduction in the maximum attenuation from 0.77 $\pm$ 0.04 (9) to 0.48 $\pm$ 0.06 (9), $p < 0.01$, and 0.40 $\pm$ 0.05 (12), $p < 0.01$, respectively. The mean K_A value (Furchgott & Bursztyn, 1967) for isoprenaline was 1.78 $\pm$ 0.32 x 10^{-7}M (21) and it was calculated that isoprenaline produced 50, 95 and 100% maximum responses by occupying 6% $\pm$ 1, 20% $\pm$ 3 and 43% $\pm$ 5 (21) of the available β_2-adrenoceptors, respectively. On the SHR portal vein, BAAM treatment at 10^{-7} or 3 x 10^{-7}M caused nonparallel rightward shifts of isoprenaline response curves and a reduction in the maximum attenuation from 0.44 $\pm$ 0.05 (8) to 0.32 $\pm$ 0.02 (7), $p < 0.01$, and 0.28 $\pm$ 0.05 (8), $p < 0.01$, respectively. The mean K_A value for isoprenaline was 1.22 $\pm$ 0.35 x 10^{-7}M (15), which was not significantly different from the WKY. On the SHR portal vein, isoprenaline produced 50, 95 and 100% of the maximum attenuation by occupying 14% $\pm$ 3, 33% $\pm$ 6 and 58% $\pm$ 7 (15) of the available β_2-adrenoceptors, respectively, which represents a significantly smaller receptor reserve, $p \leq 0.05$, at each level of response in the SHR compared to the WKY portal vein.

In conclusion there is a selective loss of efficacy and receptor reserve for isoprenaline at the β_2-adrenoceptors of the portal veins of hypertensive rats and this is not associated with hypertrophy of the SHR portal veins.

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430P THE EFFECTS OF IBERIOTOXIN ON ISOPRENALINE-INDUCED RELAXATIONS OF THE GUINEA-PIG TRACHEA

Y.D. Pendry, D. Thomas, A.T. Nials and R.A. Coleman. Pharmacology 1, Glaxo Research and Development, Park Road, Ware, Herts, SG12 0DP, England.

The large Ca^{2+}-dependent K^+-channel blocking drug, iberiotoxin (IbTX), has been reported to inhibit isoprenaline (ISO)-induced relaxation of carbachol (CCh) contracted guinea-pig trachea (GPT) (Jones *et al.*, 1993). It was thus proposed that the opening of K^+-channels is involved in this relaxant response. The present study further investigates the effects of IbTX on ISO-induced relaxations of GPT contracted with both prostaglandin $(PG)F_{2\alpha}$ and CCh at submaximally- and maximally-effective concentrations.

Strips of GPT were immersed in Krebs solution (+ indomethacin, 2.8μM) at 37°C and gassed with 95%O_2/5%CO_2. GPT were contracted with $PGF_{2\alpha}$ (1 and 10μM) or CCh (0.3 and 3μM), and cumulative concentration-effect curves (CRC) to ISO (1nM-100μM) repeated until constant. A final ISO CRC was constructed in the absence or presence of IbTX (0.3μM, 30mins). All data are the mean of at least 4 experiments.

IbTX itself caused variable contractions of GPT, equal to 32 ($\pm$17) and 44($\pm$8) % of the previous maximum to $PGF_{2\alpha}$ and CCh respectively. In GPT where the IbTX-induced contraction was $\leq$50%, it caused 12(6-25)- and 9(1-74)-fold leftward shifts in $PGF_{2\alpha}$ and CCh contractile CRC, respectively. ISO relaxed GPT contracted with $PGF_{2\alpha}$ (1 and 10μM) and CCh (0.3 and 3μM) in a concentration-dependent manner (concentrations required to relax GPT by 50% maximum (EC_{50}s) =10(6-18), 26(20-35), 60(48-76) and 184(147-229)nM, respectively). IbTX had little or no effect on ISO CRC on GPT contracted with $PGF_{2\alpha}$ (1 and 10μM) or CCh (0.3μM), concentration-ratios (CR) =3(1-8), 3(1-6) and 4(2-9) respectively, with no effect on the maximum. However, on GPT contracted with CCh (3μM), IbTX markedly depressed subsequent ISO CRC, causing an 63$\pm$7% reduction in the maximum response ($p<0.001$ compared to controls, paired Student's t-tests). Furthermore, the effects of IbTX on baseline tone, on ISO relaxant CRC on GPT contracted with CCh, and on CCh contractile CRC were markedly attenuated by preincubation with the Ca^{2+}-channel blocking drug, nifedipine (0.1μM, 30 mins).

In conclusion, the effect of IbTX on ISO-induced relaxations of the GPT depends not only on the spasmogen used, but also on its concentration. The results of the present study supports those of Huang *et al.* (1993) who suggested that the inhibitory effect of charybdotoxin on salbutamol-induced relaxation was the result of functional antagonism due to Ca^{2+} influx. We suggest that the inhibitory effects of IbTX on ISO-induced relaxation of CCh contracted GPT, observed by us and other workers, are also the result of functional antagonism, and that the opening of K^+-channels may not be involved in the relaxant activity of ISO on GPT.

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431P INVESTIGATION OF THE DIFFERENTIAL AGONIST SENSITIVITY OF A CLONED β3-ADRENOCEPTOR MEASURED IN TWO FUNCTIONAL ASSAYS

J.E. Park, J.K. Chambers, A.G. Ladurner, S. Wilson, (introduced by G.W. Price), SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow CM19 5AW

Data from a number of laboratories suggest that a series of β3-adrenoceptor agonists exhibit distinctly different rank order of potencies at the cloned human β3-adrenoceptor (expressed in CHO cells), when tested in two different second messenger assays: a membrane-based adenylyl cyclase assay and an intact cell cAMP assay (Emorine *et al*, 1994). The difference between the two assays is most clearly borne out, for a range of agonists, by the calculation of the ratio of potencies in the two assays (EC50 {cyclase}/EC50{cAMP}); ratios appear to be high for "classical" β agonists (catecholamines and BRL37344), but lower for β3-agonists which act as antagonists at β1- and β2- adrenoceptors (eg: cyanopindolol and CGP12177A). Using a clonal CHO cell line expressing the human β3-adrenoceptor (Bmax = 400 fmol/mg), we have shown that these differences in ratio are upheld when the two assays are compared within the same laboratory, using the same cell line (Table 1). Adenylyl cyclase and intact cell cAMP assays were performed as previously described (Chambers *et al.*, 1994; Pauwels *et al.*, 1991). We have carried out a number of studies to investigate the reason for these differences in ratio. Firstly, we observed that the parental cell line did not respond to isoprenaline in either assay, indicating the absence of an endogenous β- adrenoceptor which may have been activated only by "classical" agonists, and only in the more sensitive intact cell assay. Secondly, we observed that the differential potency ratios were not greatly altered in cell lines with higher or lower receptor density (130 or 3000 fmol/mg) indicating that receptor expression level is not a factor. Thirdly, additional β-adrenoceptor mediated responses which could feedback and modulate cAMP levels in intact cells but not in membrane-based assays, in an agonist specific manner, were investigated. Indomethacin (2.86μM) had no effect on the potency of either isoprenaline or CGP12177A in the intact cell assay, indicating that a potentiation of response by prostaglandins is unlikely. Likewise, the involvement of endogenous adenosine receptors was rejected on the basis that (5'-N-ethyl carboxamide)-adenosine (NECA), up to 200μM, did not elicit a response in the intact cell assay. The reason for the difference in potency order between the two assays therefore remains unclear.

Table 1. Agonist activity in two secondary messenger assays (mean EC_{50} (nM) ± s.e.mean, n≥3).

agonist	adenylyl cyclase	intact cell cAMP	EC50 ratio
(-)isoprenaline	651±64	4.2±0.4	155
(-)noradrenaline	2233±450	37±5	62
BRL37344	1300±280	23±1	57
CGP12177A	357±32	110±8	3
cyanopindolol	813±197	550±29	1.5

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432P SR 58878 IS A HIGHLY SELECTIVE β3-ADRENOCEPTOR AGONIST IN ISOLATED RAT PROXIMAL COLON AND WHITE ADIPOCYTES

M. Landi, T. Croci & L. Manara SANOFI-Midy Research Center, Milan (Italy)

Phenylethanolaminotetralins (PEAT) are novel agonists with scant or virtually no activity at conventional $ß_1$ or $ß_2$ adrenoceptors, but are highly potent at atypical ($ß_3$) sites that are abundant in the rat proximal colon (Bianchetti & Manara, 1990). SR 58611A, clinically the most advanced PEAT is remarkably free of the cardiovascular and other side effects (tremor) which occur with similar, less selective agents (Landi *et al.*, 1993). SR 58611A is rapidly and totally biotransformed to the acid metabolite SR 58878 ({(7S)-7-[(2R)-2-(3-chlorophenyl)-2-hydroxyethylamino]-5,6,7,8-tetrahydronaphthalen-2-yloxy} acetic acid).

The *in vitro* potency of SR 58878 and selectivity for atypical ß-adrenoceptors have been assessed in several isolated tissue preparations (Bianchetti & Manara, 1990; Landi *et al.*, 1993) (Table 1). ß-adrenergic responses elicited presumably by stimulation of $ß_1$- (guinea-pig right atrium, GPA), $ß_2$- (guinea-pig trachea, GPT; rat uterus, RU) or $ß_3$-adrenoceptors (rat colon, RC; rat white adipocytes, RWA) were monitored.

Unlike isoprenaline, SR 58878 selectively inhibited rat colon motility and stimulated lipolysis with virtually no effect on tissues containing either $ß_1$ or $ß_2$-adrenoceptors. Alprenolol competitively antagonized the effect of isoprenaline (in the presence of 0.1 μM propranolol) pA_2±s.e.: 6.8±0.2 (RC), 6.8±0.1 (RWA) and SR 58878 pA_2±s.e.: 6.8±0.2 (RC), 6.9±0.3 (RWA), i.e. with lower affinity than at $ß_1$ or $ß_2$ adrenoceptors (Schild slopes not significantly different from 1). Thus, SR 58878 was as potent as SR 58611A as an agonist at the atypical sites, with apparently even greater selectivity.

Table 1	GPA chronotropic action	GPT relaxation	RU motility inhibition	RC molitity inhibition	RWA lipolysis stimulation
			EC_{50}, nM		
(-)-Isoprenaline	4.8 (3.6-6.0)	1.2 (0.96-1.47)	0.2 (0.17-0.25)	0.5 (0.4-0.6)	17 (15-19)
SR 58878	> 30,000	> 10,000	~8,000	2.9 (1.9-4.5)	121 (87-169)

EC_{50} = concentration (nM) producing half-maximal response. In parentheses 95% confidence limits (n= 6-12)

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433P CHANGES IN MOUSE ADIPOCYTE LIPASE ACTIVITY AFTER CHRONIC ETHANOL ADMINISTRATION

M-F. Shih and P.V. Taberner, Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS81TD, U.K.

Chronic treatment with high levels of ethanol causes hyperlipidaemia and increased plasma lipoprotein levels, whereas lower concentrations tend to reduce circulating triglycerides and cholesterol (Baraona & Lieber, 1979). However, the molecular mechanisms of these effects are still unclear. We have already shown that chronic ethanol drinking produces adaptive increases in adipocyte fatty acid synthesis and lipolysis (Al-Qatari *et al*, 1991). Since lipoprotein lipase (LPL) is an important regulator of plasma lipoprotein levels (Taskinen *et al.*, 1987), and hormone-sensitive lipase (HSL) is the key enzyme of lipolysis, we have assayed these enzymes during two chronic ethanol treatment schedules.

Groups of 8-10 male CBA mice were given either: pelleted food and tap water or pelleted food and 20% ethanol solution (CET) for 4 weeks; or, control liquid diet (CLD) or equicaloric 7% ethanol liquid diet (ELD) (Lieber & DeCarli, 1989) for 8 days. LPL and HSL were assayed by standard procedures using acetone powder (LPL) (Carneheim *et al.*, 1984) or supernatant (HSL) (Huttunen *et al*, 1970) tissue fractions prepared from brown (BAT) and white (WAT) adipose tissue. Since HSL activity is regulated through a cAMP-dependent kinase, cAMP accumulation was measured by a saturation binding assay (Shepherd *et al*, 1977). Differences between groups (mean ± s.e.mean) were analysed by Students t-test.

Mice on CET derived 18% of their daily calorific intake from ethanol; those on ELD derived 36%. LPL activity in BAT was significantly increased from 0.16 ± 0.01 to 0.27 ± 0.05 nmol FFA h^{-1}. mg acetone $powder^{-1}$ ($P < 0.05$) after CET and from 0.39 ± 0.05 to 0.79 ± 0.14 ($P < 0.01$) after ELD. There was no difference in LPL activity in WAT between control and ethanol-treated mice. On the other hand, HSL activity in BAT decreased after CET from 4.74 ± 0.16 to 3.76 ± 0.2 nmol FFA. min^{-1}. mg $protein^{-1}$ ($P < 0.05$) and after ELD from 4.14 ± 0.53 to 2.11 ± 0.44. Interestingly, HSL activity in WAT was stimulated by CET from 5.78 ± 0.17 to 9.98 ± 0.82 ($P < 0.01$) but was unchanged after ELD. cAMP production in WAT also increased after CET, from 1.98 ± 0.2 to 2.54 ± 0.16 nmol. mg $protein^{-1}$ ($P < 0.01$), but was decreased from 1.66 ± 0.2 to 0.84 ± 0.08 after ELD ($P < 0.01$).

Control mice on the liquid diet consumed more calories than controls on pelleted food; consequently, LPL activity was higher in these mice. Removal of serum lipoproteins by adipose tissue LPL appears to be enhanced after either CET or ELD treatment. The CET schedule, in contrast to the ELD schedule, increased WAT lipolysis and cAMP production. We conclude that the differential effects of ethanol on lipid mobilisation are mediated through interactions with lipases in adipose tissue, possibly via a second messenger system.

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434P OKADAIC ACID AND QUANTAL ACETYLCHOLINE RELEASE AT THE FROG NEUROMUSCULAR JUNCTION *IN VITRO*: THE EFFECT OF NIMODIPINE

M.S. Arenson & D.S. Gill, Department of Pharmacology, Queen Mary and Westfield College, Univeristy of London, Mile End Road, London E1 4NS.

L-type Ca^{2+}-channels do not participate in the release of acetylcholine (ACh) from motor nerves (Gotgilf & Magazanik, 1978). However, intracellular (Morita & Barrett, 1989) and axial current records (Penner & Dreyer, 1986) suggest that these channels are found in the terminal or near-terminal axon membrane. Exposure of bovine adrenal medullary cells to the phosphatase inhibitor okadaic acid allows activation of L-type channels; a class which, in these cells, does not ordinarily contribute to the voltage-dependent Ca^{2+}-current (Artalejo *et al.*, 1992). Okadaic acid also increases ACh release from motor nerves (Abdul-Ghani *et al.*, 1991). The experiments reported here were carried out to ascertain whether this enhanced release can be attributed to the recruitment of otherwise silent L-type channels.

Sartorius muscles were equilibrated in Ringer solution containing Ca^{2+} 0.5 and Mg^{2+} 3.5 mM. Fibres were impaled with one or two microelectrodes filled with 3M-KCl. Control samples of mepps or mepcs (usually > 100) and epps or epcs (80 or 140) evoked at 0.33 Hz were recorded under static conditions 15 and 10 min respectively before okadaic acid was added to the chamber. Test samples of spontaneous and evoked synaptic signals were obtained 20 and 25 min respectively after addition of the inhibitor. Changes (mean ± s.e. mean %) in quantal content (M-calculated from the amplitude ratio:mean evoked/mean spontaneous) and in the frequency of spontaneous signals were compared by non-parametric statistics with those in untreated fibres or in fibres pretreated with a Ca^{2+}-channel blocking agent.

Okadaic acid (1μM) increased M by 78±16% (n=18) which was not significantly different ($P > 0.1$) from the increase (39 ± 5, n=12) calculated for untreated fibres. On washout M was sometimes reduced to below the control value. In nimodipine pretreated fibres the inhibitor-induced increase in M was 37 ± 8% (n=9). Okadaic acid increased ($P < 0.0005$) the frequency of spontaneous quantal release by 197 ±23% (n=18); the increase in untreated fibres was 43 ± 12% (n=13). Pretreatment with nimodipine (1μM) or Cd^{2+} (75μM) reduced ($P < 0.05$) the okadaic acid induced increase to 95±36% (n=9) and 80±33% (n=8) respectively. Nimodipine itself had no effect on spontaneous frequency whereas Cd^{2+} increased it 4.9-fold. Okadaic acid increased ($P < 0.02$) the amplitude of mepps by 23±5% (n=9) and mepcs by 17±4% (n=9) but neither the decline (τ) of mepcs nor the amplitude of currents evoked by iontophoretic application of carbachol was increased. The activity (Ellman *et al.*, 1961) of eel acetylcholinesterase was unaffected by okadaic acid.

The data are not inconsistent with the view that okadaic acid enhances ACh release by recruitment of L-type Ca^{2+}-channels and possibly by increasing quantal size. Failure to observe an increase in M may reflect inactivation (Werz *et al.*, 1993) of N-type channels.

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435P CHARACTERIZATION OF THE POSITIVE AND NEGATIVE INOTROPIC EFFECTS OF ACETYLCHOLINE IN THE HUMAN ISOLATED ATRIAL AND VENTRICULAR TRABECULAE

Xiao Y. Du, Regien G. Schoemaker, E. Bos & Pramod R. Saxena, Department of Pharmacology, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

We recently reported that on the human isolated myocardium acetylcholine (Ach) elicits a biphasic atrial response (a negative inotropic effect from 10^{-9}-10^{-6} M followed by a positive inotropic effect from $\geq 10^{-6}$ M), while only a positive response was observed in ventricles (Du *et al.*, 1994). We have now attempted to characterize the receptors mediating the inotropic responses to Ach in right atrial and left ventricular trabeculae (<1 mm thickness), prepared from human non-diseased hearts and mounted in organ baths (Krebs buffer containing physostigmine 10^{-6} M at 37°C, gassed with 95% O_2 and 5% CO_2 and paced at 1 Hz) for isometric tension measurement. A cumulative concentration curve was constructed with Ach (10^{-9}-10^{-3} M) in each atrial and ventricular preparation, in the absence (control) or presence of antagonists: propranolol (ß), atropine (nonselective muscarinic; M), pirenzepine ($M_1 > M_3 > M_2$), AFDX 116 ($M_2 > M_1 > M_3$), or HHSID (cyclohexylphenyl(3-piperidinopropyl)silanol HCl; $M_3 \geq M_1 >> M_2$) (Doods *et al.*, 1993). Atropine (10^{-6} M), but not propranolol (10^{-6} M), antagonized both the positive and negative inotropic effects of Ach in the atrial as well as ventricular tissue (shift approximately 3 and 4 log units, respectively). In the atrial tissue, the negative inotropic effect was most effectively antagonized by AFDX 116 (Table 1). The positive inotropic effect in the atrial tissue was not antagonized by either AFDX 116 or HHSID, but, in the presence of pirenzepine, the curve to Ach showed a rightward shift, resulting in a reduced response to Ach (10^{-3} M). In the ventricular tissue, the positive inotropic effect was best antagonized by the highest concentration of AFDX 116 (Table 1). These data show that, besides its well known negative inotropic effect, Ach can also elicit positive inotropic responses. The Ach-induced negative inotropic effect in the atrium as well as the positive inotropic effect in the ventricle, being most effectively blocked by AFDX 116, seem to be mediated by the M_2 receptor. The positive inotropic effect in atrial tissue (unmodified by AFDX 116, increased by HHSID due to blockade of the negative effect, and attenuated by pirenzepine) is not mediated by M_2 or M_3 receptors, but could involve M_1 receptor.

Table 1. Negative and positive inotropic effects of Ach (maximum % change from baseline) and apparent pD_2.

Antagonists	*µM*	*Atrium*				*Ventricle*		
		n	*Negative*	*pD_2*	*Positive*[a]	*n*	*Positive*	*pD_2*
Control		16	47±12	6.8±0.2	78±11	14	33±6	5.6±0.4
Pirenzepine	0.1	6	73±10	7.1±0.2	29±15*	5	37±5	6.3±0.2
Pirenzepine	1	8	61±7	6.4±0.2	37±11*	4	56±16	5.1±0.6
Pirenzepine	10	6	58±6	6.3±0.2	4±3*	7	50±20	4.7±1.8
Control		16	66±5	6.8±0.2	68±12	16	46±13	5.8±0.2
AFDX 116	0.1	6	67±11	6.6±0.1	50±23	6	38±9	6.1±0.1
AFDX 116	1	8	57±20	5.9±0.2*	47±37	6	26±11	4.8±0.6
AFDX 116	10	6	#*	#*	111±51	7	29±12	3.9±0.1*
Control		10	70±7	7.1±0.2	75±30	10	36±6	6.2±0.3
HHSID	1	6	69±6	6.8±0.1	51±31	6	44±12	6.0±0.2
HHSID	10	6	31±28*	6.4±0.4	106±57	5	32±10	5.3±0.4

*[a], Response at 10^{-3} M Ach, calculated as difference from the maximal negative effect (pD_2 not calculable). *, Significantly different from values in control experiments run in parallel. #, Only positive inotropic response.*

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436P INFLUENCE OF W84, AN ALLOSTERIC MODULATOR OF MUSCARINIC M_2-RECEPTORS, ON THE DISSOCIATION CHARACTERISTICS OF [^{3}H]PRAZOSIN AND [^{125}I]IODOCYANO-PINDOLOL IN RAT CARDIAC TISSUES

M.Pfaffendorf, H.D. Batink, E. Kostenis[1], C. Tränkle[1], K. Mohr[1] & P.A. van Zwieten

Dept. Pharmacotherapy, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands and [1] Dept. Pharmacology, Inst. Pharmacy, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany

The binding of ligands to muscarinic acetylcholine receptors is modulated allosterically by several compounds which is mainly indicated by a retardation of the dissociation. Among these allosteric modulators , such as alcuronium, gallamine, quinidine or verapamil, the compound W84 (N,N,N',N'-tetramethyl-N,N'-bis-(3-phtalimido-propyl)-N,N'-hexane-1,6,-diyl-bis-ammonium dibromide) is a representative of agents in which a stabilization of antagonist binding to muscarinic acetylcholine receptors is the prominent feature.

Since muscarinic receptors are members of the G-protein coupled membrane receptor family and show varying but significant homology with other receptor proteins of this type (Hulme *et al.*, 1990) the question arose whether the binding characteristics of specific ligands to α_1- and ß-adrenoceptors may be influenced likewise by W84.

The membrane fraction of homogenized rat cardiac left ventricle was incubated for 45 min. with [^{3}H]-prazosin ([^{3}H]-PRAZ, buffer: Tris 50 mM, pH 7.7 at 25°C), for one hour with (-)-[^{125}I]-iodocyanopindolol ([^{125}I]-ICYP, buffer: 10 mM Tris, 154 mM NaCl, and 0.01% ascorbic acid at pH 7.4 at 37°C) or for two hours with [^{3}H]-N-methylscopolamine ([^{3}H]-NMS, buffer: 20 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.5 at 23°C). At the end of the incubation period the dissociation was determined after the addition of an excess concentration of particular unlabelled ligands: phentolamine (α, 10 µM), (±)-CGP 12,177 (ß, 10µM) or atropine (muscarinic, 10 µM) in the presence or the absence of W84. In narrowly spaced intervals the dissociation was terminated by dilution in ice-cold incubation buffer and the bound and the free portions of the labelled ligand were separated by rapid vacuum filtration. The radioactivity on the filters was measured by standard scintillation counting ([^{3}H]-NMS, [^{3}H]-PRAZ) or standard γ-counting ([^{125}I]-ICYP) techniques. The mean data of 3-6 experiments, performed in duplicate, were analyzed.

In the experiments with [^{3}H]-NMS W84 showed a marked and concentration-dependent inhibitory effect on the dissociation rate. The dissociation half-life was increased from 2.4 ± 0.1 min under control conditions to 8.7 ± 0.6 ($P<0.05$) min in the presence of 3 µM W84 and to 29.4 ± 2.3 min ($P<0.05$) in the presence of 30 µM W84. Under the experimental conditions used, [^{3}H]-PRAZ showed a monophasic dissociation pattern with a half-life of 16.4 ± 0.6 min in the absence and of 17.4 ± 0.8 min ($P>0.05$) by 30 µM W84. The dissociation of [^{125}I]-ICYP was slow and after 3 hours more than 50% of the radioligand were still bound. Therefore, no reliable half-life could be calculated. However, at no time point the binding of ICPY in the absence and the presence of 30 µM W84 showed differences, indicating an unaltered dissociation behaviour. The results of the [^{3}H]-NMS-dissociation were very similar to data obtained in porcine left ventricular tissue (Kostenis *et al.*) and indicated that 30 µM W84 is a virtually maximal effective concentration.

From the presented data we conclude that W84, in a concentration at the far end of the concentration-response curve of [^{3}H]-NMS-dissociation retardation, does not influence the dissociation characteristics of α_1- or ß-adrenoceptors. These findings confirm the specificity of W84 as a modulator of muscarinic acetylcholine receptor binding.

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437P THE EFFECT OF ISOZYME SELECTIVE PHOSPHODIESTERASE INHIBITORS ON METHACHOLINE-INDUCED CONTRACTION OF GUINEA-PIG ILEUM

A. Tomkinson & D. Raeburn, Rhône-Poulenc Rorer Ltd., Dagenham Research Centre, Rainham Road South, Dagenham, Essex RM10 7XS.

The cyclic nucleotides cyclic AMP and cyclic GMP are now known to be important second messengers in the regulation of tone in airways, vascular and gastrointestinal smooth muscle. Elevation of cellular cyclic AMP and cyclic GMP has been associated with relaxation in several regions of the gut including the lower oesophageal sphincter, proximal colon and taenia coli (Barnette *et al.*, 1989, 1993; Joslyn *et al.*, 1990). The levels of these cyclic nucleotides are regulated by the phosphodiesterase (PDE) enzyme family. With the development of isozyme selective PDE inhibitors, the functional role of the PDE isozymes in both airways and vascular smooth muscle has been extensively investigated. As the functional role of PDE isozymes in the gut is less defined, we have used isozyme selective PDE inhibitors to determine the functional role of PDE isozymes in regulating guinea-pig ileum contractility. Lengths (15 mm) of guinea-pig ileum were suspended under an applied load (1 g) in Tyrode at 35°C and gassed with 5 % CO_2 in O_2. Following equilibration (30 min), a control cumulative concentration-response curve to methacholine (MCh) was performed on each tissue. Tissues were then pretreated (10 min) with a single PDE inhibitor (vinpocetine, type I; MEP-1, type II; siguazodan, type III; rolipram, type IV; zaprinast, type V; enprofylline, non-selective) and MCh response curves repeated. Tissues were washed and the concentration of inhibitor increased before the next MCh curve. The effect of the PDE inhibitors (10 μM - 300 μM) on the maximum response to MCh (Emax) and MCh EC_{50} (concentration to produce 50 % of the maximum response to MCh for each respective curve) are shown in table I (* $p < 0.05$ from control). All PDE inhibitors produced a concentration-dependent inhibition of the MCh response. Vinpocetine, MEP-1, zaprinast and enprofylline produced a depression of the MCh maximum only. In contrast, rolipram and siguazodan produced a rightward shift of the MCh response curve (increase in EC_{50}), with depression of the MCh maximum at higher concentrations. The results indicate that PDE inhibitors that elevate cyclic AMP produce a rightward shift of the MCh response curve, whilst those causing an elevation in cyclic GMP produce a depression of the MCh maximum response. This difference in the type of inhibition produced may reflect a different site/mechanism by which the cyclic AMP- and cyclic GMP- activated kinases may functionally antagonise contraction.

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Table I. Effects of PDE inhibitors on the MCh-induced maximum contractions and MCh EC50 values for guinea-pig ileum

		MCh Emax (% control MCh maximum)				MCh EC50 (nM)			
PDE inhibitor	n	control	10 μM	100 μM	300 μM	control	10 μM	100 μM	300 μM
vinpocetine	5	96 ± 3	81 ± 12	43 ± 8 *	40 ± 6 *	89 ± 11	58 ± 10	93 ± 28	63 ± 15
MEP-1	7	99 ± 1	78 ± 8	54 ± 11 *	30 ± 10 *	109 ± 19	89 ± 14	74 ± 10	78 ± 12
siguazodan	8	97 ± 2	98 ± 9	95 ± 11	83 ± 11	69 ± 11	90 ± 18	127 ± 23 *	150 ± 21 *
rolipram	19	93 ± 3	89 ± 4	73 ± 4 *	53 ± 3 *	82 ± 9	121 ± 9 *	272 ± 43 *	412 ± 38 *
zaprinast	7	99 ± 1	49 ± 3 *	28 ± 2 *	20 ± 3 *	63 ± 11	50 ± 8	28 ± 4 *	39 ± 8
enprofylline	4	100 ± 0	n.d.	81 ± 7 *	71 ± 2 *	125 ± 14	n.d.	138 ± 19	136 ± 19

438P EFFECT OF CIGARETTE SMOKE EXTRACT ON INTERCELLULAR ADHESION MOLECULE-1 IN HUMAN ENDOTHELIAL CELLS

A.C. Burke-Gaffney, [1]R.A. Robbins & P.G. Hellewell, Department of Applied Pharmacology, National Heart and Lung Institute, London SW3, 6LY, UK & [1]Pulmonary and Critical Care Medicine, University of Nebraska Medical Centre, Omaha, NE, USA.

Cigarette smoking is a risk factor for cardiovascular disease, however the mechanisms responsible for this have not been clearly defined. It has been shown that leukocyte adhesion to vascular endothelium (a phenomenom often associated with coronary disease) increased following exposure to cigarette smoke (Lehr *et al.*, 1993). In this study we investigated the effect of cigarette smoke extract (CSE; Laurent *et al.*, 1983) on basal or cytokine-induced intercellular adhesion molecule-1 (ICAM-1) expression in the human umbilical vein endothelial cell line, EAhy926 (Edgell *et al.*, 1983).

ICAM-1 mRNA or expression of protein were examined following exposure of EAhy926 monolayers to a combination of the cytokines tumour necrosis factor-α (TNFα), interleukin-1β (IL-1 β) and interferon-γ (IFN-γ), with or without CSE. mRNA was detected by Northern Analysis and results expressed as a ratio of mRNA for ICAM-1 to that of a non-cytokine-regulated gene, GAPDH (as determined by densitometry). Expression of ICAM-1 protein on the cell surface was detected using a three-stage streptavidin-biotin-horseradish peroxidase enzyme-linked immunosorbant assay and results expressed as absorbance ± S.E.M. (measured at 492 nm). Statistical analysis was performed using one-way ANOVA followed by the Tukey-Kramer Multiple Comparison Test. Results were deemed significant if $P < 0.05$.

ICAM-1 mRNA was undetectable in EAhy926 under control conditions or following 24h exposure to CSE (1% solution). Stimulation of monolayers with cytokine combination resulted in a significant increase in ICAM-1 mRNA by 3h that had decreased by 24h. Incubation for 24h with a combination of cytokines and CSE resulted in an mRNA ICAM-1/GAPDH ratio (0.338 ± 0.021; n=3) that was significantly greater ($P < 0.001$) than that for cytokines or CSE alone. A dose-dependent increase in cell surface expression of ICAM-1 was detected following 24h exposure to cytokines. However, in the presence of a fixed concentration of CSE (1%) the expression was significantly reduced. A significant reduction ($P < 0.001$) was also measured at 48h following either continuous exposure to cytokines/CSE or exposure to stimuli for 24h and medium alone for a further 24h. At an earlier time point (5h), no significant difference was detected between cytokine-treated monolayers either in the presence (O.D. 1.793 ± 0.049; n= 4) or absence (O.D. 1.744 ± 0.009) of CSE.

These results demonstrate that cytokine-induced ICAM-1 mRNA was prolonged in the presence of CSE, but that the prolongation did not result in an increase in surface expression of the protein above that detected with cytokines alone. Furthermore, exposure of the cells to cytokines/CSE for up to 48h decreased ICAM-1 expression which may be accounted for by CSE-induced cell death. In conclusion, under the conditions investigated CSE did not induce surface expression of ICAM-1 or enhance that stimulated by cytokines. It is possible therefore that mechanisms other than regulation of ICAM-1 account for cigarette smoke-induced leukocyte adhesion to vascular endothelium.

Support of the National Asthma Campaign is gratefully acknowledged.

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439P EFFECTS OF CIGARETTE SMOKE ON THE ABILITY OF ISOLATED RINGS OF RAT PULMONARY ARTERY TO RELAX TO ACETYLCHOLINE AND SODIUM NITROPRUSSIDE

G. Wilson, W. Martin, M. Haghighi & D. Pollock, Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ.

Cigarette smoking has been implicated in cardiovascular disease but few studies have examined the effects of cigarette smoke on the pulmonary vasculature. This is surprising since the constituents of inhaled cigarette smoke will have immediate access to the pulmonary circulation and therefore they are the vessels most likely to be affected at an early stage, since they will be exposed to the highest concentration of the constituents of cigarette smoke at their site of absorption. Morphological abnormalities in endothelial cells have been observed in humans who have smoked for long periods (Rubinstein et al, 1991) but the pathological mechanisms responsible for cigarette smoke-induced damage to blood vessels are still poorly understood. This study examined the effects of solutions of cigarette smoke on the responsiveness of the pulmonary vasculature to determine if cigarette smoke could influence the capacity of isolated rings of pulmonary artery and its branches to relax to endothelium-dependent and endothelium-independent vasorelaxants. Standard solutions of cigarette smoke (SCS) were obtained using a smoking machine, which consisted of a small pump with a piston in a calibrated barrel. This machine allowed the volume of smoke per stroke and the rate to be controlled. so that smoke could be drawn (20 strokes min^{-1}) through the lit cigarette by the pump, which then blew the smoke through a valve and a scintered glass bubbler into Krebs-Henseleit buffer, which had been gassed with 95% O_2 and 5% CO_2. Standard solutions were prepared using Players untipped cigarettes, each of which contained 1 mg nicotine and 14 mg tar. The standard solution (SCS) was prepared by bubbling the smoke from 4 cigarettes through 100 ml of buffer. Male Wistar rats (200-250g) were stunned and killed by bleeding. The main and first branch pulmonary arteries were excised and 3-4mm rings were mounted under 1.5 g tension on stainless steel hooks in 25ml organ baths containing Krebs-Henseleit buffer (37°C), Responses were recorded isometrically. Cumulative concentration-response curves were obtained in main pulmonary artery rings (MPA) and first branch pulmonary artery rings (BPA) to noradrenaline (NA,10^{-9}-10^{-5}M), and in rings precontracted with NA (EC75=10^{-7}M), cumulative inhibitory concentration-response curves were obtained to acetylcholine (ACh,10^{-9}-10^{-4}M) and to sodium nitroprusside (SNP,10^{-9}-10^{-5}M) in the absence and presence of SCS. The effects of aqueous extracts of unburned tobacco were also examined. SCS (0.1ml of standard solution) inhibited vasorelaxant responses of the BPA to ACh (maximum response: control = 73.0+0.7 %; SCS treated = 47.0+0.6, mean + s.e.mean, n =10, 0.01>P>0.001) but did not affect vasorelaxant responses to SNP (maximum response: control =100.0 + 1.0%; SCS treated = 98.0 + 1.0. Similar results were obtained in the MPA. Small volumes (0.1ml) of SCS did not affect contractile responses to NA but larger volumes (2 ml) inhibited contractile responses of the MPA to NA (maximum response: control = 0.30+0.01 g mg^{-1} tissue; SCS treated = 0.20+0.03, n=10, P<0.001). Aqueous extracts of unburned tobacco had no effect on responses of either the MPA or the BPA to vasoactive drugs. These results indicate that low concentrations of an aqueous solution of cigarette tobacco smoke inhibit ACh-induced, endothelium-dependent vasorelaxation in pulmonary artery rings but do not affect the endothelium-independent vasorelaxtion caused by SNP. The components present in SCS that are responsible for this effect are unknown but they may contribute to the toxic effects of tobacco smoke in the pulmonary vasculature and perhaps elsewhere.

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440P PREJUNCTIONAL INHIBITION OF CHOLINERGIC NEUROMUSCULAR TRANSMISSION IN THE GUINEA-PIG ILEUM BY DIADENOSINE POLYPHOSPHATES

A.U.Ziganshin[1], C.H.V.Hoyle, L.E.Ziganshina[1] & G.Burnstock, Dept. Anatomy & Developmental Biology, University College London, Gower Street, London WC1E 6BT and [1]Kazan Medical Institute, Kazan, CIS

The aim of the present study was to determine whether or not adenine dinucleotides can cause prejunctional inhibition, of cholinergic neuromuscular transmission in the guinea-pig ileum, and if so, by what type of receptor. Guinea-pigs were killed by stunning and bleeding. Isolated guinea-pig longitudinal muscle/myenteric plexus preparations were maintained in a standard Krebs' solution at 37 ± 0.5 °C, gassed with 95% O_2 / 5% CO_2 in 10 ml organ baths. Electrical field stimulation (0.3 ms, supramaximal V, 0.2 Hz), facilitated by Pt-wire ring electrodes, evoked twitch contractions that were abolished by atropine (0.3 μM) or tetrodotoxin (0.3 μM). Concentration-response relationships were determined for adenosine, ATP and a series of P^1,P^n-bis(5'-adenosinyl)oligophosphates where 'n' ranged from 2 to 6 (Ap_2A to Ap_6A). All these compounds caused a concentration-dependent reduction in the cholinergic twitch, with similar threshold concentrations (around 0.1 μM), maximum responses (almost abolition) and pD_2 (-log EC_{50}) values (Table 1, data given as mean ± s.e.mean). The P_1-purinoceptor antagonist, 8-para-sulphophenyltheophylline (8*p*SPT, 30 μM) antagonised each compound, with no significant differences amongst the pA_2 values (Table 1). Dipyridamole (50 nM) potentiated all the compounds to a similar extent (increase in pD_2, Dip ΔpD_2 in Table 1) These results indicate that α,ω–diadenosine oligophosphates inhibit cholinergic neuromuscular transmission via prejunctional P_1-purinoceptors, possibly via an indirect mechanism that involves the generation or release of adenosine.

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Table 1.

	Adenosine	ATP	Ap_2A	Ap_3A	Ap_4A	Ap_5A	Ap_6A
pD_2	5.6 ± 0.1 (6)	5.5 ± 0.2 (9)	5.7 ± 0.1 (7)	5.5 ± 0.1 (8)	5.6 ± 0.1 (8)	5.6 ± 0.1 (8)	5.6 ± 0.1 (6)
8*p*SPT pA_2	4.9 ± 0.2 (4)	5.3 ± 0.2 (4)	5.4 ± 0.2 (3)	4.8 ± 0.2 (3)	5.0 ± 0.3 (4)	5.0 ± 0.3 (4)	5.0 ± 0.1 (4)
Dip ΔpD_2	0.6 ± 0.1 (4)	0.4 ± 0.1 (4)	0.9 ± 0.1 (4)	0.6 ± 0.1 (4)	0.9 ± 0.1 (4)	0.8 ± 0.1 (4)	0.4 ± 0.1 (4)

441P CONFOCAL IMAGING OF CALCIUM ION ACTIVITY IN QUAIL FIBROBLAST CULTURES EXPRESSING NICOTINIC ACETYLCHOLINE RECEPTORS

K.M.L. Cross, S.D. Jane, A.E. Wild[1], R.C. Foreman & J.E. Chad (introduced by L. Holden-Dye), Department of Physiology & Pharmacology and [1]Department of Biology, University of Southampton, Southampton SO16 7PX.

Ratiometric confocal microscopy with the calcium-sensitive fluorescent dye Indo-1 was used to measure intracellular calcium ion ($Ca^{2+}i$) activity in quail fibroblasts (QF18 cells) stably transfected with constructs encoding muscle-type nicotinic acetylcholine receptors (AChRs; Phillips et al.,1991). $Ca^{2+}i$ was calculated from average fluorescence ratios measured from a 140μm^2 area within each cell. Application of either acetylcholine, or the nicotinic agonist suberyldicholine (SDC), to the transfected QF18 cells caused an increase in $Ca^{2+}i$. Control $Ca^{2+}i$ levels in QF18 cells were found to be 164±22nM (mean ± s.e.mean; n=40 cells; 12 experiments) rising to 600±81nM on addition of SDC (10^{-5}M; n=15 cells; 5 experiments), whereas no increase in $Ca^{2+}i$ was seen in nontransfected control fibroblasts (before 128±9nM; n=40; 12 experiments, after 113±13nM; n=15; 5 experiments; QT6 cells). The increases in $Ca^{2+}i$ were dose dependent with an EC_{50} value of 12.7±5.9μM (n=14; 5 experiments) for SDC.
The responses to SDC (10^{-5}M) in QF18 cells were blocked by prior application of 200nM α-bungarotoxin Control $Ca^{2+}i$ level was unchanged after SDC application (n=14 cells; 5 experiments), indicating that the change in $Ca^{2+}i$ involved activation of nicotinic AChRs. However, the responses to SDC were also prevented by the addition of 100μM Cd^{2+} to the bathing solution; control $Ca^{2+}i$ level, in the presence of Cd^{2+}, was 271±55nM, and after SDC application was 263±54nM (4 experiments). This implicated the involvement of voltage-dependent Ca^{2+} channels. This was further tested by experiments in which Na^+ ions were removed from the bathing solution (Tris substitution) reversing the Na^+ gradient and preventing depolarisation on activation of nicotinic AChRs. Na^+ removal prevented any change in $Ca^{2+}i$ in response to SDC (10^{-5}M). Control $Ca^{2+}i$ level was 146±21nM and following SDC application was 133±19nM (n=8 cells; 4 experiments). The three-dimensional distribution of Ca^{2+} within fibroblasts at rest and following application of SDC was determined by optically sectioning cells at 2μm intervals along their z-axis. At the sampling times used (4s per frame over 40s) no gradient in $Ca^{2+}i$ was observed.

We postulate that activation of the nicotinic AChRs leads to a Na^+ dependent depolarization and hence activation of endogenous voltage-sensitive Ca^{2+} channels in the plasma membrane.

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442P CHARACTERISATION OF NICOTINIC ACETYLCHOLINE RECEPTOR- AND P_2-PURINOCEPTOR-MEDIATED Ca^{2+}-RESPONSES IN C2C12 MYOTUBES

R.H. Henning, M. Duin, J.P. van Popta, S.A. Nelemans and A. den Hertog.
Department of Clinical Pharmacology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands.

Internal Ca^{2+}-concentration regulates important properties of skeletal muscle, including desensitisation and expression of its nicotinic acetylcholine receptors (nAChRs). Both the transmitter acetylcholine (Grassi *et al.*, 1993) and the co-transmitter ATP (Henning *et al.*, 1993) increase Ca^{2+} in C2C12 myotubes.
Stimulation of nAChRs with carbachol (0.1-100 µM) induced an initial increase in internal Ca^{2+} (1 min) followed by a sustained phase in myotubes, using fura-2 florescence (Table). Carbachol-evoked Ca^{2+}-response was blocked by pre-treatment with pancuronium (3 µM), but not affected by atropine (3 µM), or diltiazem (1 µM). Stimulation of P_2-purinoceptors with ATP (10 µM - 1 mM) induced a Ca^{2+} response qualitatively similar to that of carbachol (Table).
Under Ca^{2+}-free conditions, the sustained phase of the carbachol- and ATP-evoked Ca^{2+} response was abolished, and the amplitude was decreased to about 70% (Table). Under low Na^+ /Ca^{2+}-free conditions, carbachol-induced Ca^{2+}-mobilisation was completely abolished, while the ATP-induced response was not affected. In the presence of high K^+, the carbachol-evoked Ca^{2+}-mobilisation was abolished, in contrast to the ATP-induced response.
In ATP-stimulated cells, the sustained phase of the Ca^{2+}-response, representing Ca^{2+}-influx, was abolished by La^{3+} (50 µM) or high K^+. The sustained phase of the carbachol-induced response was not affected under these conditions, and –once developed– it was found to be insensitive to pancuronium. Low Na^+ conditions did not affect the sustained phase of either carbachol- or ATP-induced responses.
Under Ca^{2+}-free conditions, ATP (300 mM) induced a four fold increase in inositol(1,4,5)P_3 , whereas carbachol (10 mM) did not change basal inositol(1,4,5)P_3 levels (binding assay).
It is concluded that the increase in internal Ca^{2+} following stimulation of nAChRs is due to Na^+-triggered mobilisation of Ca^{2+}, and influx of Ca^{2+}, not gated via the nAChR, voltage-activated Ca^{2+}-channels or Na^+/Ca^{2+}-exchange. P_2-purinoceptor activated Ca^{2+}-response is due to activation of phospholipase C and subsequent formation of inositol(1,4,5)P_3. Despite the different mechanism involved in nAChR and P_2-purinoceptor-evoked Ca^{2+}-responses, prominent cross-talk between both receptor systems was observed (Table).

Table 1. Increase in Ca^{2+} evoked by nAChR and P_2-purinoceptor agonists. The plateau was measured at 4 min. Values are expressed as increase in fluorescence ratio (F340/380). n≥12.

	Ca^{2+} conditions		Ca^{2+}-free conditions	
agonist	*peak*	*plateau*	*peak*	*plateau*
ATP (1 mM)	1.11 ± 0.04	0.31 ± 0.05	0.80 ± 0.05	-
carb (10 µM)	0.83 ± 0.07	0.21 ± 0.02	0.56 ± 0.05	-
ATP after carb	1.02 ± 0.07	-	0.52 ± 0.06	-
carb after ATP	0.52 ± 0.05	-	0.06 ± 0.02	-
carb + ATP	1.42 ± 0.10	0.23 ± 0.05	1.04 ± 0.10	-

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443P THE EFFECTS OF CALYCULIN A UPON CALCIUM-, GUANINE NUCLEOTIDES- AND PHORBOL 12-MYRISTATE 13-ACETATE-STIMULATED ACTH SECRETION FROM AtT-20 CELLS

S.B. Guild & B.W. McFerran, Molecular Endocrinology Unit, School of Biological and Medical Sciences, University of St Andrews, St Andrews, UK.

A study of protein phosphorylation states in the mouse AtT-20/D-16-16 pituitary tumour cell line revealed that a decrease in phosphorylation state could be correlated with adrenocorticotrophin (ACTH) secretion (Bishop *et al.*, 1987). The involvement of protein phosphatases in the secretory pathway for ACTH secretion from these cells was, therefore, investigated by the use of the protein phosphatase inhibitor, calyculin A (Ishihara *et al.*, 1989). The effects of calyculin A upon calcium-, guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S)- and phorbol 12-myristate 13-acetate (PMA)-stimulated ACTH secretion from electrically permeabilised AtT-20 cells was studied as previously described (McFerran & Guild, 1994).

Calyculin A (10^{-9}-10^{-6}M) inhibited calcium (10^{-5}M, 215 ± 5% (n=3) increase over 10^{-9}M value)- and GTP-γ-S (10^{-4}M, 198 ± 5% (n=3) increase over control value)-evoked ACTH secretion from permeabilised cells in a concentration-dependent manner with a maximal effect by 10^{-7}M calyculin A (when ACTH secretion was not significantly different in the presence or absence of calcium (10^{-5}M) or GTP-γ-S (10^{-4}M)). Calcium stimulated ACTH secretion in a concentration-dependent manner, over the concentration range of 10^{-7}M to 10^{-5}M (a maximal increase to 210 ± 10% (n=3) of 10^{-9}M value). This calcium-stimulated ACTH secretion was not evident in the presence of calyculin A (10^{-7}M). GTP-γ-S stimulated ACTH secretion from permeabilised cells in a concentration dependent manner with a threshold of 10^{-6}M (a maximal increase to 205 ± 5% (n=3) of the control value at 10^{-4}M GTP-γ-S). In the presence of calyculin A (10^{-7}M), there was no significant GTP-γ-S-stimulated ACTH secretion. PMA significantly stimulated ACTH secretion from permeabilised cells, in the absence of either calcium and guanine nucleotides, at concentrations of PMA above 10^{-8}M (an increase to 128 ± 5% (n=3) of control value for 10^{-7}M PMA) and this action of PMA was potentiated by calyculin A (10^{-7}M) (an increase to 178 ± 20% (n=3) of control value for 10^{-7}M PMA). Co-incubation of PMA (10^{-7}M) with GTP-γ-S (10^{-4}M) prevented the inhibition of GTP-γ-S (10^{-4}M)-stimulated ACTH secretion observed in the presence of calyculin A (10^{-7}M) when PMA was absent.

The results indicate that phosphatases plays an important role in stimulus-secretion coupling in AtT-20 cells and may mediate the effects of guanosine 5'-triphosphate (GTP)-binding proteins upon secretion. Furthermore, a point of regulation of the secretory response by protein kinase C may lie distal to these phosphatases.

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444P COMPUTER-BASED PROBLEM-SOLVING EXERCISES TO TEACH HAEMOSTASIS TO UNDERGRADUATES

J.A. Overfield[1], D. G. Dewhurst & D. Davies, Faculty of Health & Social Care, Leeds Metropolitan University, Calverley Street, Leeds LS1 3HE & Department of Biological Sciences, [1]Manchester Metropolitan University, Chester Street, Manchester M1 5GD.

A computer-assisted learning program which adopts a case-centred approach to teaching the anaemias has been demonstrated to the Society (Dewhurst, et al, 1992) and has proved to be an extremely useful teaching resource. The program we describe here adopts a similar approach to teaching haemostasis and is aimed at undergraduate students from a range of bio-medical science courses. It extends a program (Blood Coagulation: Sheffield BioScience programs) designed to teach the principles of the tests used in the laboratory investigation of coagulation (Dewhurst, et al, 1994).

The program runs on any true IBM compatible PC (XT, AT and PS2) with approximately 400Kb of free conventional memory and graphics capability (EGA or VGA). It is written in C (Turbo C++, Borland) and utilises an easy-to-use, Windows-like menu system. It is accompanied by printed support materials (student and tutor's notes) and may be used either in association with a teacher-designed protocol or for independent learning.

The program contains a Tutorials section which uses a combination of text, colour graphics and animated graphics to describe the morphology and physiological function of platelets, the haemostatic mechanisms including platelet plug formation and the coagulation system and fibrinolysis.

A major section of the program is Clinical Cases in which students are presented with details of five different case studies of common coagulation disorders: haemophilia A; von Willebrand's disease, alcohol-induced liver disease, disseminated intravascular coagulation (DIC) and promyelocytic leukaemia. For each case study students have access to:

- a brief medical history and a haemostatic profile including test results of bleeding time, prothrombin time, thrombin time, activated partial thromboplastin time, and full blood count.

They must then study the profiles and answer an on-screen question asking them to identify which of the following are normal/abnormal: intrinsic pathway, extrinsic pathway, common pathway or haemostatic plug formation. Feedback is given for both correct and incorrect interpretations. At this stage they then have access to:

- the results of relevant further tests: for example in the case study of the patient suffering from von Willebrand's disease they are provided with a platelet aggregation profile (ristocetin and ADP) and a plasma factor VIII level.

Finally they must choose:

- the correct diagnosis from a list of possible diagnoses.

Learning is reinforced at this final stage by providing further details of the disorder. The program also contains a glossary of the medical terms used which is accessible at all times.

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445P AN INTERACTIVE, COMPUTER-BASED TUTORIAL PROGRAM TO TEACH SYNAPTIC TRANSMISSION TO UNDERGRADUATE PHARMACOLOGY STUDENTS

G.G.S. Collins[1], D. G. Dewhurst & R.T. Ullyott, Faculty of Health & Social Care, Leeds Metropolitan University, Calverley Street, Leeds LS1 3HE, and [1]Department of Medicine & Pharmacology, University of Sheffield, Western Bank, Sheffield S10 2TN.

The Teaching and Learning Technology Program (TLTP) was established with the aim of making teaching and learning in higher education more productive and efficient by harnessing modern technology. Here we describe a computer-assisted learning program: Synaptic Transmission in the Central Nervous System, which has been developed as part of the TLTP Pharma-CAL-ogy project. It is aimed at first year students and is suitable for undergraduates from science, medical and a range of biomedical courses which include basic pharmacology modules.

The CAL program was developed using Authorware Professional® (Macromedia Inc.) and both IBM PC and Macintosh versions are available. The minimum delivery platform for IBM microcomputers is a 386 SX, 25MHz PC running Windows™ (Microsoft), a sixteen colour VGA monitor and a mouse. The program will run on any Macintosh microcomputer with a hard disk and a colour monitor.

The learning package takes the form of an interactive tutorial and uses a number of features to enhance student learning. The menu has six options:

- aims and objectives;
- introduction;
- neuronal stucture;
- synaptic transmission;
- neurotransmitters and postsynaptic receptors;
- neurotransmitters and neuronal excitability.

High quality colour graphics are used extensively throughout the program and animation sequences are used to explain the steps in synaptic transmission, and the action of neurotransmitters at ligand-gated and G-protein coupled receptors. There are also several interactive features, some examples of which are given below:

- students are required to label diagrams by "dragging" labels from a list and "dropping" them into the box corresponding to the correct position on the diagram;

- in some sections information is provided in the form of multiple choice questions which give helpful feedback to incorrect answers and more detailed explanations for correct responses;

- "drag" and "drop" sections to characterise different types of neurotransmitter chemicals;

- presentation of data and recordings to explain excitatory and inhibitory postsynaptic potentials and the interaction of inhibitory and excitatory inputs.

The learning package is intended for independent study and could be used as an alternative to resource-intensive, staff-led tutorials. The program would occupy students for 1-2 hours of fairly intensive study and is suitable for primary learning or revision. The question-answer sections may also be useful for self-assessment.

Tutors with access to Authorware Professional may modify the program to tailor it to their particular needs.

446P A MULTI-TASK COMPUTER TRACKING SYSTEM FOR THE MEASUREMENT AND ANALYSIS OF BEHAVIOUR IN ANIMAL MODELS OF ANXIETY AND PANIC

S. R. G. Beckett, R. S. Kulkarni and C. A. Marsden. Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham, NG7 2UH.

Computer aided tracking systems are an important addition to most behavioural pharmacology laboratories providing reliable and accurate measurement of locomotor behaviour. Different behaviours and test environments often demand the use of specifically designed systems. For example the elevated plus maze is used to measure aversion by comparing open to closed arm entries and time (Pellow *et al.* 1985) while activation of the periaqueductal grey (PAG) results in defence behaviour often assessed by duration (Beckett *et al.* 1992). We have recently developed a computer based method of tracking which can be readily applied to behavioural models as diverse as those described above.

VideoTrack, developed in conjunction with CPL Systems, Cambridge, runs under windows on IBM compatible PC's using a standard video camera and digitising board. Tracking is achieved by calculating the 'difference' between two sequential pictures. As the animal is the only moving object in the field of view then any difference detected is attributable to movement of the animal. Test apparatus are divided into recording zones allowing different locomotor patterns to be examined.

The effect of diazepam on the plus maze was determined by computer analysis and the results compared to those obtained by manual scoring. Similarly, the effect of locally applied 8-hydroxy 2-(di-n-propylamino)tetralin (8-OHDPAT) on PAG induced locomotor behaviour was measured and compared using both computer and manual methods. Both manual and computer methods revealed a significant increase in the amount of time and entries in plus maze open arms produced by diazepam (3mg/kg i.p.) indicating an anxiolytic effect (table 1).

Table 1. Manual and computer measures on the plus maze.

	vehicle (n=9)		diazepam 3mg/kg (n=8)	
	%open arm entries	time in open arms (s)	%open arm entries	time in open arms (s)
manual	31.7±2.4	46.8±8.1	52.5±6.5*	74.4±9.9*
computer	31.4±2.2	53.1±7.0	49.0±5.2**	90.7±19.5*

(*P<0.05, **P<0.01 one way ANOVA post hoc Duncan NMR)

Observer rated measurements of defence behaviour showed a significant decrease in response duration produced by intra-PAG 8-OHDPAT from 36.8±8s to 4.5±3s. Computer tracking of the same behaviour revealed a significant reduction in the distance travelled during the first 40 seconds of the response by 8-OHDPAT (table 2).

Table 2. Computer measure of PAG induced defence behaviour.

time period	distance travelled (m) DLH alone	8-OHDPAT+DLH(n=11)
0-20 s	9.5±2.3	3.1±1.1*
20-40s	4.5±0.8	1.5±.03*

(*P<0.05 two way ANOVA, post hoc Duncan NMR)

The data demonstrate that it is possible to reliably track forms of behaviour using a system based on differential image analysis. Plus maze behaviour can be accurately assessed by measuring zone transitions whilst distance and speed values provide a method of quantifying rapid defence behaviour. Non-parametric behavioural measures however, such as the number of head dips, stretch attends rears or jumps still had to be scored manually.

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447P INHIBITION OF FOOT TAPPING IN GERBILS BY CNS PENETRANT TACHYKININ NK_1 RECEPTOR ANTAGONISTS

A. R. Williams and N.M.J. Rupniak, Neuroscience Research Centre, Merck Sharp & Dohme, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR

448P NOVEL CYCLIC PSEUDOPEPTIDE ANTAGONISTS OF TACHYKININ NK_1 AND NK_2 RECEPTORS

R. Patacchini, L. Quartara[1], M. Astolfi[2], C. Goso[2], A. Giachetti & C.A. Maggi. Pharmacology and [1]Chemistry Department, A. Menarini Pharmaceuticals, Florence Italy. [2]Pharmacology Department, Menarini Ricerche Sud, Pomezia, Rome.

ORAL COMMUNICATIONS

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1P **Grimwood S, Barton CL, Hutson PH, Myers J, Le Bourdèlles B & Whiting PJ** The pharmacological characterisation of a stably transfected cell line expressing human NMDAR1a/NMDAR2A receptors, using radioligand binding techniques

2P **Danks P, Le Bourdelles B, Myers J, Whiting P, Kemp JA & Priestley T** Pharmacological profile of recombinant human NR1a+NR2A and native rat cerebellar granule cell NMDA receptors

3P **Chazot PL, Cik M, Coleman S & Stephenson FA** Characterisation of the polyamine modulatory site on recombinant NR1/NR2A receptors

4P **Chapman AG, Millan MH, Kingsley C & Meldrum BS** Anticonvulsant activity of a novel competitive NMDA antagonist, the S-enantiomer SDZ EAB 515

5P **Rowley HL, Martin KF & Marsden CA** Generalised seizures inhibit the release of GABA but augment the release of glutamate from rat hippocampus *in vivo*

6P **Williams RJ, Glowinski J & Prémont J** Glucose deprivation potentiates glutamatergic receptor-mediated arachidonic acid release from cultured striatal neurones

7P **Saulskaya N & Marsden CA** Changes in extracellular glutamate in the nucleus accumbens during a conditioned emotional response

8P **Gray DW, Challiss RAJ & Nahorski RAJ** Effects of ionotropic glutamate receptor agonists on the activation of phosphoinositide C by metabotropic glutamate receptors in rat cerebellar granule cells

9P **Thomas NK, Jane DE, Pittaway K, Sunter DC & Watkins JC** Antagonism of L-AP4- and (1S,3S)-ACPD-induced depression of dorsal root-evoked monosynaptic excitation of neonatal motoneurones by the novel antagonists MSPG and MPPG

10P **Thompson GA, Chalmers DJ, Jane DE, Sunter DC, Udvarhelyi PM, Kilpatrick IC & Watkins JC** Identification of the stereoisomers of 3-(4-chlorophenyl)-glutamate that potentiate L-homocysteic acid-evoked depolarisations in neonatal rat motoneurones

11P **Hawkins LM, Roberts PJ, Beaver KM, Jane DE, Taylor PM & Sunter DC** Pharmacology and regional distribution of (S)-[^{3}H]5-fluorowillardiine binding in rat brain, a new radioligand for the study of AMPA receptors

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14P **Cao CQ, Jane DE, Tse H-W, Headley PM & Evans RH** Enhancement of spinal excitatory postsynaptic currents (EPSCs) by α-methyl-(2S,3S,4S)-α-carboxycyclopropylglycine (MCCG) *in vitro*

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17P **Davia K, Wynne DW & Harding SE** Control of relaxation in cardiac myocytes isolated from the ventricles of noradrenaline-treated guinea-pigs and patients with heart failure

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149P **Gartside SE, Umbers V, Hajós M & Sharp T** 5-HT_{1A} receptor blockade prevents the inhibitory effect of paroxetine on 5-HT cell firing: relationship to changes in brain extracellular 5-HT

150P **Parkins KJ, Cheng CHK, Costall B & Naylor RJ** Role of the 5-HT_{1A} receptor on *in vivo* acetylcholine release in rat hippocampus

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152P **Schoeffter P & Bobirnac I** 5-HT_{1D} receptors mediate inhibition of cyclic AMP accumulation in MDCK cells

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154P **Maurel-Remy S, Audinot V, Lejeune F, Bervoets K & Millan MJ** Blockade of phencyclidine-induced hyperlocomotion in rats by clozapine, MDL 100,907 and other antipsychotics correlates with affinity at 5-HT_{2A} receptors

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156P **Millan MJ, Audinot V, Brocco M, Maurel-Remy S, Joly F, Newman-Tancredi A, Candon H, Hautefoye P, Spedding M, Muller O & Lavielle G** S 16924: a pyrrolidine-substituted benzodioxane with higher affinity at 5-$HT1_{A/2A/2C}$ than D_2 receptors, and an 'atypical' antipsychotic profile in the rat

157P **Baxter GS, Ellis ES, Forbes IT, Jones GE, Kennett GA, Murphy OE & Tilford N** SB 204741: a potent and selective antagonist at 5-HT_{2B} receptors

158P **Adham N, Gerald C, Vaysse PJ-J, Weinshank RL & Branchek TA** Characterization of the functional responses mediated by two splice variants of the cloned rat 5-HT_4 receptor in heterologous expression systems

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12 Woolmer Way
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