1P ON THE QUANTIFICATION OF RECEPTOR FUNCTION AND THE MOLECULAR MECHANISMS OF AGONIST ACTION AT G-PROTEIN LINKED RECEPTORS

B.R. Gardner & P.G. Strange, Research School of Biosciences, The University, Canterbury, Kent CT2 7NJ

There are several models that attempt to explain agonism, the preeminent theory amongst these being occupancy theory. An alternative model that has gained considerable acceptance in recent years is the operational model (Black & Leff, 1983), which has particular relevance for G-protein linked receptors (GPLR). According to both of these models, as the strength of the stimulus increases the intrinsic activity of agonists should increase until limited by the tissue's maximal response (Stephenson, 1956; Black & Leff, 1983).
In this study we have determined agonist affinities (Table 1) for the D_{2L} dopamine receptor, a GPLR, by employing a radioligand binding assay. Agonist potencies and intrinsic activities were also determined for the primary response of G-protein activation (Table 1) using a [^{35}S]GTPγS binding assay (Gardner & Strange, 1994). We also calculated the operational efficacies, τ, of agonists from the observed affinities and potencies using the formula;

$$\tau = \frac{K_A}{EC_{50}} - 1$$

It is clear from these results that the predicted correlation between τ and intrinsic activity is not observed. This discrepancy can be accounted for if efficacy is composed of two components, an activational component and a stimulatory component. A molecular model of agonism that divides efficacy into two components is the extended ternary complex model (Samama *et al.*, 1993). In this model the receptor must undergo an isomerisation from an inactive to an active state before it can interact with a G-protein. The isomerisation event (activational component) can be described by a two state model (Leff, 1995), while the interaction between the activated receptor and G-protein (stimulatory component) can be described by the operational model (Black & Leff, 1983). For this model of agonism to explain the observed results it must be assumed that the ability of an agonist to cause the isomerisation of the receptor to the active state is independent of the ability of the agonist to increase the affinity of the receptor for G-protein.

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Table 1: Pharmacological characteristics of dopamine agonists acting on membrane preparations from a CHO cell line stably expressing D_{2L} dopamine receptors. The agonist affinities and potencies are mean values ± SEM (n≥3) or mean values ± range (n=2). The value in parenthesis is the number of determinations. I.A. represents the percentage intrinsic activity relative to dopamine.

	pK_{iGTP}	pEC_{50}	τ	I.A.		pK_{iGTP}	pEC_{50}	τ	I.A.
Dopamine	5.21±0.12 (5)	6.05±0.09 (5)	6.0	100	(-)-3-PPP	6.07±0.12 (3)	6.62±0.19 (2)	2.5	10±2
Quinpirole	5.20±0.08 (4)	5.85±0.10 (6)	3.6	96±5	Bromocriptine	8.01±0.07 (2)	8.12±0.06 (5)	0.3	56±5
Apomorphine	6.78±0.02 (3)	6.76±0.12 (3)	0.0	90±18	Dihydroergocristine	8.20±0.02 (2)	8.86±0.13 (2)	3.4	16±8
NPA	7.73±0.04 (3)	7.76±0.15 (4)	0.1	114±7					

2P MODULATION OF INS(1,4,5)P_3-INDUCED Ca^{2+} RELEASE FROM PERMEABILISED PLATELETS BY cGMP-DEPENDENT KINASE ACTIVITY

C.T.Murphy & J.Westwick, Department of Pharmacology, School of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY

It is now well established that D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is responsible for the release of Ca^{2+} from intracellular stores. It has previously been demonstrated that elevation of intracellular levels of cGMP causes inhibition of agonist-induced platelet activation which may be due to the regulation of intracellular levels of Ca^{2+}. One possible site of action for cGMP-dependent kinase activity is at the level of the Ins(1,4,5)P_3-receptor [Ins(1,4,5)P_3-R]. This possibility has been examined by investigating the regulatory properties of cGMP and the nitric oxide donor sodium nitroprusside (SNP) on Ins(1,4,5)P_3-induced Ca^{2+} release in permeabilised platelets.

Rabbit platelets were prepared as previously described (Murphy *et al.*, 1991). Mobilisation of Ca^{2+} from the intracellular stores of permeabilised platelets was determined using the Ca^{2+} specific probe fura-2 (free acid) in the extracellular buffer and changes in fluorescence monitored by spectrophotofluorimetry (20°C) (Guillemette *et al.*, 1989). Metabolism of [^{3}H]Ins(1,4,5)P_3 to [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,3,4,5)P_4 in permeabilised platelets at 20°C was determined by anion exchange HPLC (Ward *et al.*, 1992).

The D-isomer (0.1-9 μM) but not the L-isomer (0.1-9 μM) of Ins(1,4,5)P_3 caused a dose-dependent release of Ca^{2+} from the intracellular stores of permeabilised platelets with an EC_{50} of 0.4 ± 0.08 μM (mean ± S.E.M) (n=9). Treatment of platelets with D-Ins(1,4,5)P_3 did not desensitise the Ins(1,4,5)P_3-R to subsequent additions of D-Ins(1,4,5)P_3. Treatment of platelets with heparin for 3 min inhibited Ca^{2+} release induced by 1 μM D-Ins(1,4,5)P_3 with an IC_{50} of 16.8 ± 2.4 μM (n=4).

Treatment of permeabilised platelets for 10 min with either 3-100 μM of cGMP or 3-30 μM of SNP caused a dose-dependent inhibition of Ca^{2+} release induced by 1 μM D-Ins(1,4,5)P_3 (IC_{50} = 46 ± 11.4 μM for cGMP; IC_{50} = 18.6 ± 4.8 μM for SNP; n=4). Inhibition of Ca^{2+} release by a submaximal concentration of cGMP (30 μM) was further enhanced from 68.1 ± 5.8% of vehicle control to 3.9 ± 0.34% of vehicle control (n=4) by a 2 min pretreatment with 10 μM of the selective type IV cGMP-specific phosphodiesterase inhibitor M&B 22948 (Radomski *et al.*, 1990).

To establish whether the observed inhibitory effects of cGMP and SNP on Ca^{2+} release were due to cGMP-dependent kinase activation, platelets were treated with the protein kinase inhibitor staurosporine (2 μM, 2 min) before treatment with either 30 μM SNP or 100 μM cGMP (10 min) followed by 1 μM Ins(1,4,5)P_3. Treatment with staurosporine resulted in complete reversal of the inhibition caused by either 30 μM SNP or 100 μM cGMP (n=3). Neither cGMP (100 μM) nor SNP (30 μM) had any effect on the rate of metabolism of [^{3}H]Ins(1,4,5)P_3 added to permeabilised platelets, nor individually on the production of either of the resulting two metabolites [^{3}H]Ins(1,4)P_2 or [^{3}H]Ins(1,3,4,5)P_4 when compared to vehicle controls ($P > 0.1$ at all time points; one-way ANOVA).

The findings of this study therefore suggest a role for cGMP-dependent kinase activity in the regulation of Ins(1,4,5)P_3 mediated release of Ca^{2+} from the intracellular stores of platelets.

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3P Ca^{2+}-REGULATED CALMODULIN BINDING TO PURIFIED INOSITOL 1,4,5-TRISPHOSPHATE ($InsP_3$) RECEPTORS

Sandip Patel, Alison Harris* and Colin W. Taylor, Department of Pharmacology, Tennis Court Road, Cambridge CB2 1QJ and *Amersham International plc, Whitchurch, Cardiff, Wales, CF4 7YT.

In many cells, $InsP_3$ mediates the release of Ca^{2+} from intracellular stores. Ca^{2+} exerts its effects by either direct interaction with target proteins or indirectly through Ca^{2+}-binding proteins such as calmodulin. The role of calmodulin in mediating the effects of Ca^{2+} on $InsP_3$ receptors is unclear. Here, we use Scintillation Proximity Assays (SPA†) to examine calmodulin binding to pure $InsP_3$ receptors.

Purified $InsP_3$ receptors (40 μg/ml) were attached to wheat germ agglutinin-coated SPA beads (1.25 mg beads/ml) by incubation for 2.5 hours at 2°C (Patel *et al.*, 1995). Binding of [^{125}I]calmodulin (3 - 4 nM; 35 μCi/μg) to the $InsP_3$ receptor-beads (0.625 mg beads/ml) was performed at 2°C in a cytosol-like medium (CLM) with the free $[Ca^{2+}]$ ($[Ca^{2+}]_m$) buffered between 2 nM and 30 μM. Non-specific binding (typically 10% of total binding) was determined in the presence of 50 μM calmodulin. Samples (200 μl) were counted for 60 s at 2°C

In Ca^{2+}-free CLM, [^{125}I]calmodulin bound specifically to $InsP_3$-receptor beads, but not to the beads alone. Ca^{2+} ($[Ca^{2+}]_m$ = 30 μM) reversibly stimulated calmodulin binding by 4.0 ± 0.6 fold (n = 10) and the effect was half-maximal when $[Ca^{2+}]_m$ was 184 ± 14 nM (n = 3). Association of calmodulin to the receptor-beads was faster in the presence of Ca^{2+} than in Ca^{2+}-free CLM; k_{obs} was 0.05 ± 0.009 min.$^{-1}$ (n = 3) when $[Ca^{2+}]_m$ was 2 nM and 0.18 ± 0.009 min.$^{-1}$ (n = 3) when $[Ca^{2+}]_m$ was 30 μM. From competition-equilibrium binding analyses, the K_d for calmodulin was 219 ± 42 nM (n_H = 0.75 ± 0.16; n = 3) when $[Ca^{2+}]_m$ was 2 nM and 355 ± 45 nM (n_H = 0.65 ± 0.02; n = 3) when $[Ca^{2+}]_m$ was 30 μM. Using standard ^{3}H and ^{125}I-SPA beads to establish counting efficiencies, the B_{max} for calmodulin was 1.0 ± 0.3 pmol/mg bead when $[Ca^{2+}]_m$ was 2 nM and 5.7 ± 0.3 pmol/mg bead when $[Ca^{2+}]_m$ was 30 μM; the B_{max} for $InsP_3$ was 13 ± 0.6 pmol/mg bead. Each tetrameric $InsP_3$ receptor therefore appears to bind ~0.31 and ~1.75 calmodulin molecules in the absence and presence of Ca^{2+}, respectively. W7 (500 μM) abolished Ca^{2+}-stimulated calmodulin binding, but not its Ca^{2+}-independent binding. $InsP_3$ (1 μM) did not affect calmodulin binding in either the absence or presence of Ca^{2+}.

We conclude that calmodulin binds to purified $InsP_3$ receptors and that physiological increases in intracellular $[Ca^{2+}]$ increase its binding by exposing additional binding sites.

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4P ACTIONS OF PHORBOL DIBUTYRATE ON SPONTANEOUS ELECTRICAL ACTIVITY IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

Connors, S.C., Mamas, M.A. & Terrar, D.A. University Dept of Pharmacology, Mansfield Road Oxford OX1 3QT and Nuffield Dept of Anaesthetics, Woodstock Road, Oxford OX2 6HE.

Phorbol esters have been found to exert negative inotropic effects at doses greater than 10^{-8} M in Langendorff perfused hearts, with a decline in the calcium transient measured in isolated myocytes using fura-2 (Ward & Moffat, 1992). The purpose of the present experiments was to investigate whether phorbol dibutyrate might influence spontaneous activity thought to be related to calcium release from the sarcoplasmic reticulum in ventricular myocytes.

Myocytes were isolated from guinea-pig ventricular muscle and superfused with a balanced salt solution containing 2.5 mM Ca and 5.4 mM K (36°C). Membrane potential was recorded with conventional intracellular microelectrodes containing 1M $KMeSO_4$ with 10 mM KCl (resistance 25-50 MΩ). In some experiments a 'switched' voltage-clamp was applied (clamp at -70 mV and trains of 15 depolarizations to +40 mV, interval 300 ms, were applied with a cycle time of 10s).

In cells stimulated to fire action potentials at 1 Hz, exposure to isoprenaline (20-100 nM) provoked delayed after-depolarisations, and in most cells additional spontaneous action potentials were also observed. Exposure to 10^{-7}M phorbol dibutyrate markedly suppressed this spontaneous activity in 6 of 6 cells (in 5 cells spontaneous action potentials were abolished, and in the remaining cell, their frequency was greatly reduced). In a further series of experiments exposure to 10^{-6} M phorbol dibutyrate also supressed isoprenaline-induced delayed after-depolarizations and spontaneous action potentials (in 6 of 6 cells). These effects were significant ($P<0.05$, Wilcoxon signed-ranks test). When spontaneous transient inward currents were provoked by exposure to ouabain (10^{-6} M for 5 to 8 min) with the voltage-clamp protocol described above, exposure to 10^{-7} M phorbol dibutyrate supressed the spontaneous activity in 4 of 5 cells. In the absence of either isoprenaline or ouabain, calcium transients (constructed from the magnitude of calcium-activated tail currents following interruption of action potentials by voltage clamp to -70 mV, Terrar & White 1989) were significantly reduced following exposure of the cells to 10^{-7} M phorbol dibutyrate, so that at 10 min the peak amplitude was reduced by 38±2 %, n=9 ($P<0.05$, paired 't' test).

In conclusion, phorbol dibutyrate applied under the conditions described here can reduce spontaneous activity associated with calcium-induced calcium release from sarcoplasmic reticulum stores. Since we have observed a reduced calcium transient in the presence of phorbol dibutyrate, the observed suppresion in spontaneous activity following exposure to phorbol dibutyrate may be associated with a reduced loading and/or release of calcium from SR stores.

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5P DISPARATE SIGNALLING PATHWAYS UTILISED BY HEK 293 CELLS TRANSFECTED WITH MCP-1 TYPE A OR TYPE B RECEPTORS

Turner, S. J., Ward, S. G., Smith, G., Raport,C.J.[1], Schweickart, V.[1], and Westwick, J., Pharmacology Dept., University of Bath, Claverton Down, Bath, U.K.; [1]ICOS Corp., 20th Ave SE, Bothell, WA 98021, U.S.A.

Monocyte chemotactic peptide-1 (MCP-1), a member of the C-C chemokine family, is a potent activator of human monocytes (Yoshimura *et al.*, 1994). Three MCP-1 receptors have been characterised to date, two high affinity receptors, namely type A and type B, and a low affinity receptor (Charo *et al.*, 1994; Neote *et al.*, 1993).
We have looked at the binding of MCP-1 to the two high affinity receptors when transfected into HEK 293 cells, as well as the changes in $[Ca^{2+}]_i$ and activation of the signal transduction pathway phosphoinositide 3-kinase (PI 3-kinase) in response to MCP-1.
Biotinylated MCP-1 was incubated with the cells and then an avidin-FITC conjugate was used to allow analysis of MCP-1 binding using a fluorescence activated cell sorter (FACS). The data showed specific MCP-1 binding to both receptor types as seen by the increase in fluorescence intensity and inhibition by MCP-1-antibody (see table I).

Table I-MCP-1 binding in HEK 293 cells expressing the type A or type B MCP-1 receptor (data±sem.,n=3 *p<0.05 compared to control)

	Mean fluorescence intensity		
Receptor Type	Control	MCP-1	MCP-1 antibody
Type A	18.93±1.42	36.0±4.0 *	22.96±1.21
Type B	25.22±3.2	57.65±12.4 *	25.4±2.4

Changes in cytosolic calcium levels were studied in fura-2 loaded HEK 293 cells. MCP-1 type A receptor transfected cells showed no change in $[Ca^{2+}]_i$ in response to up to 60nM MCP-1, RANTES, MIP-1α, MIP-1β or the C-X-C chemokine IL-8.
The type B receptor transfected cells showed a dose dependent increase in $[Ca^{2+}]_i$ in response to 1.25-60nM MCP-1, which was due to both calcium mobilisation and calcium influx. The response to 12.5nM MCP-1 was inhibited by the PLC inhibitor U73122 (100% at 10μM, n=3), 1mM Ni^{2+} (62.75±7.9%, n=4) and 1mM EGTA (62.1±5.79%, n=7); readdition of 1mM Ca^{2+} after EGTA treatment restored the $[Ca^{2+}]_i$ response. The use of 300μM Mn^{2+} as a surrogate calcium ion revealed an MCP-1-induced Mn^{2+} influx (42.25±7.1% at 12.5nM MCP-1, n=3). 100% Mn^{2+} influx was induced by 10μM ionomycin. All these $[Ca^{2+}]_i$ changes in type B receptor transfected cells were also inhibited by 16 hours pre-treatment with 250ng/ml pertussis toxin (73.9±8.7%, n=4).
To study PI 3-kinase activity, the HEK 293 cells were labelled with ^{32}P-orthophosphate, stimulated and the lipids extracted as described previously (Jackson *et al.*, 1992). The MCP-1 type A receptor transfected cells showed an increase in PI 3-kinase activity, as defined by the increase in the levels of the product, phosphatidylinositol (3,4,5) trisphosphate, following 180nM MCP-1 stimulation for 30 secs (157.8±44.7% above control, n=3) but the type B receptor transfected cells showed no increase in PI 3-kinase activity (4.83±4.8% above control, n=3).
This study has shown that, although MCP-1 binds to both of its receptors, only the type A receptors show PI 3-kinase activation and only the type B receptors induce an increase in $[Ca^{2+}]_i$ which is apparently linked to both calcium mobilisation and calcium influx.

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6P CHARACTERISATION OF CHO CELLS EXPRESSING THE CLONED RAT μ OPIOID RECEPTOR

D.G. Lambert, R.A. Hirst, D. Smart and D.K. Grandy[1] University Department of Anaesthesia, Leicester Royal Infirmary, Leicester. LE1 5WW. [1]Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201-3089. USA.

We have previously reported in SH-SY5Y human neuroblastoma cells (expressing both μ and δ receptors) that μ-opioids inhibit basal and forskolin stimulated cAMP formation (Lambert et al., 1993). In addition, μ- but not δ-opioids stimulate $Ins(1,4,5)P_3$ formation in a Ca^{2+} dependent manner. (Smart et al, 1994; 1995). In this study we have examined the effects of opioids on cAMP and $Ins(1,4,5)P_3$ formation in CHO cells expressing the cloned rat μ-opioid receptor as an homogenous expression system.

Stock cultures of CHOμ cells were maintained in Hams F12 medium supplemented with 10% foetal calf serum, 100iu/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml fungizone and 70μg/ml G418. Cells used for experimentation were cultured free of G418 for 7 days. The binding of [^{3}H]diprenorphine (DPN) to CHOμ cell membranes was performed essentially as described (Bunzow et al., 1995). cAMP and $Ins(1,4,5)P_3$ measurements were made in whole CHOμ cell suspensions at 37°C in Krebs/HEPES buffer, pH 7.4 as described (Lambert et al., 1993; Smart et al., 1994). Data are mean±s.e.mean. Data were analysed using Student's t-test and considered significant when $p<0.05$.

All experiments were performed with CHOμ cells of passage 12-17 inclusive. Over this range the maximum number of receptors (B_{max}) and their affinity for DPN (K_d) remained relatively constant. (B_{max} and K_d at passage 12, 13 and 15 were 266±10, 287±19 and 235±4fmol/mg protein and 0.19±0.02, 0.20±0.02 and 0.29±0.02nM respectively, n=3). Fentanyl (36.4±6.1% inhibition at 1μM, n=4, $p<0.05$) and DAMGO (41.4±4.8% inhibition at 1μM, n=5, $p<0.05$) but not DPDPE (8.6±3.6% inhibition at 1μM, n=5) inhibited forskolin-stimulated cAMP formation. Fentanyl did not affect basal cAMP formation. Fentanyl (100nM) also produced a time dependent stimulation of $Ins(1,4,5)P_3$ formation peaking at around 2min (basal, 57±5; fentanyl 2min, 136±17pmol/mg protein, n=3, $p<0.05$)

These data clearly demonstrate that CHOμ transfects behave in a similar manner to SH-SY5Y cells expressing endogenous μ opioid receptors. In both cell types an inhibition of forskolin stimulated cAMP formation (without stimulating basal) and a stimulation of $Ins(1,4,5)P_3$ formation is observed. Further studies to compare desensitisation mechanisms to SH-SY5Y cells for cAMP and $Ins(1,4,5)P_3$ formation are currently underway.

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7P ROLE OF Ca^{2+} IONS IN THE STIMULATION OF cAMP ACCUMULATION BY HISTAMINE IN CHO-K1 CELLS TRANSFECTED WITH THE BOVINE H_1 RECEPTOR

E.M. Sanderson, *P.A. Iredale & S.J. Hill, Department of Pharmacology and Physiology, Queen's Medical Centre, Nottingham, NG7 2UH. *Department of Psychiatry, Yale University School of Medicine, New Haven, USA.

In mammalian brain slices, histamine (HA) potently stimulates cAMP accumulation by the activation of both H_2 and H_1 HA receptors. Whilst H_2 receptors directly activate adenylyl cyclase (AC), H_1 receptors appear to act indirectly, by augmenting AC activity stimulated by G_s linked receptors *eg.* H_2 or A_{2b} receptors (Al-Gadi & Hill 1985). In these tissues, influx of extracellular Ca^{2+} has been implicated in the H_1 receptor effect on cAMP production (Donaldson *et al* 1998). In this study, we have examined the role of Ca^{2+} mobilization, on H_1 receptor mediated cAMP accumulation in CHO-K1 cells transfected with the bovine H_1 receptor.

Accumulation of cAMP and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in H_1 receptor transfected CHO-K1 (CHO-H_1) cells were measured as previously described (Megson *et al* 1995, Iredale & Hill 1993). All data are expressed as mean ± s.e.mean. In CHO-H_1 cells, HA (1 mM) augmented forskolin (1 μM)-stimulated cAMP accumulation (4.1 ± 0.6 fold; n = 6). However, HA (1 mM) also stimulated basal cAMP levels (3.5 ± 0.7 fold; n = 6). Both responses were blocked by the H_1 receptor antagonist mepyramine (pK_D's = 8.75 ± 0.16 and 9.08 ± 0.33 for HA-forskolin, n = 5, and HA-alone, n = 4), These responses were well maintained in Ca^{2+}-free media (+0.1mM EGTA, HA-forskolin = 3.4 ± 0.9 times the forskolin response: HA = 3.9 ± 0.8 fold over basal). In Ca^{2+}-free media, the Ca^{2+} ionophore ionomycin (1 μM) transiently raised $[Ca^{2+}]_i$ to the same extent as HA (100 μM) (579 ± 96 nM Ca^{2+}, n = 7;. 425 ± 99 nM Ca^{2+}, n = 4, respectively), and these responses could be completely abolished by preincubation with the Ca^{2+} chelator BAPTA/AM (50 μM). However, 1 μM ionomycin was unable to mimic the effects of HA on cAMP production (n = 3). Furthermore, BAPTA/AM (50 μM) was ineffective in modifying the cAMP response to HA (n = 3).

The results of this study suggest that in CHO-H_1 cells, neither intracellular or extracellular Ca^{2+} is involved in the mechanisms linking H_1 receptor activation to changes in the accumulation of intracellular cAMP.

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8P IMMUNOHISTOCHEMICAL LOCALISATION OF THE SOMATOSTATIN sst_2 RECEPTOR IN THE RAT BRAIN AND SPINAL CORD

M. Schindler[1,2], P.P.A. Humphrey[2] and P.C. Emson[1]. 1) MRC Molecular Neuroscience Group, Department of Neurobiology, Babraham Institute, Cambridge CB2 4AT and 2) Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge CB2 1QJ.

Somatostatin is widely distributed in the mammalian brain and is believed to play a role as a neurotransmitter/neuromodulator. The genes for five types of high affinity somatostatin receptor (sst_1-sst_5) have been cloned from rat and human tissues. Northern blotting and in situ hybridisation have shown the presence of the transcripts for all the cloned receptors in the CNS. As part of a study to investigate the distribution of the corresponding proteins, we have raised polyclonal antibodies specific for the carboxy-terminus of sst_2 and used them in immunohistochemistry and Western blotting.

A peptide corresponding to amino acids 347-366 of the rat sst_2 receptor (Kluxen et al., 1992) was coupled to bovine serum albumin using glutaraldehyde. Aliquots of the conjugate (500μg per boost) were emulsified in Freund's Adjuvant and injected into sheep. IgG fractions were purified from the immune serum and affinity purified against a recombinant fusion protein incorporating the entire carboxy-terminus of the sst_2 receptor. Specific antibodies were eluted and used for immunohistochemistry on sections (30μm) of fixed rat brain.

In Western blotting experiments, the antibody specifically detected fusion proteins incorporating the carboxy-terminus of the sst_2 receptor, whilst no cross-reactivity was observed with other recombinant receptor proteins. All specific bands could be blocked by pre-incubating the antibody (1:1000 dilution) with 100μM peptide. Immunohistochemistry on rat brain sections showed a distinct distribution of the sst_2 protein. The signal was located especially on fibres and processes in the deeper layers of the cerebral cortex, the endopiriform cortex, the amygdala (Fig. 1A), the claustrum, the subiculum, the locus coeruleus, the dorsal central grey, the islands of Calleja and the arcuate nucleus. Fibres were also observed in the interpeduncular nucleus and the medial habenula, whereas fibres as well as cell bodies were identified in the hippocampal formation. In the spinal cord, fibres and cell bodies were labelled exclusively in the substantia gelatinosa (dorsal horn). Controls included the use of pre-immune serum, omission of the primary antibody, and pre-blocking of the antibody with 100μM peptide (Fig. 1B). The data obtained are in accordance with the distribution of the mRNA for sst_2 (Kong et al., 1993; Señaris et al., 1994) and an autoradiography study employing the sst_2 receptor selective radioligand, ^{125}I-BIM 23027 (Holloway et al., this meeting).

Figure 1A

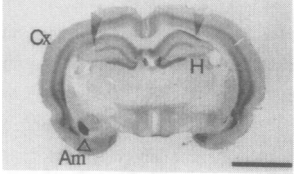

Figure 1B

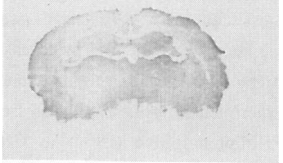

Figure 1A: Immunohistochemical localisation of sst_2 receptor protein in a coronal section of rat brain. Calibration bar 3mm. Cx cerebral cortex; H Hippocampus; Am amygdala.

Figure 1B: Pre-adsorption control using 100μM of peptide.

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9P HUMAN RECOMBINANT sst_5 RECEPTORS EXPRESSED IN CHO-K1 CELLS MEDIATE INCREASES IN EXTRACELLULAR ACIDIFICATION BY PERTUSSIS TOXIN-SENSITIVE AND -INSENSITIVE PATHWAYS

R.J. Thurlow, L. Sellers, J.E. Coote[1], W. Feniuk and P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ; [1]Glaxo-Wellcome Research and Development, Gunnels Wood Road, Stevenage, SG1 2NY.

The functional characteristics of the human recombinant somatostatin sst_5 receptor expressed in CHO-K1 cells were investigated by measuring changes in extracellular acidification rates (EARs).

Cells were seeded at 5 x 10^5 cells per cup 18h prior to experimentation. Bicarbonate-free HAMS F-12/DMEM 1:1 media (pH 7.4) containing bacitracin (0.2mg ml^{-1}) was perfused over the cells at 120µl min^{-1} in the Cytosensor microphysiometer. EARs were measured for 10s of a total perfusion cycle time of 1min. Basal rates were between 100-250 µV s^{-1} (0.1-0.25 pH units/min). Increasing concentrations of somatostatin (SRIF) or SRIF receptor selective analogues (Raynor *et al*, 1993a, b) were administered at hourly intervals with a contact time of 3 min 40s. Responses were expressed as a percentage of the maximum increase in EAR produced by a maximally effective concentration (0.1µM) of SRIF (61.6 ± 3.5% above basal, n=24) administered at the start of each experiment.

All of the analogues examined caused concentration-dependent increases in EAR. L-362,855 was the most potent whilst BIM 23027 and BIM 23056 were the weakest agonists studied (Table 1). The maximum response to L-362,855 was lower ($P<0.05$; unpaired t-test, two tail) than that produced by SRIF. Treatment of cells (16h prior to study, n=6) with pertussis toxin (PTX; 50ng ml^{-1}) caused a significant decrease ($P<0.05$; paired t-test, two tail) in the acidification response to 0.1µM SRIF (Control increase in EAR, 114 ± 5 µV s^{-1}; PTX treatment 53 ± 4 µV s^{-1}). Responses to UTP (0.1mM) were unchanged in the two groups (Control 61± 8 µV s^{-1}; PTX treatment 64 ± 9 µV s^{-1}). Higher concentrations of PTX (100 and 500ng ml^{-1}) caused no further inhibition, responses to $SRIF_{14}$ (0.1µM) were 55 ± 4 µV s^{-1} and 57 ± 5 µV s^{-1}, respectively.

Table 1. Potencies of SRIF analogues at increasing EAR in CHO-K1 cells expressing human sst_5 receptors (n=4)

	EC_{50} (nM) Geom. mean [95% C.L.]	Hill slope mean ± s.e.mean	% SRIF max mean ± s.e.mean
SRIF	0.54 [0.13-2.26]	0.63 ± 0.11	125.3 ± 24.6
$SRIF_{28}$	0.41 [0.18-0.97]	1.05 ± 0.23	94.8 ± 7.6
BIM 23027	116.6 [64.8-210.0]	0.70 ± 0.04	132.8 ± 5.0
L-362,855	0.09 [0.04-0.21]	1.10 ± 0.18	69.1 ± 10.1
BIM 23056	—	—	50% at 1µM
MK 678	41.5 [19.0-90.6]	0.84 ± 0.07	95.0 ± 19.6
CGP 23996	6.90 [2.57-18.5]	0.77 ± 0.13	132.7 ± 12.3
Octreotide	32.8 [20.2-53.3]	0.67 ± 0.09	100.5 ± 6.4

The results for this study show that the sst_5 receptor selective ligand L-362,855 is a potent agonist, albeit partial, at human sst_5 receptors expressed in CHO-K1 cells that mediate increases in EAR. The rank order of agonist potencies (L-362,855>SRIF>>BIM 23027) observed in this study is the reverse of that obtained in a similar study in Ltk⁻ cells expressing human recombinant sst_2 receptors (Castro *et al.*, 1995). The marked but incomplete inhibition of the response to SRIF by a supramaximal concentration of PTX suggest that responses to SRIF in this cell line are mediated by both a PTX-sensitive and insensitive pathways.

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10P CHARACTERISATION OF THE HUMAN RECOMBINANT sst_5 RECEPTOR IN CHO-K1 CELLS BY QUANTIFICATION OF GUANOSINE-5'-O-(3-[^{35}]THIO)TRIPHOSPHATE BINDING

A.J. Williams, A.D. Michel, J.E. Coote[1], L. Sellers, W. Feniuk and P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ; [1]Glaxo Wellcome Research and Development, Gunnels Wood Road, Stevenage, SG1 2NY.

Receptor-stimulated GTPγS binding is a quantitative method of characterising the functional activity of some G protein-coupled receptors (Lazareno & Birdsall, 1993). We have examined whether somatostatin (SRIF) sst_5 receptor activation increases GTPγS binding, in the presence and absence of sodium. SRIF and the selective peptide analogues, L-362,855 (sst_5) and BIM-23027 (sst_2), were used to characterise the response (Raynor *et al.*, 1993).

Membranes, from CHO-K1 cells transfected with the human sst_5 receptor, were prepared as described (Castro *et al.*, 1994) and suspended in 50mM Tris buffer, 11mM $MgCl_2$, 1mM EDTA (pH 7.4 at 21°C) either in the presence or absence of 100mM NaCl. Competition binding experiments using [^{125}I]Tyr^{11}-SRIF were performed at 21°C for 2h with non specific binding determined in the presence of 1µM SRIF. Binding of [^{35}S]-GTPγS in the presence of 1µM GDP was measured after a 90 min pre-incubation with agonist followed by co-incubation with [^{35}S]-GTPγS (30 min in the presence or 15 min in the absence of 100mM NaCl). All experiments were terminated by vacuum filtration. Curve maxima are % stimulation over basal [^{35}S]-GTPγS binding. Data are the mean ± s.e.m, n ≥ 3.

In the presence of NaCl, pEC_{50} values were markedly lower than the corresponding pIC_{50} values in its absence ($P<0.05$; unpaired t-test, two tail). In the presence of NaCl the maximum stimulation of [^{35}S]-GTPγS binding produced by L-362,855 was much less than that produced by the other agonists (see table; $P<0.05$, ANOVA). Furthermore, L-362,855 (0.3 µM) surmountably antagonized responses to SRIF (55.7 ± 3.5 % inhibition of response to 1 µM, n=3; $P<0.01$; Student's t-test, two tail). [^{35}S]-GTPγS binding was carried out in the absence of NaCl to compare agonist potencies with their binding affinities under similar conditions. The pEC_{50} values were significantly less than in the presence of NaCl ($P<0.05$; unpaired t-test, two tail) by approximately 10-fold and closer to the corresponding pIC_{50} values. The maximum effect produced by L-362,855 in the absence of NaCl was not significantly different from that of the other agonists (see table).

This study shows that GTPγS binding can be used to measure the functional activity of sst_5 receptors. Both functional and binding experiments produce rank orders of agonist potencies which agree with previously reported data for the sst_5 receptor (Raynor *et al*, 1993b). In addition, L-362,855 behaves as a partial agonist at the sst_5 receptor transfected into CHO-K1 cells.

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Raynor, K. *et al.*, (1993) *Mol. Pharmacol.* 44, 385-392.

Agonist affinities (pIC_{50}) for [^{125}I]Tyr^{11}-SRIF binding sites and potencies (pEC_{50}) at stimulating [^{35}S]-GTPγS binding.

	Experiments performed in the absence of NaCl					Experiments performed in 100mM NaCl		
	pIC_{50}	n_H	pEC_{50}	n_H	Maximum	pEC_{50}	n_H	Maximum
L-362,855	9.43 ± 0.08	1.11 ± 0.21	8.10 ± 0.13	1.28 ± 0.45	197 ± 4.3	7.91 ± 0.24	1.15 ± 0.63	159 ± 5.6
$SRIF_{28}$	9.31 ± 0.06	0.77 ± 0.08	8.36 ± 0.23	0.92 ± 0.36	220 ± 5.4	7.12 ± 0.15	0.84 ± 0.24	358 ± 19.0
SRIF	8.99 ± 0.08	0.80 ± 0.13	7.70 ± 0.12	0.90 ± 0.22	218 ± 5.4	6.85 ± 0.13	0.98 ± 0.27	324 ± 16.7
BIM-23027	7.71 ± 0.14	0.71 ± 0.08	6.30 ± 0.11	1.03 ± 0.24	179 ± 6.9	<6	-	-

11P SOMATOSTATIN AND NEUROPEPTIDE Y ELEVATE INTRACELLULAR CALCIUM IN SH-SY5Y CELLS WHEN APPLIED IN THE PRESENCE OF CARBACHOL

M. A. Connor, A. Jones & G. Henderson, Dept. Pharmacology, University of Bristol, University Walk, Bristol, BS8 1TD.

In the human neuroblastoma cell line SH-SY5Y, δ and μ opioid receptor agonists mobilize intracellular Ca^{2+} when applied in the presence of carbachol (Connor & Henderson 1994). Both somatostatin (Friederich *et al.* 1993) and neuropeptide Y (NPY) (McDonald *et al.* 1994) have been reported to inhibit voltage-dependent Ca^{2+} currents in SH-SY5Y cells, we have now examined whether these agents can also elevate Ca^{2+} in SH-SY5Y cells.

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) were measured in confluent monolayers of undifferentiated SH-SY5Y cells loaded with the Ca^{2+} sensitive dye Fura 2. Standard ratiometric measurements of $[Ca^{2+}]i$ were made. Data are presented as mean ± s.e.mean.

Somatostatin (100 nM, n=4) never elevated $[Ca^{2+}]_i$ when applied alone. However, when applied in the continued presence of carbachol (1 μM), somatostatin elevated $[Ca^{2+}]_i$ in a concentration-dependent manner. The EC_{50} for somatostatin elevating $[Ca^{2+}]_i$ in the presence of carbachol was 6 nM (95 % confidence limits 3-13 nM, n=5). Pretreatment of the cells with pertussis toxin (200 ng/ml, 16 hrs) completely abolished the increases in $[Ca^{2+}]_i$ caused by somatostatin (n=5). An initial somatostatin application resulted in profound desensitization of subsequent responses. A submaximal concentration of somatostatin (30 nM) was applied for 30s, at an interval of 15 min. The elevation of $[Ca^{2+}]_i$ caused by the second application of somatostatin was only 32 ± 3 % of the first ($P<0.01$, paired t-test, n=5). This desensitization is in contrast to that observed with the δ opioid agonist [D-$Pen^{2,5}$]enkephalin (DPDPE). When a submaximal concentration of DPDPE (30 nM) was applied for 30s, at an interval of 15 min, the elevation of $[Ca^{2+}]_i$ caused by the second application was 94 ± 4 % of the first ($P>0.2$, paired t-test, n=5).

NPY (30-100 nM, n=4) also never elevated $[Ca^{2+}]_i$ when applied alone. However, when applied in the continued presence of carbachol, NPY elevated $[Ca^{2+}]_i$ in a concentration-dependent manner. The EC_{50} for NPY elevating $[Ca^{2+}]_i$ in the presence of carbachol was 20 nM (95 % confidence limits 12-34 nM, n=5). Pretreatment of the cells with pertussis toxin (200 ng/ml, 16 hrs) completely abolished the increases in $[Ca^{2+}]_i$ caused by NPY (n=5). When a submaximal concentration of NPY (30 nM) was applied for 30s, at an interval of 15 min, the elevation of $[Ca^{2+}]_i$ caused by the second application of NPY was 88 ± 2% of the first ($P<0.025$, paired t-test, n=5).

It has been reported that the NPY receptor responsible for inhibition of the voltage-dependent Ca^{2+} currents in SH-SY5Y cells may be of the Y2 type (McDonald *et al.* 1994). We found that PYY(3-36), a selective Y2 receptor agonist (Dumont *et al.* 1994), also elevated $[Ca^{2+}]_i$ when applied in the presence of carbachol. The EC_{50} for PYY(3-36) was 13 nM (95 % confidence interval 9-19 nM, n=5); PYY(3-36) (100 nM) never elevated $[Ca^{2+}]_i$ when applied alone (n=4).

We have previously shown that δ and μ opioid receptors elevate $[Ca^{2+}]_i$ in SH-SY5Y cells when applied in the continued presence of an agonist which activates phospholipase C-coupled receptors. This study demonstrates that both somatostatin and NPY can also couple to the elevation of $[Ca^{2+}]_i$ via pertussis toxin-sensitive G-proteins. Thus elevation of $[Ca^{2+}]_i$ may represent a common effector pathway for Gi/Go coupled agonists in SH-SY5Y cells, in addition to the previously described inhibition of voltage-dependent Ca^{2+} channels.

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12P ENDOTHELIN-1 AND RAT PERIAQUEDUCTAL GRAY AREA: AN AUTORADIOGRAPHIC AND FUNCTIONAL PHARMACOLOGICAL STUDY

[1,2,3]M. D'Amico, [2]M.R. Dashwood and [3]T.D. Warner. [1]Institute of Pharmacology and Toxicology, 2nd University of Naples, Italy; [2]Department of Physiology, Royal Free Hospital, Rowland Hill Street, London; [3]The William Harvey Research Institute, Charterhouse Square, London.

ET-1 injected into the periaqueductal gray (PAG) area of rats induces pressor responses (D'Amico *et al.*, 1995). As two ET receptor subtypes have been identified (ET_A and ET_B), we have identified, by means of *in vivo* and *in vitro* studies, the central receptor(s) mediating this pressor response and the associated haemodynamic changes. Thus, we have examined the effects of FR 139317, an ET_A receptor selective antagonist, SB 209670, an ET_A/ET_B receptor non-selective antagonist, and BQ-788, an ET_B receptor selective antagonist, on the responses to ET-1. By *in vitro* autoradiographic studies we have also identified binding sites for ET-1 in the PAG.

Male Wistar rats (250-300 g) were anaesthetised with urethane ethyl carbamate (1.2 g/kg i.p.) and their femoral arteries cannulated for measurement of mean arterial blood pressure (MAP). The spontaneously breathing animals were then placed in a stereotaxic head frame and the dorsal surface of the brain exposed by a craniotomy to permit intracerebral microinjections. The coordinates of the atlas of Paxinos and Watson (1986) (measured in mm from the bregma: posteriorly, -7.8; laterally, 0.8; vertically, 4.5) were used to position the microsyringe. Total peripheral resistance (TPR) and cardiac output (CO) were evaluated by administration of ^{57}Co-labelled microspheres into the left ventricle (McDevitt and Nies, 1976). In vitro autoradiography of the PAG was performed using 10 μm sections of tissue incubated with [^{125}I]-PD151242 or [^{125}I]-BQ3020 to identify ET_A and ET_B binding sites, respectively.

In vitro autoradiography showed dense binding of [^{125}]-PD151242 in the PAG area, with the binding sites being evenly distributed within the dorsal, lateral and ventral subregions. Tissues incubated with [^{125}]-BQ3020 showed little binding. All injections of ET-1 and antagonists were made into the PAG area. Injection of ET-1 (0.1 - 10 pmol/rat) significantly and dose-dependently increased the MAP. These increases in MAP were greatly reduced by FR 139317 (5 nmol/rat) or SB 209670 (3 nmol/rat), but were unaffected by BQ-788 (5 nmol/rat) (e.g. ΔMAP ET-1, 1 pmol; 31 ± 6.6 mmHg: plus FR 139317; 2 ± 1 mmHg: plus SB 209670; 5 ± 0.8 mmHg: plus BQ-788; 27 ± 5 mmHg. ET-1 0.1, 1 or 10 pmol reduced left renal blood flow by 13 ± 1%, 20 ± 5% and 27 ± 9%, respectively (control; 9.9 ± 0.8 ml min^{-1}) and increased renal vascular resistance (RVR) by 2 ± 1%, 20 ± 4% and 27 ± 3% (control; 11.7 ± 1.4 mmHg ml min^{-1}). Following pretreatment with FR 139317, SB 209670 or BQ-788 ET-1 (1 pmol) increased RVR by 3 ± 1%, 10 ± 2% and 21 ± 4%, respectively. ET-1 (10 pmol) also increased TPR by 100% and decreased CO by approximately 30% (control, 2.39 ± 0.2 mmHg ml^{-1} min 100g body weight, and 94.7 ± 3.1 ml min^{-1}). Vascular resistances were also increased in many other organs. For instance in skeletal muscle the vascular resistance increased by 88%, in the colon by 55% and in the stomach by 47%. In contrast, no significant changes were observed in the brain, spleen, caecum, liver or lungs. Pretreatment of the PAG with FR 139317 or SB 209670 reduced the increases in TPR and the reduction in CO. BQ-788 did not influence the haemodynamic changes induced by ET-1.

Thus, there are predominantly ET_A binding sites within the PAG, with a subpopulation of ET_B binding sites, and ET-1 injected into the PAG causes complex haemodynamic changes which are sensitive to ET_A receptor antagonism. Thus, ET_A receptors are most probably the predominant mediator of the actions of ET-1 in the PAG of the rat.

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13P ANTISENSE CONSTRUCTS DIRECTED AGAINS $G_{\alpha q/11}$ ATTENUATE G PROTEIN AND REDUCE MUSCARINIC M-CURRENT INHIBITION FOLLOWING INTRANUCLEAR INJECTION IN RAT SYMPATHETIC NEURONES

M.P. Caulfield, J.E. Haley, F.C. Abogadie, Y. Vallis & N.J. Buckley, Wellcome Laboratory for Molecular Pharmacology, Dept of Pharmacology, University College London, Gower Street, London. WC1E 6BT

Initial studies with injections of antisera raised against the C-terminal decapeptide of G proteins established that $G_{\alpha q}$ and/or $G_{\alpha 11}$ are involved in the transduction of M-current inhibition by muscarinic receptor agonists (Caulfield *et al.*, 1994). Such antisera do not discriminate functionally between $G_{\alpha q}$ and $G_{\alpha 11}$, so we have explored the use of DNA constructs expressing sequences that are antisense to parts of G-protein gene sequences (Abogadie *et al.*, 1995). These should provide more specific tools to define G-protein involvement in cell function. In this study we have assessed the ability of these antisense constructs to reduce both G protein levels and muscarinic receptor modulation of the potassium M-current $I_{K(M)}$.

Rat superior cervical ganglion (SCG) neurones were cultured for 48 h and then injected intranuclearly with a sharp microelectrode (about 60MΩ) using a variant of our method for injection of antisera (Caulfield *et al.*, 1994; Abogadie *et al*, 1995). We verified successful injection of either antisense or sense constructs by inclusion of fluorescein-isothiocyanate labeled dextran (0.5%, mw 10kD), a fluorescent label which also enabled identification of injected cells 1 or 2 days later. The injection of an antisense construct against $G_{\alpha q/11}$ resulted in an abolition of immunocytochemically-detectable $G_{\alpha q/11}$ at both 24 and 48 h after intranuclear injection (Abogadie *et al.*, 1995).

Muscarinic inhibition of the M-current was assessed in cellsinjected with either antisense and sense (control) constructs 24 or 48 h previously. The SCG neurones were superfused with a modified Krebs solution (mM: 120 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 23 $NaHCO_3$, 11 glucose, 5 Hepes) plus 500nM tetrodotoxin and bubbled with 95%O_2-5%CO_2 at 32°C. M-current was recorded using the amphotericin B perforated patch method (access resistance 10-30MΩ). Cells were voltage-clamped at about -25mV and stepped to about -55mV for 1s and the total M-current deactivation relaxation during this step was measured. A significant reduction in muscarinic mediated inhibition occurred in antisense injected cells 48 h after injection: Oxotremorine-M resulted in mean ± s.e.mean inhibitions of 29 ± 4% (n=4) of total M-current relaxation in antisense injected cells compared to sense-injected cells, 64 ± 9% with 1μM (n=4, P=0.012 Students 2-tailed t-test). Increasing the dose of agonist to 3μM overcame the antisense block (54 ± 4%, inhibition, n=3), was also seen with antibody injection and may reflect spare G-protein capacity (Caulfield *et al.*, 1994).

Thus, injection of antisense, but not sense, constructs in SCG cells results in a decrease in the $G_{\alpha q/11}$ subunit and parallel inhibition of a $G_{\alpha q/11}$ mediated response. Further studies are in progress to investigate more specific antisense constructs against $G_{\alpha q}$ and $G_{\alpha 11}$ and assess their functional role in mediating muscarinic responses.

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14P USE OF ANTISENSE CONSTRUCTS DIRECTED AGAINST G_α SUBUNITS TO ATTENUATE G-PROTEIN EXPRESSION

F.C. Abogadie, J.E. Haley, Y. Vallis, M.P. Caulfield and N.J. Buckley. Wellcome Laboratory for Molecular Pharmacology, Dept. of Pharmacology, University College London, Gower Street, London. WC1E 6BT

The use of antisense oligodeoxynucleotides has considerably advanced the understanding of the roles of various proteins in neuronal function. This approach has several inherent disadvantages which we have tried to eliminate by generating antisense constructs in a mammalian expression vector. These constructs are more stable, can be used to achieve a high intracellular concentration of antisense transcripts and can be used to stably transfect cell lines. We describe here a methodology for the cloning and delivery of such antisense constructs against specific G-protein α–subunits to neurons.

The large degree of homology amongst the coding sequences of G-protein α–subunits precludes their use to derive subunit-specific probes. In contrast, there is little homology, either within or across species, amongst the 3'untranslated regions of mouse α–subunit cDNAs. Because of the likely disparity between published mouse sequences and the targeted rat sequence we first cloned the 3'untranslated regions of rat α-subunit cDNAs using primers based on mouse sequences and a low stringency PCR cloning strategy. These domains were subcloned into the mammalian expression vector pBK-CMV (Stratagene) and the resultant constructs used to transfect NG108-15 neuroblastoma x glioma cells. Transfectants were analyzed by PCR to confirm expression of the antisense cRNA.

These constructs (100 μg/ml in Ca^{2+}-free Krebs solution) were delivered to cultured SCGs by intranuclear microinjection with a sharp electrode (about 60 MΩ) or to differentiated NG108-15s by lipofection. Injected SCGs were identified by co-injection with FITC-conjugated dextran (10kD) and transfected NG108-15s by co-transfection with a CD8 expression plasmid. Binding with coloured magnetic beads labelled with anti-CD8 antibody identified the transfected cells (Jurman *et al,.,* 1994). The construct:CD8 ratio was 4:1. To confirm that most cells expressing CD8 also expressed the construct, a CMV-gal plasmid was used in the transfection and the cells were stained for ß-galactosidase. Bead binding was assessed after 48 hours and only cells that bound several beads both on the soma and along the processes were included in the counts. 96%±1% of the cells that bound beads also expressed ß-galactosidase (n=3). It is also possible to separate transfected from untransfected cells by incubating the cells with the beads, washing them off of the culture dish and passing the cell suspension through a magnetic separator. It is then possible to perform Western blots to quantitate the reduction in protein. The effects of the constructs were functionally assessed in SCGs (Caulfield *et al.*, 1995). Using indirect immunocytochemistry, we have shown that G-protein staining was totally abolished in the SCGs at 48 hours.

Antisense constructs against specific G-proteins can be used to selectively block the expression of these proteins in neurons. These constructs are powerful tools that may offer significant advantages over antisense oligodeoxynucleotides for dissecting the roles of different G-proteins in signal transduction.

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15P DIFFERENTIAL EFFECTS OF CHRONIC TREATMENT WITH PHENELZINE, IMIPRAMINE OR BUSPIRONE ON $GABA_A$ RECEPTOR SUBUNIT GENE EXPRESSION IN RAT BRAIN

V.A.-M.I. Tanay*[1], G.B. Baker[2,3], A.J. Greenshaw[2,3] & A.N. Bateson[1,3], Depts of Pharmacology[1] and Psychiatry[2], and Division of Neuroscience[3], Faculty of Medicine, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.
Introduced by Dr W.F. Dryden.

The tricyclic antidepressant imipramine (a monoamine reuptake inhibitor) and the monoamine oxidase inhibitor phenelzine are both used to treat major depressive disorder and panic disorder (Rosenberg, 1993). It is unclear how these drugs produce such a common therapeutic effect given their different primary sites of action. Further, their rapid effects on aminergic transmission contrast with their slow onset of therapeutic action. These drugs, however, do increase GABA levels (phenelzine; Baker *et al.*, 1991) or GABA release (imipramine; Korf & Venema, 1983), thereby enhancing GABAergic transmission. Chronic treatment with $GABA_A$ receptor agonists (such as GABA or muscimol) or modulators (for example diazepam) produce specific changes in receptor gene expression (Heninger *et al.*, 1990). We therefore hypothesized that long-term treatment with phenelzine or imipramine would alter specific $GABA_A$ receptor subunit steady-state mRNA levels. Buspirone (an anxiolytic with no anti-panic activity that does not directly act via a GABAergic mechanism) was used for comparison.

Adult male rats (Sprague-Dawley, weight 200-250g) were anaesthetised with methoxyflurane and osmotic minipumps were subcutaneously implanted to deliver imipramine, phenelzine, buspirone (at 20, 15 and 4 mg/kg/day, respectively) or vehicle (treatment groups, n=6; vehicle groups, n=3). After 21 days rats were decapitated. Brains were dissected on ice and frozen in an ethanol/dry ice bath. Total RNA was isolated from cerebellum and cortex with Trizol reagent® (Gibco-BRL). Multiprobe quantitative solution hybridization assays (O'Donovan *et al.*, 1991) were performed to measure $GABA_A$ receptor subunit mRNA levels using radiolabelled subunit-specific oligonucleotides. A β-actin oligonucleotide probe served as internal standard. Densitometric analysis of autoradiographic images was performed on a Macintosh LCIII using the NIH image software. For each sample, ratios of $GABA_A$ receptor subunit mRNA levels to the β-actin mRNA level were calculated. Statistical analysis was by arcsine transformation and 2-way ANOVA.

When data were expressed as percent of vehicle controls, imipramine and phenelzine both increased $GABA_A$ receptor subunit mRNA levels in the cerebellum (imipramine: α1, 178 ± 24%; β2, 146 ± 15%; γ2, 120 ± 19%; phenelzine: α1, 182 ± 39%; β2, 198 ± 48%; γ2, 207 ± 45%), but not in the cortex. In contrast, buspirone increased $GABA_A$ receptor subunit mRNA levels only in the cortex (α1, 132 ± 14%; β2, 147 ± 11%; γ2, 145 ± 28%).

These data support our hypothesis that chronic treatment with antipanic drugs modulates the expression of specific $GABA_A$ receptor subunit genes. Other subunits and brain regions are currently under investigation.

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16P THE 5-$HT_{2C/2B}$ RECEPTOR ANTAGONIST, SB 206553, HAS ANXIOLYTIC-LIKE PROPERTIES IN TWO RAT MODELS

G.A. Kennett, F. Bright, S. D'Arcy, T.P. Blackburn, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow, Essex CM19 5AW, U.K.

The 5-$HT_{2C/2B}$ receptor antagonist, SB 200646A, has anxiolytic-like actions in rats and marmosets (Kennett et al., 1994, 1995). We now report the effects of a more selective and potent 5-$HT_{2C/2B}$ receptor antagonist, SB 206553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo-[2,3-fr]indole (Forbes et al., 1995).

Male Sprague-Dawley rats (200-250 g) were held under a 12 h light cycle (lights on 0700 h) with free access to food and water. They were dosed with SB 206553 p.o. 1 h pretest and mCPP (7 mg/kg i.p. 20 min pretest). At test time, they were placed in automated locomotor boxes for 10 min and the number of transits recorded (see Kennett et al., 1994). Other rats were singly housed on day 1 and given vehicle, SB 2066553 or chlordiazepoxide (CDP) on day 5. One h later pairs of like-treated rats were placed in a brightly lit, white perspex, test arena for 15 min and their social interaction (SI) (grooming, following, sniffing, crawling over or under, boxing, biting) was scored by an observer, blinded to treatments, via a video monitor (see Kennett et al., 1994). In the Geller-Seifter (GS) test, rats (400-500 g) fed a restricted diet, were trained to press a lever for a food reward, and to associate a light cue with both a high level of reward and a contingent footshock, in 5 x 3 min periods with rewards at variable intervals (see Kennett et al., 1995). SB 206553 and CDP were given as suspensions in 1% methyl cellulose (2 ml/kg) with , in SI studies, the addition of 10 mg/ml BaSO4 and a drop of yellow food colourant. Results are cited as means ± s.e.m. and analysed by 1-way ANOVA and Dunnett's test (SI, locomotion) or 2-way ANOVA (treatments x subjects) in the GS test where comparisons were made with levels of responding on two preceding vehicle treated days and expressed as % change. ID_{50} estimates were made by the iteritive curve fitting program "Allfit".

SB 206553 inhibited mCPP-induced hypolocomotion, a model of 5-HT_{2C} receptor function in vivo (Trail et al., 1995, Kennett et al., 1994) with an ID_{50} of 5.5 ± 1.69 mg/kg p.o. (confidence limits) but alone had no effect on locomotion at up to 40 mg/kg. SB 206553 increased time (secs) spent in SI (saline 49.2 ± 6.6, SB 206553 2 mg/kg 92.9 ± 10.1, $p<0.01$, 20 mg/kg 136.6 ± 10.2, $p<0.01$) as did CDP 5 mg/kg p.o. 114.4 ± 10.8, $p<0.01$). In this test, locomotion was modestly increased by SB 206553 (10 and 20 mg/kg), but the effect was less than that of CDP (5 mg/kg p.o.). In the GS procedure, SB 206553 had little effect on unpunished, but increased punished responding (SB 206553 2 mg/kg +68.1 ± 29.6%, $p<0.05$, 20 mg/kg +177 ± 55.7% $p<0.01$, 40 mg/kg +225.3 ± 124.3% $p<0.01$), as did CDP (5 mg/kg) (+335.9 ± 101.3% $p<0.01$).

In conclusion, SB 206553 is a potent antagonist of 5-HT_{2C} receptor function in vivo and like SB 200646A, (Kennett et al., 1994), has anxiolytic-like effects in rats.

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17P THE EFFECT OF mCPP AND YOHIMBINE ON 20 kHz ULTRASOUND-INDUCED DEFENCE BEHAVIOUR

S. R. G. Beckett,, S. Aspley, & C. A. Marsden. Department of Physiology & Pharmacology, Queen's Medical Centre, Nottingham, NG7 2UH.

In rats, predator threat and aversive stimuli cause the production of 20-32 kHz ultrasound calls (Blanchard *et al.*, 1991). Recently we have shown that rats exposed to 20 kHz ultrasound display flight behaviour, identical to behaviour produced by electrical or chemical stimulation of brain regions associated with anxiety and defence. This response can be attenuated by pretreatment with diazepam (Beckett *et al.*, 1995a). The present study examines the effect the α2 antagonist yohimbine, and the 5-$HT_{2C/2B}$ agonist 1-(3-chlorophenyl)piperazine (mCPP), on ultrasound induced defence behaviour.

Male Lister hooded rats (250-300g, n=7-8 per dose) pretreated (20min i.p.) with mCPP (0.5, 1.0 & 2.0mg/kg) in saline (0.9%), yohimbine HCl (0.5, 2.0 & 5.0mg/kg), in sterile water or vehicle were placed in an open arena (75 cm diameter) with a wall mounted piezo-electric speaker. After 2 min they were exposed to a 1 min 20 kHz square wave ultrasound tone (60, 72 & 75dB intensity, randomised) followed by a further 2 min without sound. This procedure was repeated for each intensity with a 1 min inter-procedure interval. Behaviours was analysed via a computer tracking system (Beckett *et al.*, 1995b) as distance travelled and speed.

Exposure to ultrasound produced intensity related locomotor behaviour, similar to that previously reported (Beckett *et al.* 1995). Treatment with mCPP caused a significant reduction in maximum speed at all 3 doses tested (Fig 1A) without a hypolocomotor effect. Yohimbine (0.5mg/kg) significantly increased the response, whilst 2.0 and 5.0mg/kg caused an attenuation (Fig 1B), although a hypolocomotor effect was evident at these higher doses. Values obtained for distance travelled and average speed revealed similar changes.

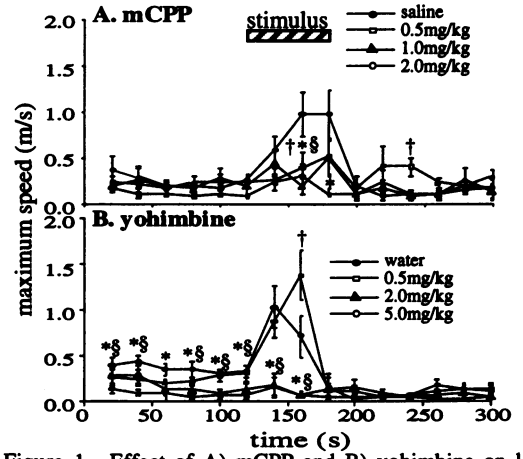

Figure 1. Effect of A) mCPP and B) yohimbine on locomotor speed per 20s time bin in response to 20kHz ultrasound (72dB) (mean ± s.e.m $p<.05$ vs vehicle (0.5†, 1.0§ & 2.0* & 0.5†, 2.0§ & 5.0* mg/kg) One way ANOVA, post-hoc Duncans NMR).

Response enhancement by yohimbine may indicate α_2 adrenoceptor control of aversion. mCPP reduced the response supporting previous studies showing unconditioned aversion can be restrained by 5-$HT_{2B/2C}$ receptor activation (Jenck et al. 1991).

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18P CHANGES IN BEHAVIOUR, CORTICOSTERONE RELEASE AND 5-HT_{2C} RECEPTOR LEVELS FOLLOWING CHRONIC m-CPP INFUSION IN THE RAT

K.C.F. Fone, R.H. Austin & T. Punhani, Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham University, Nottingham, NG7 2UH

Acute injection of the 5-hydroxytrypamine$_{2C}$ (5-HT_{2C}) agonist meta-chlorophenylpiperazine (m-CPP) causes hypolocomotion, hypophagia and corticosterone release in rats. However, the hypolocomotion and hypophagia attenuate with chronic injection (Ulrichsen *et al.*, 1992), suggesting that receptor down-regulation occurs. This study examines the effect of 14 days m-CPP infusion on m-CPP-induced behaviour, corticosterone release and cortical [^{3}H] mesulergine binding to correlate functional tolerance with change in rat brain 5-HT_{2C} receptor levels.

Male Hooded Lister rats (480-580g) were divided into two groups; either housed together and given food and water *ad libitum* (n=12) or separately and food restricted (10.00-14.00h or 40 min post-injection, n=16). Both groups received 0.154 M saline (1 ml kg^{-1}, i.p., d 7) and m-CPP (2.5 mg kg^{-1}, d 14 and 28, except half the group housed rats received saline on all days) and the locomotor activity and rears were scored manually (20 to 40 min post-injection) in a novel 45 cm^3 arena. In addition, food restricted rats were implanted (under halothane anaesthesia, d 15) with minipumps to continuously infuse saline (4.41 μl h^{-1}, s.c.) or m-CPP (10 mg kg^{-1} day^{-1}). All rats were decapitated one day after the last behavioural test (d 29) to assay plasma corticosterone (Immunodiagnostic Ltd radioimmunoassay kit) and cortical [^{3}H] mesulergine binding (Pranzatelli *et al.*, 1992) using 10 μM ketanserin to define non-specific binding. Results are given as mean ± s.e.mean and analysed by Student's *t*-test unless otherwise stated.

Chronic m-CPP infusion significantly reduced daily food intake compared with that of saline controls ($P<0.05$ from d 17 to 29 inclusive, except d 19, 22, 24 and 26 n.s.). Furthermore, irrespective of whether rats received chronic m-CPP or saline, acute m-CPP injection significantly reduced food consumption compared with that on the previous day, both on d 14, prior to, (by 56 and 65% in saline and m-CPP infusion groups respectively, $P<0.01$) and on d 28 (by 43 and 30%, $P<0.01$) after drug infusion. In the uninfused rat group, m-CPP significantly attenuated the number of rears ($P<0.05$ d 14 and $P<0.01$ d 28, Duncan's New Multiple Range following ANOVA) and 90° turns (n.s. and $P<0.02$) compared with that of saline, showing that m-CPP-induced hypolocomotion was sustained over three consecutive open field trials. In contrast, the reductions in rears and turns produced by acute m-CPP on d 28 were significantly attenuated in rats receiving chronic m-CPP (14 ± 4 and 42 ± 11 in 20 min, respectively) compared with those given saline infusion (3 ± 1, $P<0.05$ and 12 ± 7, $P<0.01$). Furthermore, plasma corticosterone was significantly higher ($P<0.01$ Duncan's New Multiple Range after ANOVA) when acute m-CPP was given to uninfused rats than to those given 14 days m-CPP. Chronic m-CPP infusion significantly reduced the Bmax of [^{3}H] mesulergine in cortical homogenates (10.80 ± 1.24) compared with that in controls (14.81 ± 0.96 fmol mg $protein^{-1}$, $P<0.02$, without altering the Kd) suggesting that 5-HT_{2C} receptor down-regulation had occured.

This study confirms that acute m-CPP produces hypolocomotion and hypophagia. A more marked tolerance to the hypolocomotor than the hypophagic and corticosterone responses occured with 14 days 5-HT_{2C} agonist administration, suggesting that the extent of receptor down-regulation may vary in different brain regions.

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19P ANTIDEPRESSANT DRUG EFFECTS ON DIFFERENTIAL REINFORCEMENT OF LOW RATE RESPONDING 72 secs (DRL) AND LEARNING ASSESSED BY AUTOSHAPING (AUTO) USING LONG EVANS RATS

J.S. Andrews, Th. de Boer & C.D. Nicholson, Scientific Development Group, NV Organon, P.O.B. 20, 5340 BH Oss, NL

Cognitive disruption induced by tricyclic antidepressants is presumed to depend on their anticholinergic properties. Many newer antidepressants have less negative effects on cognition despite similar anticholinergic properties to the tricyclics. Thus it has been suggested that differences in the antihistaminergic effects might account for the impairments seen. Antidepressants with varying degrees of anticholinergic and antihistaminergic properties were compared in two operant tasks where such effects might be expected to depress responding: the DRL, used to predict antidepressant potential (Marek & Seiden 1988), and AUTO used to quantify learning ability (Andrews et al 1995).

Male rats (250-350g) were trained to a stable level of responding in the DRL before drug testing; naive rats were tested for speed of AUTO. In both experiments separate groups (N=10-12, DRL; 11-24, AUTO) were injected i.p. (min placebo & three doses) 30 mins before the session with known antidepressants, ORG 4428 (Ruigt & Proosdij 1990) and scopolamine. The minimum effective dose (MED) to effect lever pressing and pellets earned per session was identified by ANOVA/Tukey tests in the DRL, and to impair AUTO with Freidman Willis/Mann Whitney tests. Antagonism at muscarinic (MUSC) and histaminergic (HIST) receptors was assessed using isolated guinea pig ileum as described previously (de Boer et al 1988).

All antidepressants affected the DRL in the expected manner i.e. decreased lever pressing but increased the food pellets obtained.

Table 1 Summary: effects on operant performance and *in vitro*
NT= Not Tested

	MED (mg/kg) AUTO	DRL ↑ Pellets	DRL ↓ Response	pA2 MUSC	pA2 HIST
Imipramine	≤ 10	5	5	6.5	7.8
Desipramine	≤ 10	5	1.25	6.3	6.9
Fluoxetine	20	20	20	5.8	5.5
Mirtazapine	>20	1.25	20	6.1	9.3
ORG 4428	>10	5	5	4	5.3
Scopolamine	0.2	>0.8	>0.8	9.3	NT

All compounds except mirtazapine and ORG 4428 disrupted learning. Fluoxetine treated rats failed to consume pellets during AUTO, and thus failure here may be a nonspecific effect. Although scopolamine impairs acquisition, the anticholinergic properties of the other compounds are weaker, similar to one another and cannot account for differences in AUTO. Antihistaminergic activity does not predict the ability to disrupt new learning in rats, or decrease responding in the DRL, and depression of responding in the DRL does not predict impaired lever press acquisition. Finally, mirtazapine is equipotent to tricyclics in the DRL but less likely to disrupt learning.

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20P THE EFFECT OF DIZOCILPINE AND SELECTIVE HIPPOCAMPAL LESIONS IN THE ELEVATED PLUS-MAZE

T.K. Murray and A.J. Cross, Astra Neuroscience Research Unit, 1, Wakefield Street, London, WC1N 1PJ.

The hippocampus has a high density of NMDA receptors (Monaghan & Cotman, 1985) and both hippocampal lesions (see Gray, 1982) and NMDA-receptor antagonism produce anxiolytic-like behaviour (Clineschmidt *et al.*, 1982). The NMDA antagonist, dizocilpine, was tested in the elevated plus-maze model of anxiety (Pellow *et al*, 1985), using a dose known to produce a selective learning impairment, which was not due to a performance deficit (Murray *et al*, 1995). The effect of dizocilpine was compared to that of selective hippocampal lesions. In addition the effect of previous exposure to a maze - learning situation was investigated to determine subsequent changes in plus-maze exploration.

Male Lister Hooded rats (250-300g) were either injected with dizocilpine ($0.075 mgkg^{-1}$, s.c.) 20 min prior to testing (controls received saline), or had previously received excitotoxic hippocampal lesions. Lesions were produced using 0.5µl 0.1M NMDA injected into three sites per hemisphere. Diazepam ($10 mgkg^{-1}$) was administered (i.p.) to prevent seizures following surgery. Prior to plus-maze testing, lesion and sham-operated rats had been trained in Y-maze learning tasks. Thus an additional maze-naive group were also tested. All rats were tested in an elevated plus-maze under high-light (anxiogenic) conditions for 5 min, and the number of open and closed arm entries recorded.

Total arm entries were unaffected by dizocilpine, thus this dose did not induce hyperactivity (Table 1), but significantly increased open arm entries, suggesting an anxiolytic effect. Total arm entries were significantly greater in sham and lesioned rats compared to naive, but no different from each other.

Table 1. Effect of dizocilpine and excitotoxic hippocampal lesions on elevated plus-maze exploration.

Group	Total Arm Entries	% Open Arm Entries
Saline (n=9)	14.6±0.6	21.8±2.0
Dizocilpine (n=9)	14±1.7	40.7±5.5 *
Naive (n=10)	13.7±0.8 +	20.7±2.1 +
Sham (n=9)	19.1±1.2 *	39.5±1.9 *
Lesion (n=9)	21±1.4 *	64.7±4.0 * +

values are mean±s.e.mean
*p<0.01 compared to saline or naive, +p<0.01 compared to sham

Open arm entries were significantly greater in sham compared to naive, and in lesioned compared to sham. Thus pre-exposure to a Y-maze increased exploration in the plus-maze. In addition, NMDA lesions of the hippocampus produced an anxiolytic-like effect.

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21P ALTERED STRIATAL DOPAMINE EFFLUX PRODUCED BY HIGH CONCENTRATIONS OF L-ARGININE IS NOT DEPENDENT ON NITRIC OXIDE FORMATION

M.T. Silva, S. Rose, J.G. Hindmarsh, P. Jenner and C.D. Marsden. Neurodegenerative Diseases Research Centre, Pharmacology Group, Biomedical Sciences Division, King's College London, Manresa Rd, London, UK.

Inhibition of striatal nitric oxide synthase (NOS) produces an increase in dopamine (DA) efflux *in vivo*, suggesting an inhibitory effect of nitric oxide (NO) on DA release (Silva *et al.*, 1995). Conversely, using L-arginine (L-ARG), the NO precursor, Strasser *et al.*, (1994) demonstrated increased striatal DA efflux. However, the concentration of L-ARG used, (71mM), was considerably greater than that used by others (Silva *et al.*, 1995), throwing doubt on the mechanism of action. For this reason we report the effect of a range of L-ARG concentrations on striatal DA efflux *in vivo*.

Male Wistar rats (280-320g) were anaesthetised with chloral hydrate (500mg/kg i.p.). DA efflux was measured in the striatum by *in vivo* microdialysis as previously described (Silva *et al.*, 1995). Probes were continuously perfused with artificial CSF (aCSF) at a rate of 2μl min^{-1}. At least 1 h following implantation, 10 min fractions of dialysate (20μl) were collected into 0.25M acetic acid (25μl). Following a 1h period to determine basal dopamine efflux, L-ARG (10-100mM), D-arginine (100mM) or L-citrulline (100mM), were included in the aCSF for a period of 150min. Control animals received only aCSF. In a further series of experiments investigating the role of NO, probes were perfused with the NOS inhibitors N^{G}-nitro-L-arginine methyl ester (L-NAME;1mM) or the monosodium salt of 7-nitro indazole (7-NINA;1mM) for 1h prior to and for 90min following perfusion with L-arginine (50mM). Dialysate samples were analysed for DA by h.p.l.c. with electrochemical detection (all groups n=5).

L-ARG (10mM) had no effect on striatal DA efflux compared to basal or control values. However, at higher concentrations (50 and 100mM) there was a biphasic change in DA efflux lasting up to 110min ; an initial reduction lasting 40min, followed by a dramatic increase (Figure). Both the initial reduction and the secondary increase in DA efflux were concentration dependent, ($p<0.05$, one way ANOVA). D-arginine and L-citrulline had no effect on DA efflux ($P<0.05$ vs control ; Dunnett's test). The biphasic effect of L-ARG (50mM) on DA efflux was not altered by the incorporation of 7-NINA or L-NAME.

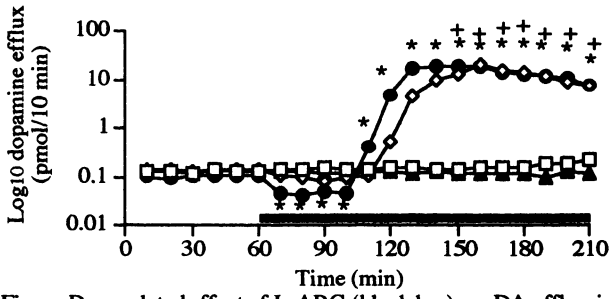

Figure Dose related effect of L-ARG (black bar) on DA efflux in the rat striatum. (▲ = control, □ = 10mM, L-ARG, ◇ = 50mM L-arginine, ● = 100mM L-ARG). $^{+}$ $p<0.05$ L-ARG (50mM) vs control. * $p<0.05$, L-ARG (100mM) vs control (Dunnett's test).

These data suggest that, at high concentrations, L-ARG produces a biphasic effect on DA efflux in the striatum *in vivo*. This effect appears to be specific to L-ARG but, contrary to the findings of Strasser *et al.* (1994), independent of NO.

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22P L-NAME DOES NOT PROTECT AGAINST MPTP TOXICITY IN THE COMMON MARMOSET

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

Excitotoxicity may contribute to nigral cell loss in Parkinson's disease (PD). For example, the NMDA receptor antagonist, MK-801 prevents MPTP-induced toxicity in the cynomologus monkey (Zuddas *et al.*, 1992). Since nitric oxide (NO) is produced following activation of NMDA receptors and exhibits potent neurotoxic effects *in vitro* (Dawson *et al.*, 1992), NO may contribute to nigral toxicity. To investigate the involvement of NO in such toxicity occurring in a model of PD, the effect of L-N^{G} nitro arginine methyl ester (L-NAME) on MPTP toxicity in the common marmoset (*Callithrix jacchus*) was determined.

Animals were divided into 4 groups and treated as follows: Group 1, 0.9 % (w/v) saline (1ml/kg, s.c., once daily on days 2-6; Group 2, MPTP (2mg/kg, s.c., once daily on days 2-6); Group 3, (L-NAME, 50mg/kg, s.c., twice daily on days 1-7); Group 4, MPTP (2mg/kg, s.c., once daily on days 2-6) and L-NAME (50mg/kg, s.c., twice daily on days 1-7). Neuronal loss in nigral sections (30μm) of animals killed 18 days after MPTP treatment was determined by immunohistochemical staining of tyrosine hydroxylase (TH)-containing neurones. TH positive neurones were counted in the substantia nigra pars compacta (SNc) at the level of the rootlets of the third nerve. Dopamine uptake sites present in the laterodorsal (LD) and medioventral (MV) regions of the caudate nucleus (Cd) and putamen (Pt) were identified by autoradiography of [^{3}H]-mazindol.

MPTP caused a marked loss of both TH positive neurones in the SNc and specific [^{3}H]-mazindol binding in the Cd and Pt compared to saline-treated animals. L-NAME treatment alone did not alter the number of TH positive neurones or [^{3}H]-mazindol binding. Furthermore, L-NAME did not affect the loss of TH-containing neurones and loss of [^{3}H]-mazindol binding in the Cd and Pt induced by MPTP (Table).

This data contrasts with another study which reports that the NO synthase inhibitor, 7-nitro indazole protects against MPTP-induced loss of striatal dopamine in mice (Schulz *et al.*, 1995). However, these authors did not measure loss of neuronal cell bodies. The present results suggest that NO does not play a major role in MPTP neurotoxicity at least in the marmoset.

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Table: Effects of L-NAME on MPTP-induced toxicity in the marmoset.

Treatment	Number of TH cells	[^{3}H]-Mazindol binding (fmol/mg) Cd-LD	Cd-MV	Pt-LD	Pt-MV
Saline	370±10	298±10	351±15	323±12	330±11
MPTP	89±13*	14±2*	23±3*	11±2*	15±2*
L-NAME	406±42	301±14	370±19	348±12	332±18
MPTP/L-NAME	90±3*	16±5*	23±4*	11±2*	17±3*

*$P<0.01$ compared with saline and L-NAME, Dunn's test (n=4).

23P THE EFFECT OF CHRONIC GLUTATHIONE DEPLETION INDUCED BY L-BUTHIONINE-(S,R)-SULPHOXIMINE (BSO) ON THE NIGROSTRIATAL PATHWAY IN RATS

G.M. Kunikowska, S. Toffa, B-Y. Zeng, P. Jenner & C.D. Marsden. Neurodegenerative Diseases Research Centre, Pharmacology Group, Biochemical Sciences Division, King's College London, London SW3 6LX, UK.

Nigral cell death in Parkinson's disease (PD) may involve oxidative stress. In particular, levels of reduced glutathione (GSH) are decreased in substantia nigra in both the early and late stages of PD, forming the earliest biochemical marker of nigral cell death (Dexter et al., 1994; Sian et al., 1994). We now investigate whether L-buthionine-(S,R)-sulfoximine (BSO)-induced depletion of GSH in brain leads to nigrostriatal degeneration in rats.

BSO, an irreversible inhibitor of γ-glutamyl cysteine synthetase, the rate-limiting enzyme in GSH synthesis, was infused via a stainless steel cannula, stereotaxically implanted into the left lateral ventricle of adult male Wistar rats (200±10g). Osmotic mini-pumps (Alzet) were subcutaneously implanted to deliver 9.6mg/kg/day BSO through the cannula over a period of 28 days. Animals were then killed under terminal anaesthesia and brains removed for analysis of GSH using HPLC. Nigrostriatal integrity was determined by tyrosine hydroxylase (TH) immunohistochemistry in the substantia nigra and [^{3}H]-mazindol autoradiography in the striatum.

BSO infusion for 28 days markedly reduced GSH concentrations in the substantia nigra (approx. 65%) and the striatum (approx. 80%) (Table 1). The number of TH-positive cells in substantia nigra was not altered by BSO-treatment compared to control animals. Similarly, there was no difference in specific [^{3}H]-mazindol binding in the striatum of BSO-treated or controls rats (Table 2).

The lack of dopaminergic cell loss suggests that GSH depletion alone is not the cause of nigrostriatal damage in PD. However, chronic GSH deficiency may increase the vulnerability of the substantia nigra to a toxic insult. Indeed, the toxicity of 6-hydroxydopamine to the nigrostriatal pathway in rats is potentiated by GSH depletion (Pileblad et al., 1989). Similarly GSH depletion potentiates MPP^+ toxicity in mice (Wullner et al., 1995).

Table 1. GSH concentrations (nmol/mg) in striatum and substantia nigra of control and chronically BSO-infused rats. Mean ± SD for n=6 for all groups. *p<0.05 compared to control; Student's t-test.

	Control	BSO
Striatum (left)	3.49 ± 0.22	0.38 ± 0.18*
Striatum (right)	3.71 ± 0.87	0.97 ± 0.15*
Substantia nigra	2.75 ± 0.43	0.95 ± 0.09*

Table 2. Numbers of TH-positive cells in substantia nigra (SN) of control and chronically BSO-infused rat. Mean ± SD for 4 areas per rat (n=4-9).

	Control	BSO
SN (left)	343.0 ± 54.3	330.0 ± 49.4
SN (right)	336.0 ± 59.6	357.0 ± 63.6

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24P CHOLINERGIC MANIPULATION OF L-DOPA-INDUCED DYSKINESIAS IN THE MPTP-TREATED COMMON MARMOSET (*CALLITHRIX JACCHUS*)

T. Banerji, R.K.B. Pearce, M.J. Jackson, P. Jenner & C.D. Marsden, Neurodegenerative Diseases Research Centre, Pharmacology Group, King's College, London SW3 6LX, UK.

The common marmoset (*Callithrix jacchus*) shows parkinsonian motor disability following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure and develops dyskinesias during chronic L-DOPA dosing (Pearce *et al.*, 1994). We now report the effect of cholinergic drugs, alone and in combination with L-DOPA upon L-DOPA-induced dyskinesias and parkinsonian disability in this model.

Animals were divided into 3 groups of 4: naive controls; MPTP-treated, L-DOPA-naive; MPTP-treated, L-DOPA-primed (12.5 mg/kg L-DOPA po with 12.5 mg/kg carbidopa po) to show dyskinesias. Each group received the anticholinergic drugs atropine (0.5 and 1.0 mg/kg sc) and trihexyphenidyl (0.5 and 1.0 mg/kg po), the acetylcholinesterase inhibitor physostigmine (0.2 mg/kg), and the muscarinic agonist oxotremorine (0.1 mg/kg). The L-DOPA-primed group also received a combination of L-DOPA (12.5 mg/kg po) with carbidopa (12.5 mg/kg po) plus each experimental drug. Activity levels were monitored in locomotor cages and dyskinesias were rated on a semiquantitative dyskinesia scoring system (0 = absent to 4 = severe).

L-DOPA was highly effective, and the anticholinergic drugs atropine and trihexyphenidyl were moderately effective in reversing MPTP-induced akinesia. The procholinergic drugs physostigmine and oxotremorine worsened MPTP-induced parkinsonism, reducing activity below baseline levels. Oxotremorine increased tremor in MPTP-treated animals. In the L-DOPA-primed animals, L-DOPA administration produced marked dyskinesias, with prominent limb chorea and dystonia and a driven hyperkinesis. In this group, the anticholinergic drugs produced a higher proportion of chorea, both alone and in combination with L-DOPA, and the procholinergic drugs produced proportionally more dystonia, both alone and in combination with L-DOPA (Figure 1). In all cases, the mean dyskinesia scores were reduced when compared to those induced by L-DOPA alone.

This is the first report of a provocation of choreic dyskinesias without concurrent L-DOPA therapy in MPTP-treated, L-DOPA-primed marmosets. We also confirm the effects of cholinergic manipulation upon parkinsonian disability (Close *et al.*, 1990) and dyskinesia (Bédard *et al.*, 1993) in this model. The differential effects upon choreic and dystonic components of L-DOPA-induced dyskinesias produced by these drugs may relate to imbalanced dopamine-acetylcholine interaction in the striatum after nigral damage.

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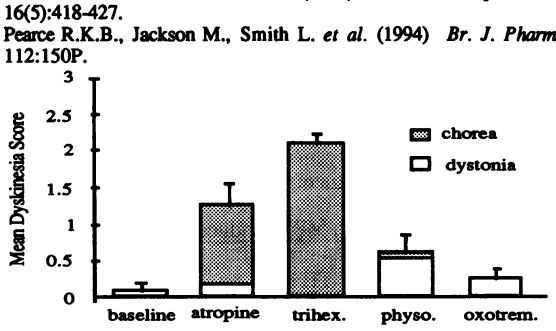

Figure 1. Mean dyskinesia scores in MPTP-treated, L-DOPA-primed common marmosets at baseline and receiving atropine 1 mg/kg sc, trihexyphenidyl (trihex.) 1 mg/kg sc., physostigmine (physo.) 0.2 mg/kg sc and oxotremorine (oxotrem.) 0.1 mg/kg sc. (Bars = S.E.M.)

25P THE EFFECTS OF THE NITRIC OXIDE-DONORS SODIUM NITROPRUSSIDE (SNP) AND S-NITROSOGLUTATHIONE (GSNO) ON RAT VENTROBASAL THALAMUS NEURONES *IN VIVO*

P.J. Shaw & T.E. Salt, Institute of Ophthalmology, University College London, Bath Street, London. EC1V 9EL

The ventrobasal thalamus (VB) is the relay nucleus for somatosensory information. Iontophoretic application of L-arginine, the precursor of nitric oxide (NO), potentiates responses of neurones in VB to excitatory amino acids and physiological stimuli (Do *et al*, 1994), and L-arginine is released in VB upon sensory stimulation. To further investigate the possible role of NO in thalamic sensory transmission, we have tested the effect of two NO-donor compounds, SNP and GSNO (Radomski *et al*, 1992) on VB neurones.

Multi-barrelled glass electrodes were used to record extracellularly from 36 VB neurones in male Wistar rats (210-450g) anaesthetised with urethane (1.2g/kg, i.p.), and to apply drugs by iontophoresis. Cycles of physiological stimulation (10ms or 1000ms air-jet displacement of a single whisker) and ejection of N-methyl-D-aspartate (NMDA), (R,S)-α-amino-3-hydroxy-5-isoxazolepropionate (AMPA) and carbachol were established and single cell spike data recorded. Reproducible controls were tested in the presence of SNP, GSNO or glutathione (ejected from 10mM, pH 8 aqueous solutions).

SNP (-5nA) selectively inhibited the responses to NMDA (by 77 ± 6.6%, mean ± s.e.m; n=8; $p<0.05$ Wilcoxon Signed Rank). In the same cells, SNP caused statistically significant reductions in the responses to 10ms air-jet pulses (by 40 ± 12.2%, $p<0.01$) and 1000ms air pulses (by 40 ± 8.1%, $p<0.01$). Responses to AMPA and carbachol were relatively unaffected, being reduced by only 8 ± 9.8% and 6 ± 17.0% respectively. GSNO (-40 to -200nA) however, when applied to a separate population of 12 VB neurones, caused a significant ($p<0.05$) potentiation of responses to NMDA (by 23 ± 12.4%), AMPA (23 ± 10.2%) and both 10ms (24 ± 7.2%) and 1000ms (26 ± 8.0%) air-jet pulses. The responses to carbachol remained unaffected, being reduced by only 1 ± 11.5%.

To determine whether the effect of GSNO was mediated by NO or by the molecule's glutathione moiety, we applied glutathione in its reduced (GSH) and oxidised forms (GSSG) to VB neurones. GSH (-5 to -100nA) was found to reduce the response to NMDA by 49 ± 6.5% ($p<0.05$; n=6) and AMPA by 52 ± 11.2% ($p<0.05$). 10ms air-jet responses were also inhibited (by 23 ± 5.6%, $p<0.05$) but responses to 1000ms air-jets and carbachol were unchanged (reductions of 8 ± 4.2% and 48 ± 21.0% respectively, both non-significant). GSSG (-5 to -60nA) inhibited only the response to NMDA, by 30 ± 8.5% ($p<0.05$; n=10). From these data we conclude that glutathione does not produce the potentiation observed with GSNO.

Manzoni *et al* (1992) suggested that the inhibition of responses to NMDA by SNP may be mediated by ferrocyanide ions, a by-product of its breakdown, rather than an effect of NO. For this reason, SNP would seem to be unsuitable for use as an NO-donor in systems involving NMDA receptors. In contrast, GSNO appears to potentiate NMDA, AMPA and sensory responses in an NO mediated mechanism, and this supports the suggestion of a role for NO in the modulation of thalamic sensory transmission.

This work was supported by the Medical Research Council.

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26P EFFECTS OF L-701,324, A NOVEL ANTAGONIST AT THE GLYCINE SITE OF THE *N*-METHYL-D-ASPARTATE (NMDA) RECEPTOR, ON THE ELECTROENCEPHALOGRAM AND NMDA-EVOKED RESPONSES IN THE RAT STRIATUM

T.P. Obrenovitch, A.M. Hardy & E. Zilkha, Dept of Neurological Surgery, Institute of Neurology, Queen Square, London, WC1N 3BG

L-701,324 (7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2-(1H)-quinolone), a high affinity antagonist at the glycine site of the *N*-methyl-D-aspartate (NMDA)-receptor (Leeson & Iversen, 1994), inhibited K^+-induced spreading depression (SD) without any obvious change in the electroencephalogram (EEG) (Obrenovitch & Zilkha, 1995). Here, we have examined whether L-701,324 alters the EEG at doses which effectively reduce NMDA-evoked responses *in vivo*.

Microdialysis probes incorporating an electrode were implanted in the striatum of male Sprague-Dawley rats (270-420 g), and perfused at 1 μl min^{-1} with artificial CSF (ACSF) (Obrenovitch *et al.*, 1994). Under halothane anaesthesia, 12 consecutive depolarizations were elicited by switching to ACSF containing 200 μM NMDA for 2 or 3 min every 20 min. NMDA-evoked depolarizations and EEG were recorded between the microdialysis electrode and a reference electrode placed under the scalp. Spectral analysis of the EEG in the 0.25-21 Hz window was performed using data acquired during consecutive 4-sec periods. L-701,324 (5 or 10 mg kg^{-1}) or vehicle (10 % polyethylene glycol 300) was administered i.v. 5 min after the end of the 3rd NMDA-evoked stimulus (Figure 1).

L-701,324 dose-dependently inhibited the amplitude of depolarisations evoked by 200 μM NMDA (Figure 1), 10 mg kg^{-1} reducing these responses by around half. Despite its strong binding to plasma proteins (> 99.9 %), L-701,324 was rapidly effective following i.v. administration.

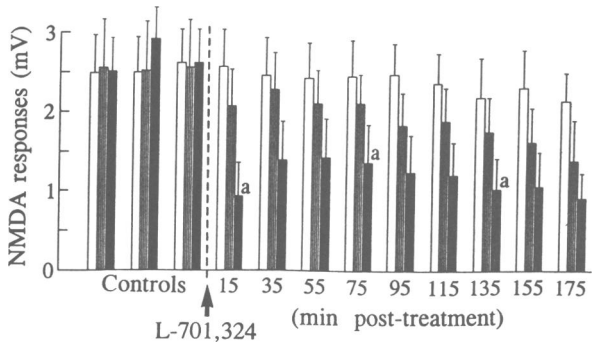

Figure 1. Effects of L-701,324 on NMDA-evoked responses. Values are mean ± s.e.mean (open bars, control group, $n = 5$; dashed bars, 5 mg kg^{-1} L-701,324, $n = 6$; solid bars, 10 mg kg^{-1} L-701,324, $n = 8$). **a** $P < 0.001$, comparison to the response obtained prior to treatment (Student's paired *t*-test).

The average amplitude of the EEG in the frequency windows 0.25-6 and 6-21 Hz did not change in the control group. At the dose of 10 mg kg^{-1}, L-701,324 transiently increased the low-frequency amplitude by around 20 %. In contrast, both doses significantly reduced the high-frequencies to around 70 % of control, and this effect was sustained with the higher dose. In comparison to other NMDA-receptor antagonists, these effects of L-701,324 on the EEG appear minor.

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27P CHARACTERISATION OF THE DEVELOPMENTAL CHANGES IN NEUROTENSIN METABOLISM IN THE RAT TELENCEPHALON

S.H. Moss, G.W. Bennett & C.A. Marsden, Department of Physiology and Pharmacology, Nottingham University Medical School, Queens Medical Centre, Nottingham NG7 2UH

We have previously characterised the ontogeny of neurotensin-like immunoreactivity (NT-LI) in the rat brain (Moss *et al.*, 1994), by radioimmunoassay (RIA), using a specific N-terminally directed antibody. Combination of high pressure liquid chromatography (HPLC) with RIA (Moss *et al.*, 1995) enabled separate measurement of NT and its N-terminal metabolites, in order to evaluate developmental changes in NT metabolism within the perinatal rat telencephalon (the primordia of several adult brain regions, including the striatum and cortex).

Telencephalons were dissected bilaterally from Lister hooded rat embryos of 16, 18 and 20 embryonic (E) days, at birth (P0; 22nd day of gestation) and from 2 and 5 day old postnatal rats (P2 and P5). Telencephalon extracts were separated by HPLC after manual sample injection using a Beckman System Gold, collecting one minute HPLC fractions as described previously (Moss *et al.*, 1995). NT and its N-terminal metabolites were measured by RIA in each fraction and identified using NT, NT1-12, NT1-11, NT1-10, NT1-8 and NT1-7 synthetic standards.

At all ages studied, NT demonstrated the highest levels, with measurable quantities of NT 1-10 and NT 1-12 (Figure 1) and low levels (<0.04 pg/µg protein) of NT1-8 and NT1-11. A prominent transient increase in NT levels occurred at birth, whilst NT1-8 (data not shown) and NT1-10 peaked later, on P2 (Figure 1). NT1-12 levels increased with age, although no peak was observed during the period studied. The fact that NT1-10 peaked after NT suggests that NT1-10 results from the metabolism of NT. This may occur by the action of endopeptidases (EP)24.11 (Checler *et al.*, 1984) and/or EP24.16 (Mentlein and Dahms, 1994), which cleave the Pro^{10}-Tyr^{11} bond of NT in the adult rat brain. There is little information, however, concerning the enzyme(s) responsible for the formation of NT1-12.

The results suggest that NT metabolising enzymes appear before birth in the telencephalon, indicating that the NT system is initially established prenatally. Furthermore these transient ontogenic changes in NT precede the postnatal increase in dopamine (DA) (Santana *et al.*, 1992), implicating a neurotrophic role for NT in development of the DAergic system, which is consistent with the established association between NT and DA systems in the adult rat brain.

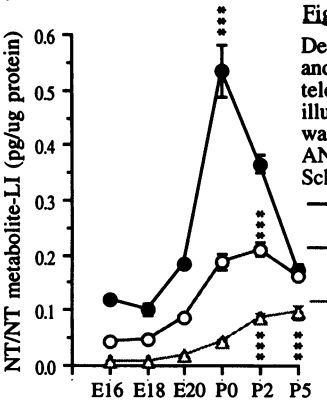

Figure 1.
Developmental changes in NT- and NT metabolite-LI in the rat telencephalon (n=5-10). Data illustrates mean ± s.e.mean and was analysed by a one way ANOVA followed by post hoc Scheffe F test.
NT: ***p<0.001 vs E16, 18,20 & P5 (F=42.55)
NT1-10: ***p<0.001 vs E16-E20 (F=42.63)
NT1-12: ***p<0.001 vs E16-P0 (F=56.67)
(Data for NT1-8 and NT1-11 are not shown).

Acknowledgements: We thank D. Forster for technical assistance, Dr. A. Boyd for NT1-12 and Dr. F. Checler for NT1-10.

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28P A CCK_B ANTAGONIST REDUCES SIGNS OF ANXIETY-RELATED BEHAVIOUR IN THE ELEVATED PLUS-MAZE DURING WITHDRAWAL FROM CHRONIC ETHANOL TREATMENT

Wilson, J., Watson, W.P. and Little, H.J., Pharmacology Department, Medical School, University Walk, Bristol BS8 1TD.

Withdrawal from prolonged ethanol intake in humans results in a syndrome which includes anxiety, tremor and convulsions. Current treatment of the ethanol withdrawal syndrome involves drugs such as benzodiazepines, which cause sedation and ataxia. The dihydropyridine calcium channel antagonists decrease the convulsive, but not the anxiety-related behaviour (File et al., 1989). Antagonists at the CCKB receptor have anxiolytic actions (Singh et al. 1991). At high doses, they gave some protection against the convulsive aspects of the ethanol withdrawal syndrome (Wilson and Little, 1995). We now investigate the effects of CAM1028, a CCKB receptor antagonist (Hughes et al., 1990), on the anxiety-related behaviour in the elevated plus-maze following withdrawal from chronic ethanol administration.

Male TO mice (25-30g) were given liquid diet with 8% ethanol for 7 days; ethanol intake = 25-30 g/kg/day. Controls were pair-fed an isocalorific diet with no ethanol. Mice were withdrawn from the chronic treatment between 7 and 9 am, then injected (n=12 per group) s.c. with CAM1028, at 0.1 and 1 mg/kg, or saline, on withdrawal and 40 min before the test. At 16 h after cessation of the ethanol treatment, the mice were placed on the plus-maze for 5 min. Videotapes of the behaviour were analysed by a trained observer, blind to the drug treatment.

Results are shown in table 1 as mean ± s.e.m. The most prominent effect of ethanol withdrawal was a decrease in the percentage time spent on the open arms of the maze. This effect was completely blocked by CAM1028 (P<0.05 saline vs CAM1028). CAM1028 also blocked the decreased frequency of open arm entries, at 1 mg/kg (P<0.05 saline vs CAM1028) and the increased number of stretch attend postures, at 0.1 and 1 mg/kg (P<0.05 saline vs CAM1028) observed during ethanol withdrawal. Mice undergoing withdrawal showed a significant increase in head dips from the closed arms (protected head dips; P<0.05 compared with controls) and a decreased general activity (number of line crossings; P<0.05 compared with controls); these effects were not altered by CAM1028. CAM1028 had no effect on behaviour of control animals in the plus-maze.

CCKB antagonists may therefore have a place in the treatment of withdrawal and study of their effects may provide information about the mechanisms involved in the syndrome.

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Table 1: Effects of ethanol withdrawal and CAM1028 on behaviour in the elevated plus-maze.

Chronic/Acute treatment	% time on open arms	frequency of open arm entries	stretch-attend postures	% protected head dips	general activity
Control/Saline	21.3±2.5	10.1±1.6	6.3±0.9	37.4±7.7	51.3±5.2
Ethanol/Saline	8.2±2.0 **	4.6±1.2 *	16.9±2.7 **	59.8±6.4 *	35.4±4.4 *
Ethanol/CAM1028 0.1mg/kg	18.4±4.0 †	7.4±0.9	10.5±1.5 †	56.8±7.5	37.8±3.4
Ethanol/CAM1028 1mg/kg	17.5±3.6 †	8.0±0.8 †	9.7±1.1 †	49.0±5.1	31.0±4.8

* P < 0.05, **P < 0.001 compared with control/saline, † P < 0.05 compared with ethanol/saline (Student's t-test)

29P THE ANTIEPILEPTIC, GABAPENTIN, AND THE CCK_B ANTAGONIST, C1988, DECREASE HYPEREXCITABILITY IN HIPPOCAMPAL SLICES DURING ETHANOL WITHDRAWAL

Bailey, C. P., Molleman, A. and Little, H. J., Pharmacology Department, Medical School, University Walk, Bristol BS8 1TD.

The antiepileptic, gabapentin, prevents some behavioural signs of ethanol withdrawal, including seizures (Watson and Little, 1995) and anxiety related behaviour (Watson et al, 1995). The CCKB antagonist, CI988 (Hughes et al, 1990), has anxiolytic actions (Singh et al, 1991) and, at higher doses, decreases some of the convulsive aspects of ethanol withdrawal (J. Wilson, unpublished results). Isolated mouse hippocampal slices prepared after prolonged administration of ethanol in vivo show clear signs of hyperexcitability (Whittington & Little, 1990). We now investigate the effects of the above drugs in hippocampal slices.

Male C57 mice (20-25g.) were given ethanol, 24% as sole fluid for 7-9 months; ethanol intake was 12-13g/kg/day. Diet was laboratory chow and control drank tap water. Hippocampal slices were prepared between 9 am and 9.30 am each day without prior withdrawal of the mice from ethanol treatment (lights on at 9 am). The slices were allowed to equilibrate in a recording chamber for 105 min, then extracellular recordings made until 7h from preparation of the slices (i.e. from cessation of the ethanol treatment). The drugs were included in the bathing medium for the whole recording period.

Single and multiple spike thresholds for elicitation of population spikes were decreased in slices prepared after the ethanol treatment, as shown previously. Multiple spike thresholds from the second of two pulses, pulse interval 70 ms, were also decreased. In the presence of gabapentin, 1 μM, these changes were significantly reduced. At 100 nM, gabapentin did not affect these changes. CI988, when included in the bathing medium at 1 μM, decreased the lowering of single and multiple spike thresholds. Gabapentin and CI988, at 1 μM, and gabapentin at 100 nM, had no effect on any of the thresholds in slices prepared from control animals.

The results indicate that both these compounds decrease signs of ethanol withdrawal hyperexcitability in isolated hippocampal slices. More detailed analysis of these effects will provide information about the causes of the withdrawal hyperexcitability.

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We thank Parke Davis Neuroscience Unit for gifts of drugs and financial support for this project.

Table 1. The thresholds (μA) for single (mean ± s.e.m.) and multiple (medians with interquartile ranges) population spikes, at 5.5h after slice preparation. Cut-off = 350 μA. Statistical comparisons by Student's t-test (single spike thresholds), Mann-Whitney U test (multiple spike thresholds). * $P < 0.01$, ** $P < 0.0001$ vs controls; † $P < 0.05$, †† $P < 0.01$ vs ethanol + no drug. n = 4-6.

Chronic treatment	Bathing medium	Single thresholds		Multiple (single pulse)			Multiple (paired pulse)		
Control	Control	57.0 ± 0.9		350	(350, 350)		226	(96, 350)	
Ethanol	Control	35.2 ± 0.9	**	71	(45, 115)	*	36	(30, 42)	*
Ethanol	Gabapentin 100 nM	34.3 ± 2.9		132	(94, 135)		40	(39, 40)	
Ethanol	Gabapentin 1μM	47.3 ± 1.1	††	350	(350, 350)	†	79	(71, 215)	†
Ethanol	CI988 1μM	44.0 ± 3.4	†	350	(296, 350)	†	52	(48, 56)	†

30P PROLONGED CHANGES IN EFFECTS OF COCAINE AFTER CHRONIC ETHANOL TREATMENT

Manley, S. J. & Little, H.J., Pharmacology Department, Medical School, University Walk, Bristol BS8 1TD.

Long-term changes in brain function may be responsible for craving and the relapse back into drug taking which is seen in addicts. We have investigated the effects of chronic ethanol administration and subsequent long-term withdrawal on the effects of cocaine on locomotor activity.

Male TO mice (25-30g) were administered ethanol by liquid diet. Ethanol-treated mice first received control diet for 3 days, followed by 2 days 3.5% v/v ethanol in the diet, then 9 days 5% ethanol, followed by 9 days 8% ethanol (average daily intake was 31g/kg). Control groups were pair-fed control diet. Locomotor activity was measured for 10 min, in groups of mice (n=8), 20 min after administration of either cocaine, 10 mg/kg or 20 mg/kg or saline. Initial measurements were made after cocaine or saline administration , 2 months after cessation of the ethanol treatment. Animals then received injections of cocaine, 20 mg/kg or saline once daily and the effects on locomotor activity were measured again, 24h after the last injections, after 8 and after 15 days treatment.

When tested for the first time, cocaine 10mg/kg caused a significant increase in locomotor activity in ethanol treated animals, but not in control animals. The higher dose of cocaine significantly increased locomotor activity in both ethanol treated and control animals. After 8 days of daily injections, the same pattern was observed. When tested after 15 days treatment, cocaine, 10 mg/kg caused a significant increase in locomotor activity in control animals but not in ethanol treated animals and the response to the higher dose of cocaine was significantly reduced in ethanol treated animals when compared with the response obtained upon first administration. No significant differences were seen in the locomotor activity of controls or ethanol treated mice after administration of saline at any of the test times.

The results show that chronic ethanol intake can cause an increase in the effects of cocaine and subsequent tolerance to the effects on repeated administration. The prolonged changes in the effects of cocaine compare with previous findings (Manley and Little, 1995) which showed increases in the effects of amphetamine, 2 months after ethanol treatment.

Manley, & Little, (1995) Br. J. Pharmacol., in press.

Table 1. Locomotor activity measurements, mean ± s.e.m., comparisons by Student's t-test. Con = controls; Eth. = chronic ethanol; Coc = cocaine; 10 = 10 mg/kg; 20 = 20 mg/kg. 2nd and 3rd test made after 8 and 15 daily injections.

	Con/Sal	Eth/Sal	Con/Coc 10	Eth/Coc 10	Con/Coc 20	Eth/Coc 20
1st test	2365±169	2581±203	2889±362	3589±273 •	4647±428 **	4336±287 ••
2nd test	2307±227	2334±295	2737±301	3364±129 •	4435±303 **	3811±382 ••
3rd test	2055±252	2561±265	3160±310 *	3048±194	3983±245 **	3538±177••†

•P<0.05, ••P<0.01 cf. Eth/Sal; *P<0.05, **P<0.01 cf. Con/Sal; †P<0.05 cf. Eth/Coc 20, 1st test.

31P ENDOTHELIN-1 ENHANCES THE SYNTHESIS OF TUMOUR-NECROSIS FACTOR-α IN CULTURED MACROPHAGES AND IN THE ANAESTHETISED RAT

H. Ruetten , C. Thiemermann & J.R. Vane, The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1 6BQ.

There is good evidense that proinflammatory cytokines including tumour necrosis factor-α (TNFα), interleukin (IL)-1 or IL-2 stimulate the biosynthesis of endothelin-1 (ET-1) *in vitro* and *in vivo*. Interestingly, ET-1 also enhances the formsation of IL-6 *in vitro* (Xin et al., 1995). Here we investigate (i) whether ET-1 stimulates the formation of TNFα or interferin-γ (IFN) in cultured macrophages or in the anaesthetised rat and (ii) whether the signal transduction pathway of the stimulation of the biosynthesis of TNFα caused by ET-1 involves the activation of tyrosine kinase and/or the transcription factor NF-κB.

Murine macrophages (J774.2) were cultured in Dulbecco's modified Eagle's medium containing L-glutamine (3.5 mM) and 10% foetal calf serum. To induce the biosynthesis of TNFα, fresh culture medium containing ET-1 (0.01 - 1 μM, Peptide Inst.) was added to the cells. The accumulation of TNFα was measured at 6, 12 or 24 h later by ELISA. Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mossman, 1983). Male Wistar rats were anaesthetised with thiopentone (120 mg kg^{-1} i.p.). Blood samples were obtained prior to and every 15 min after injection of ET-1 (1 nmol kg^{-1} i.v.) to determine the plasma levels of TNFα and IFNγ.

Incubation of J774.2 macrophages with ET-1 (0.01 - 1 μM) caused a concentration- and time-dependent increase in the concentration of TNFα, but not of IFNγ, in the culture medium. For instance, incubation of macrophages for 24 h with ET-1 (1 μM) caused an increase in the levels of TNFα from 0 (control, n=9) to 149+12 pg ml^{-1} ($P<0.05$, n=9). Pretreatment of the cells with the tyrosine kinase inhibitor tyrphostin (AG-126, 30 μM) significantly attenuated the increase in tne formation of TNFα afforded by ET-1 (46±18 pg ml^{-1}, n=5, $p<0.05$). Inhibition of the activation of the nuclear transcription factor NF-κB with pyrrolidine dithiocarbamate (PDTC, 25 μM, an antioxidant and metal chelator; Bauerle & Henkel, 1994) significantly reduced the increase in TNFα biosynthesis afforded by ET-1 (27±7 pg ml^{-1}, n=5, $p<0.05$). Similarly, inhibition of IkB-protease, which is essential for the activation of NF-κB, by L-1-tosylamido-2-phenylethyl-chloromethyl-ketone (TPCK, 100 μM) also significantly attenuated the formation of TNFα caused by ET-1 ((24±10 pg ml^{-1}, n=5, $p<0.05$). Neither ET-1 alone nor ET-1 in the presence of tyrphostin, PDTC or TPCK caused a significant decrease in cell viability (data not shown). In anaesthetised rats, injection of ET-1 (1 nmol kg^{-1} i.v.) caused within 15 min a significant rise in the plasma levels of TNFα (baseline: 10±5 pg ml^{-1}, n=6), which was maximal at 45 min after injection of ET-1 (414±28 pg ml^{-1}, n=6; $P<0.05$).

Thus, activation of murine macrophages with ET-1 results in a concentration-dependent increase in the biosynthesis of TNFα, but not IFN. In addition, our results strongly suggest that the signal transduction mechanisms leading to the enhanced biosynthesis of TNFα by ET-1 involve the activation of tyrosine kinase and the nuclear transcription factor NF-κB.

HR is a fellow of the Deutsche Forschungsgemeinschaft (Ru595/1-1)

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32P THE ENDOTHELIN ANTAGONIST TAK-044 INCREASES HUMAN PLASMA IMMUNOREACTIVE ENDOTHELIN BUT NOT BIG ENDOTHELIN-1 OR C-TERMINAL FRAGMENT OF BIG ENDOTHELIN-1

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

Our aim was to determine whether systemic infusion of the endothelin (ET) antagonist TAK-044 (Kusumoto *et al*, 1994) altered levels of immunoreactive (IR) mature ET in human plasma. Secondly, in order to distinguish between receptor-bound ET and newly synthesised peptide, we also measured the concentrations of the precursor big ET-1 and the other product of ET synthesis, the C-terminal fragment of big ET-1 (CTF).

With the approval of the local ethical committee, TAK-044 or placebo were administered via a left forearm vein. Blood samples were withdrawn from a right antecubital vein prior to infusion and after 15min of infusion of either 50ml placebo or TAK-044. The two doses, 30 or 750mg, resulted in plasma levels of 2 and 80nM TAK-044 after 15min. These concentrations were calculated from binding studies to achieve about 95/5% and 99/75% ET_A/ET_B receptor occupation, respectively. Plasma IR ET, big ET-1 and CTF were determined by selective solid phase extraction and radioimmunoassays (Plumpton *et al*, 1995). Results are mean of six individuals ± s.e.mean. Data were analysed by ANOVA followed by Student's *t*-tests.

Infusion of placebo resulted in no change in the IR levels of the three peptides after 15min when compared to pre-infusion levels. Infusion of 30mg TAK-044 also resulted in no significant changes. However, the higher dose of TAK-044 produced a significant increase in IR mature ET compared with pre-infusion levels ($P< 0.01$) but no change in the levels of either IR big ET-1 or CTF.

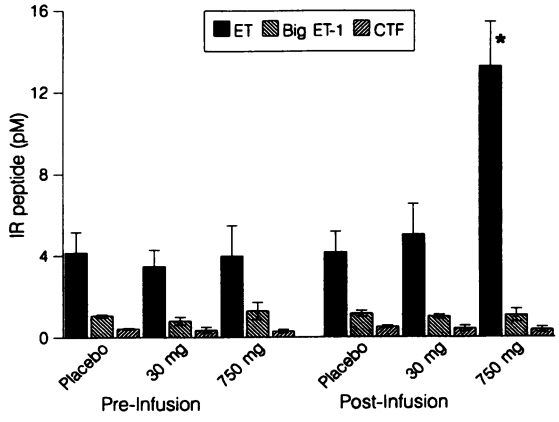

Figure 1 Measurement of IR ET, big ET-1 and CTF in human plasma. * $P<0.01$ significant change compared to placebo.

One explanation of these results is that TAK-044 had no affect on either the synthesis or conversion of big ET-1 after 15min of infusion. These data suggest that the increase in human plasma IR ET following the infusion of 750 mg TAK-044 may be a result of displacement of ET_B receptor-bound peptide and/or blockade of clearance receptors.

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33P INVESTIGATION OF ENDOTHELIN RECEPTOR SUBTYPES IN HUMAN ISOLATED MIDDLE MENINGEAL ARTERY

L.N.Pierre & A.P.Davenport. Clinical Pharmacology Unit, University of Cambridge, Box 110, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK.

Endothelin (ET) is a potent constrictor of both animal and human cerebral vasculature (Papadopoulos *et al.*, 1990; Salom *et al.*, 1993). It has been suggested that ET may be involved in the pathophysiology of cerebrovascular diseases such as stroke and the delayed vasospasm associated with subarachnoid haemorrhage. We have investigated the nature of ET receptors present on human isolated middle meningeal artery.

Middle meningeal artery was obtained from 19 patients undergoing surgery for the treatment of cerebral aneurysms or tumours.

Autoradiography: Slide mounted cryostat sections (10μm) were incubated for 2h with buffer containing 0.1nM of either [^{125}I]PD151242 (ET_A selective) or [^{125}I]BQ3020 (ET_B selective) (Davenport *et al.*, 1994) both 2000 Ci.mmol^{-1}.

In vitro pharmacology: 2 mm rings were set up in a wire-myograph containing oxygenated Krebs-Henseleit solution (37°C). Each preparation was set to 0.9 of the internal diameter (i.d.) the vessel would have if under a transmural pressure of 100mmHg (mean± s.e.mean i.d.=624.5±42.1 μm).

Following stimulation with potassium rich solution (95 mM) an initial response to 1μM 5-hydroxytryptamine (5-HT) was obtained. Cumulative concentration response curves were constructed to either ET-1, ET-3 or the ET_B receptor selective agonist sarafotoxin S6c, one curve was constructed per preparation. The effects of the ET_A receptor selective antagonists, PD156707 (Reynolds *et al.*, 1995) (non-peptide;30nM-1μM) and FR139317 (Davenport *et al.*, 1994) (peptide; 1μM) on responses to ET-1 were investigated. The antagonists were added 30 min prior to ET-1. All agonist responses were expressed as a percentage of the initial 5-HT-induced contraction. Antagonist potency (pA_2) was estimated from the Gaddum-Schild equation, assuming slope of one.

Specific high density binding of [^{125}I]PD151242 was observed on the vascular smooth muscle of middle meningeal artery (9.55±1.36 amol mm^{-1}). In contrast, any specific [^{125}I]BQ3020 binding in these blood vessels was below the level of detection (n=6).

ET-1 was 60 fold more potent than ET-3 as a vasoconstrictor, both agonists elicited similar maximal responses. EC_{50} values (geometric mean with 95% C.I.) were 3.82 nM (0.93-15nM) and 229 nM (110-470 nM) respectively (n=4-5). S6c (1pM-30nM) failed to elicit any contractile response in these blood vessels. Both PD156707 and FR139317 caused parallel rightward shift of the concentration response curve to ET-1 yielding pA_2 values of 8.27±0.15 (n=9) and 7.48±0.22 (n=4), respectively.

The present data suggests that ET_A receptors mediate ET-induced vasoconstriction in human middle meningeal artery. There was no evidence for the presence of ET_B receptors on the vascular smooth muscle of these blood vessels.

We are grateful to Ms L Maskell and the neurosurgeons, Dept of Neurosurgery, Addenbrooke's for collection of human tissue.

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34P ACTION OF ENDOTHELIN ET_B RECEPTOR ANTAGONISTS ON ENDOTHELIN-3-INDUCED PROLIFERATION OF WOUNDED ENDOTHELIAL CELLS

H. Lauder, T.-P.D. Fan & C.R. Hiley, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

Endothelin-1 (ET-1; Yanagisawa *et al.*, 1988) is mitogenic in several cell types including vascular smooth muscle (Komuro *et al.*, 1988) and brain endothelial cells (Vigne *et al.*, 1990). Endothelins may therefore act as vascular growth regulators and we have previously used an *in vitro* multichannel wounding device to study repair of endothelial cell injury in the presence of endothelins in human umbilical vein endothelial cells (HUVEC). In the presence of 1-1000 nM ET-1 there was no significant change in wound repair of HUVEC (Lauder *et al*, 1995). In contrast, the isopeptide ET-3 facilitated the recovery of HUVEC in a concentration-dependent fashion over 18 h with an $EC_{50} \approx 2$ nM but did not accelerate mitogenesis in sub-confluent cells. Thus it appears that recovery from injury is accelerated by ET-3 by means of a receptor which is insensitive to ET-1 at concentrations up to 1 μM. The role of ET_A and ET_B receptors in the repair response of HUVEC to ET-3 has therefore been investigated using antagonists.

HUVEC (pooled cells from Clonetics: TCS Biologicals, Buckingham) were grown to confluence on 13 mm diameter Thermanox™ coverslips. A wounder was used to produce 11 parallel, 400 μm wide, wounds across the monolayer of cells (Fan & Frost, 1990). The coverslips were rinsed and placed in a new well containing 450 μl complete medium and 50 μl drug or vehicle (medium E199). After 18 h incubation, the regeneration of HUVEC into the denuded area was quantified using a Seescan (Cambridge) semi-automated computerised image analysis system and the percentage recovery after wounding was calculated. Statistical comparison was by analysis of variance followed by Fisher's PLSD test using $P < 0.05$ as the level of significance.

Neither BQ-123 (21.9±1.3 % recovery), the ET_A receptor antagonist, nor BQ-788 (21.5±1.3 % recovery), the ET_B receptor antagonist, or the mixed receptor antagonist SB 209670 (20.3±1.4 % recovery), affected the basal rate of recovery from wounding in the absence of ET-3, although ET-3 (100 nM) increased recovery to 38.8±1.4 % from a control of 20.7±1.0 % ($n = 3$ for all, $P<0.001$ ET-3 versus control). In the presence of BQ-123 (500 nM), no difference was detected compared to ET-3 alone (38.4±2.3 % recovery, $n = 3$). However, BQ-788 (200 nM), suppressed the ET-3 response (recovery = 24.9±1.4 %; $n = 3$, $P<0.001$ relative to ET-3 alone). SB 209670 (5 nM) also suppressed the response (recovery = 19.8±1.4 %; $n = 3$, $P<0.001$ relative to ET-3 alone).

These results show that the repair response of wounded endothelial cell monolayers to ET-3 is sensitive to antagonists at the ET_B receptor, even though the response cannot be elicited by ET-1 which is classically an agonist at ET_B receptors. This may indicate that a novel subtype of receptor mediates the ET-3 enhancement of recovery from wounding in HUVEC.

HL is an MRC Research Student.

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35P AFFINITY OF THE ET_A SELECTIVE NON-PEPTIDE ANTAGONIST PD156707 FOR ENDOTHELIN RECEPTORS IN HUMAN RENAL AND CARDIOVASCULAR TISSUES

J.J. Maguire, R.E. Kuc, A.M. Doherty* & A.P. Davenport. Clinical Pharmacology Unit, University of Cambridge, Box 110, Addenbrooke's Hospital, Cambridge, CB2 2QQ, U.K. *Park- Davis Pharmaceutical Research Division, Ann Arbor, MI. 48105, USA.

PD156707 is a recently described non-peptide endothelin antagonist which appears to have good selectivity and potency for ET_A receptors and importantly shows activity following oral administration (Reynolds *et al.*, 1995). We have determined the affinity of this compound for endothelin receptors in human heart, kidney, coronary artery (CA) and saphenous vein (SV). Additionally we tested the ability of PD156707 to antagonise ET-1 mediated vasoconstriction in human isolated coronary artery, saphenous vein and left internal mammary artery (LIMA).

Competition Experiments: Sections (10μm) of human kidney, left ventricle and CA (media only) and aliquots of SV homogenate were incubated with 0.1nM [^{125}I]ET-1, for 2h at 23°C, in the presence of increasing concentrations of PD156707 (20pM-100μM). Non-specific binding was determined by the inclusion of 1μM ET-1. Sections/aliquots were washed in ice-cold Tris-HCl and counted for [^{125}I] content.

Tissue bath experiments: 3mm rings of endothelium-denuded CA, SV or LIMA were suspended in organ baths containing oxygenated Krebs soln. (37°C). Cumulative concentration-response curves were constructed to ET-1 (10^{-10}-10^{-6}M) in the absence and presence of PD156707 (30 and 100nM) added 30 min prior to ET-1. Experiments were terminated by addition of 50mM KCl and ET-1 responses expressed as a percentage of this maximum response. Values of pA_2 were estimated from the Gaddum-Schild equation assuming slope of one.

In competition experiments PD156707 competed with subnanomolar affinity for ET_A receptors in human tissues (Table 1). The compound exhibited at least 1000 fold selectivity for this subtype compared to the ET_B receptor.

Table 1 Affinity (K_D) of PD156707 for human endothelin receptors

	ET_AK_D (nM)	ET_BK_D (μM)	ET_A:ET_B
Coronary artery	0.15±0.06*	-	100:0
Saphenous vein	0.50±0.13	1.42±0.02	83:17
Left ventricle	0.92±0.38	13.3±2.09	75:25
Kidney	0.53±0.49	0.57±0.04	22:78

* One site fit preferred, no detectable ET_B binding. Values are the mean±s.e.mean (n=3) generated by the iterative programme Ligand.

PD156707 antagonised ET-1 responses in isolated CA, SV and LIMA with estimated pA_2 values of 8.14±0.11 (n=16), 8.49±0.34 (n=8) and 8.18±0.14 (n=8), respectively. These values are an order of magnitude greater than those we obtained for the peptide antagonist BQ123. Additionally, PD156707 (300nM) reversed an established ET-1 contraction in CA.

These data indicate that in binding and functional assays PD156707 is one of the most potent and selective non-peptide antagonists yet described for ET_A receptors in human tissue.

We are grateful to the Theatre staff at Addenbrooke's and Papworth Hospitals for permission to collect tissue samples.

Reynolds, E.E., Keiser, J.A., Haleen, S.J. *et al.*, (1995) *J. Pharmacol., Exp. Therap.* 273(3), 1410-1417.

36P BINDING CHARACTERISTICS OF ET_B SELECTIVE COMPOUNDS IN HUMAN AND RAT HEART

F.D. Russell & A.P. Davenport, Clinical Pharmacology Unit, University of Cambridge, Box 110, Addenbrooke's Hospital, Cambridge CB2 2QQ, U.K.

Species differences should be considered in studies that utilise previously determined endothelin (ET) receptor subtype selectivity ratios. The aim of the present study was to determine inhibition binding constants for ET_B selective compounds at native human and rat ET receptors. Left ventricle obtained from patients with idiopathic dilated cardiomyopathy undergoing cardiac transplantation (HLV; 6 males, 23-64 years) and from American Cancer Institute (ACI) rats (RLV; 7 females, 175-240g) were frozen immediately in liquid nitrogen and cut on a cryostat at -22°C. Sections (10 μm) were labelled with [^{125}I]-ET-1 (non-selective), [^{125}I]-BQ3020 (ET_B selective) or [^{125}I]-PD151242 (ET_A selective) (0.1 nM, 2h, 23°C) in the presence of increasing concentrations of ET_B selective compounds. Sections were also labelled with [^{125}I]-ET-1 and FR139317 (ET_A antagonist, 0.1 μM; Aramori *et al.*, 1993) to help delineate binding to ET_B receptors. Sections were washed, wiped from the slides and counted.

Sarafotoxin S6c (S6c) inhibited [^{125}I]-ET-1 binding in HLV with a selectivity ratio for ET_B over ET_A receptors (3700 fold) similar to that reported for CHO cells transfected with human ET receptors (4100 fold; Buchan *et al.*, 1994) and different to that in RLV (114,000 fold, Table 1) and in other rat tissues (>174,000 fold; Williams *et al.*, 1991). The affinity of ET_B receptors for BQ788 and ET-3 was similar for both human and rat left ventricular tissues (Table 1). Interestingly the affinity of BQ788 at HLV ET_B receptors was 33 fold lower than in human Girardi heart cells (Ishikawa *et al.*, 1994). IRL1038 competed against [^{125}I]-ET-1 in HLV with low affinity at ET_A and ET_B receptors (data not shown). The affinity of S6c, BQ788 and ET-3 against [^{125}I]-BQ3020 in HLV was high and low against [^{125}I]-PD151242, confirming the ET_B selectivity of these compounds in this tissue (data not shown).

We conclude that species differences can account for marked discrepancies in the binding affinity and receptor subtype selectivity ratio for S6c. The affinity of ET_B receptors for BQ788 in HLV and RLV was lower than that reported for human Girardi heart cells indicating possible differences between binding in native tissue and cultured cells.

Table 1. Inhibition constants for ET_B selective compounds against [^{125}I]-ET-1 in human (HLV) and rat left ventricle (RLV)

Competitor	Tissue	K_DET_A (nM)	K_DET_B (nM)	n
S6c	HLV	2120±252	0.57±0.68	6
S6c+FR139317	HLV	*	0.31±0.17	3
S6c	RLV	3880±295	ND	4
S6c+FR139317	RLV	*	0.034±0.039	4
BQ788	HLV	1347±157	39.9±29.3	5
BQ788+FR139317	HLV	*	19.5±9.3	5
BQ788	RLV	1230±199	29.3±57.0	4
BQ788+FR139317	RLV	*	29.5±9.3	4
ET-3	HLV	80.6±13.7	1.93±1.12	4
ET-3+FR139317	HLV	*	0.32±0.11	5
ET-3	RLV	1816±2768	19.0±4.4	4
ET-3+FR139317	RLV	*	0.33±0.32	3

Values are expressed as mean ± s.e. mean. *, K_D not shown because of reduced Bmax with FR139317. ND, Not determinable

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37P CHARACTERISATION OF ENDOTHELIN RECEPTORS IN PORCINE PULMONARY TISSUE

C.A. Sargent, M Birrell, J McCormick, T.J. Brown & A.G. Roach. Rhône-Poulenc Rorer, Dagenham, Essex RM10 7XS.

In porcine pulmonary artery (PPA), ET-1 has been reported to mediate relaxation via activation of ETB receptors in endothelium intact vessels (+E) and contraction via ETA receptors in tissues with endothelium removed (-E) (Sudjarwo et al, 1993, Fukuroda et al, 1992). In porcine pulmonary vein (PPV), ET-1 has been reported to mediate contraction via a novel ETB receptor (Sudjarwo et al, 1993). In this study, we determined the effect of the selective ETB receptor agonist, BQ 3020, on PPA (-E and +E) and PPV (-E). The contractile response to ET-1 was also determined in PPA (-E). The pKB values for the ETA-receptor antagonists (BQ 123 and PD 156707), ETB-receptor antagonist BQ 788 and the ETA/ETB receptor antagonist bosentan (Battistini et al, 1995) were determined against contraction to ET-1 and BQ 3020 in PPA (-E) and BQ 3020 in PPV (-E). The pKB values for PD 156707 and bosentan on BQ 3020 mediated relaxation were also calculated.

Male Large White pigs (30-40 kg) were given an overdose of anaesthetic and lungs were removed. The secondary branch of the PPA and PPV were isolated, tissues cut into 3 mm rings and endothelium removed if required. The rings were placed in an organ bath and equilibrated for 60 min at 37°C under 1g of tension. A reference response to 30 μM phenylephrine (PE) was obtained in PPA (-E) and PPV (-E). In PPA (+E) tissues were preconstricted with 300 nM PE and reference relaxation to 3 μM acetylcholine obtained. PPA was incubated with vehicle or antagonist for 30 min. The contractile response to BQ 3020 and ET-1 was determined in PPA (-E). In PPA (+E) the relaxant response to BQ 3020 was measured in preconstricted tissue. PPV was incubated with vehicle and the contractile curve to BQ 3020 measured. PPV was washed, incubated with vehicle or antagonist for 30 min and a second response to BQ 3020 measured. The potency of the ET antagonists were expressed as percentage of reference agent in PPA and grams tension in PPV.

In PPA (-E) constriction was observed with ET-1 but not BQ 3020. In this tissue ET antagonists caused parallel rightward shifts with equivalent maximums (Table 1). BQ 3020 caused constriction in PPV and relaxation in PPA (+E) preconstricted with PE. In PPV and PPA (+E) all antagonists caused parallel shifts to the right and augmented maximum responses. Estimated pKB values were calculated for theses tissues using the individual maximums for vehicle and antagonist (Table 1). The pKB values for PD 156707 and bosentan in PPA (+E) were the same as those in PPV.

The ETB selective agonist BQ 3020 had no effect on PPA (-E) while ET-1 induced contraction. This coupled with the low potency of the ETB selective antagonist BQ 788 suggest that contraction in this tissue is mediated by ETA receptors. A contractile response in PPV was obtained with BQ3020 which was antagonised by BQ 788, indicative of classical ETB mediated contraction. BQ 3020 produced relaxation of PPA (+E) preconstricted with PE, suggesting ETB mediated relaxation was present. In both these ETB tissues all antagonists caused an augmented maximum response. In conclusion, porcine pulmonary tissue contains receptors which could be classified as ETA contractile, ETB contractile and ETB relaxant. However, the augmented maximum responses observed with ET antagonists in ETB preparations suggest the ETB receptors may be atypical.

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Table 1. pKB values of ET antagonists in PPA (-E) and estimated pKB in PPV.

Compound	PD 156707	Bosentan	BQ123	BQ788
pKB pulmonary artery (ETA)	6.9	6.3	6.3	5.3
pKB pulmonary vein (ETB)	6.3	6.3	5.0	7.5

All values are expressed as geometric mean (n=4-6 per group)

38P GROWTH-INDUCED NATRIURETIC PEPTIDE RECEPTOR SUBTYPE SWITCHING AND NATRIURETIC PEPTIDE EXPRESSION IN PRIMARY CULTURES OF RAT PROXIMAL TUBULAR CELLS

S.K. Mistry, P.K. Chatterjee, R.P. Weerackody, N.G. McKay, R.M. Knott, G.M. Hawksworth and J.S. McLay
Departments of Medicine & Therapeutics and Biomedical Sciences and Ophthalmology, University of Aberdeen, Scotland, UK.

The natriuretic peptide receptors (NPR-A,B,C) are widely distributed throughout the kidney, and are involved in the modulation of salt and water transport and possibly cell growth (Appel, 1992) and immune response (McLay, *et al*, 1995). There is increasing evidence to suggest that the natriuretic peptides (ANF,BNP,CNP) are also synthesised within the kidney, in areas such as the distal and proximal tubules (Totsune, *et al*, 1994). The function of these locally synthesised natriuretic peptides is not clear but they may be involved in the regulation of electrolyte transport, cell growth and response to an immune challenge.

The objectives of the present study were to use RT-PCR analysis and cGMP response to ANF to (i) identify natriuretic peptide and NPR expression in freshly isolated primary cultures of rat proximal tubular (RPT) cells and (ii) to determine how cell growth and time in culture might affect NPR and peptide expression.

Renal proximal tubular cells were isolated from Sprague Dawley rat kidney cortex by collagenase digestion, sieving and Percoll density centrifugation. Cells were grown in 80cm^2 flasks in DMEM/Hams F12 (10% FCS). Total RNA was prepared from freshly isolated RPT cells and then following 4, 5 and 7 days in culture. Natriuretic peptide and NPR expression were determined using RT-PCR and specific pairs of primers for ANF, BNP, CNP, NPR-A, NPR-B and NPR-C. The presence of the guanylate cyclase-linked ANF receptors (NPR-A,B) was confirmed by measurement of rat-ANF (10^{-6}M) stimulated cGMP production. All PCR products for the natriuretic peptides and NPR were sequenced and compared with those deposited in the GENBANK database.

RT-PCR analysis of freshly isolated RPT cells revealed expression of mRNA for NPR-C, however no mRNA for NPR-A or B could be detected. After 5 days in culture the expression of NPR-C disappeared, however mRNA for both NPR-A and B became evident. Similarly no mRNA for the natriuretic peptides could be detected in freshly isolated RPT cells. However after 4 days in culture the mRNA for both ANF and BNP became apparent, preceding the expression of receptor mRNA by 24 hours.

Although RT-PCR analysis did not identify mRNA for the NPR-A or B in freshly isolated RPT cells, rat-ANF stimulation produced a significant increase in cGMP production from 0.032 ± 0.017 (control) to 1.57 ± 0.07 pmol/mg protein/hour (n=4, P<0.01). Following 5 days in culture, by which time RT-PCR had identified mRNA for both NPR-A and B, the magnitude of the cGMP response had increased approximately 5 fold (day 7) from 0.063 ± 0.015 (control) to 8.84 ± 0.47 pmol/mg protein/hour (n=4, P<0.01). This suggests that the identification of mRNA for the NPR-A and B was associated with a true increase in functional guanylate cyclase-linked receptor number.

The results of this study clearly demonstrate the presence of guanylate cyclase-linked ANF receptors on both freshly isolated and cultured RPT cells. The expression and functional activity of NPR-A and B increased with time in culture, while the expression of the NPR-C disappeared completely. This study also demonstrates that the NPR subtype expression does change significantly during cell growth and time in culture. Finally, we have demonstrated that RPT cells express the mRNA for both ANF and BNP during active cell growth. This suggests that proximal tubular cells can produce natriuretic peptides at a local level, with a possible autocrine/paracrine function.

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39P ALDOSTERONE STIMULATES DNA, RNA AND PROTEIN SYNTHESIS IN PRIMARY CULTURES OF RAT PROXIMAL TUBULAR CELLS

R.P. Weerackody, P.K. Chatterjee, S.K. Mistry, G.M. Hawksworth and J.S. McLay
Departments of Medicine & Therapeutics and Biomedical Sciences, University of Aberdeen, Scotland, U.K.

Hypertension and resultant renal damage are common clinical features associated with primary and secondary hyperaldosteronism. Recent reports have suggested that aldosterone may affect cardiovascular and renal cell growth. Aldosterone may also stimulate collagen deposition by myocytes and enhance angiotensin II-induced hypertrophy of vascular smooth muscle cells (Hatakeyama *et al* 1994). However little information is available on growth effects of aldosterone within the kidney. The proliferation and hypertrophy of renal proximal tubular cells are known to be associated with tubulointerstitial fibrosis leading to end stage renal failure.

RPT cells were prepared by collagenase digestion, sieving and Percoll density centrifugation of freshly isolated kidney cortex obtained from Sprague-Dawley rats. Cells were cultured on 35mm collagen-coated plates in DMEM/Ham's F12 media supplemented with 10% FCS for 3 days and made quiescent in DMEM/Ham's F12 containing insulin, hydrocortisone, sodium selenite, transferrin and tri-iodo thyronine for 24hr. Quiescent cells were incubated with aldosterone (10^{-10}, 10^{-8} and 10^{-6} M) for 24 and 72 hours. DNA synthesis was assessed using [^{3}H] thymidine uptake, protein synthesis was determined using the uptake [^{3}H] uridine and [^{3}H] leucine and measurement of total protein content.

Incubation with aldosterone produced a significant increase in DNA and protein synthesis in RPT cells. The maximal effect upon [^{3}H] thymidine uptake was obtained after 24 hrs, whereas the maximal increase in protein synthesis occurred at 72hr. These results are summarised in the following table.

The effect of aldosterone upon RPT cell growth

	[^{3}H] thymidine uptake (dpm/plate/hour)	Protein content (µg protein/plate)
	24hr incubation	72hr incubation
Control	3584±505	503+24
10^{-10} M Aldosterone	3696±636	498+29
10^{-8} M Aldosterone	3678±471	591+31*
10^{-6} M Aldosterone	4671±521*	551+28*
	[^{3}H] uridine uptake (dpm/plate/hour)	[^{3}H] leucine uptake (dpm/plate/hour)
	72hr incubation	72hr incubation
Control	19117±2158	7317+1483
10^{-10} M Aldosterone	18425±1692	7886+1565
10^{-8} M Aldosterone	25225±1810*	12589+1485*
10^{-6} M Aldosterone	21020±1696*	10678+1624*

n=3, *P<0.01 compared with control

This study clearly demonstrates that aldosterone stimulates both DNA synthesis and protein synthesis and adds further evidence to the hypothesis that the renin-angiotensin-aldosterone system may be a principal modulator of pathological cell growth.

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40P ANGIOTENSIN II-STIMULATED DNA, RNA AND PROTEIN SYNTHESIS IN HUMAN PROXIMAL TUBULAR CELLS IS INHIBITED BY THE AT_1 RECEPTOR ANTAGONIST DuP753

P.K. Chatterjee, R.P. Weerackody, S.K. Mistry, G.M. Hawksworth & J.S. McLay, Departments of Medicine & Therapeutics and Biomedical Sciences, University of Aberdeen, Scotland, U.K.

The proliferation and hypertrophy of renal proximal tubular cells observed during renal disease or following acute renal failure indicates a poor prognosis for recovery (Nath, 1992). Evidence from both *in vitro* and *in vivo* studies suggests that angiotensin II (AngII) may play an important role in the stimulation of cellular proliferation and hypertrophy observed in many disease states (Wolf, 1993).

The aims of this study were to investigate the growth-promoting effects of AngII upon primary cultures of human proximal tubular (HPT) cells and secondly, which AngII receptor subtypes and second messenger systems might be involved.

Primary cultures of HPT cells were prepared using collagenase digestion, sieving and Percoll density centrifugation of cortex obtained from nephrectomy specimens. HPT cells were cultured on 6 well plates ($1x10^6$ cells/well) in DMEM/Ham's F12 medium containing 10% fetal calf serum. Cultures were grown in media containing AngII (10^{-10},10^{-8}, 10^{-6}M) either alone or in combination with 10^{-6}M Losartan, the AT_2 receptor antagonist PD123319 (10^{-6}M) (Dudley *et al.*, 1990) or 10^{-6}M 8-Bromo-cAMP. DNA synthesis was assessed using [^{3}H]thymidine incorporation, RNA synthesis determined using the uptake of [^{3}H]uridine, *de novo* protein synthesis measured using [^{3}H]leucine uptake and measurement of total protein content.

Incubation with both 10^{-10} and 10^{-8}M AngII produced significant increases in HPT cell DNA synthesis, with maximal values occurring after 5 days in culture (19 and 42% respectively, n=4, P<0.01). HPT cell RNA and protein synthesis and total protein content increased significantly in a dose-dependent manner with a maximal response occurring after 10 days incubation with 10^{-6}M AngII. At this concentration of AngII, [^{3}H]uridine, [^{3}H]leucine uptake and protein content, increased by 46%, 27% and 30% respectively (n=4, P<0.01). Coincubation with AngII and Losartan produced a significant inhibitory effect on both AngII-stimulated DNA, RNA and protein synthesis as well as total protein content. A similar response was observed following coincubation with AngII and 8-Bromo cAMP. However, incubation with PD123319 had no effect on the AngII stimulation of HPT cell growth.

Effect of Losartan, PD123319 and 8-Bromo-cAMP upon AngII-stimulated HPT cell DNA synthesis and protein content

	Thymidine Uptake (5 day cultures) x10^3 dpm/mg prot/hr	Protein Content (10 day cultures) µg prot/plate
Control	621±39	805±60
10^{-8}M AngII only	882±72*	1035±20*
10^{-6}M Losartan only	602±79	795±40
10^{-6}M PD123319 only	676±66	715±100
10^{-6}M 8-Bromo-cAMP only	644±81	896±77
10^{-8}M AII+10^{-6}M Losartan	626±93+	825±90+
10^{-8}M AII+10^{-6}M PD123319	828±38	1208±38
10^{-8}M AII+10^{-6}M 8-Br-cAMP	625±50+	784±56+

n=4, *P<0.01 vs. control, +P<0.01 compared to 10^{-8}M AngII only

The ability of Losartan to block the AngII mediated stimulation of HPT cell DNA synthesis and increase in protein content suggests the effects of AngII are mediated via the AT_1 receptor. The similar effect obtained using 8-Bromo-cAMP further suggests that cAMP may be involved.

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41P ANGIOTENSIN II STIMULATION OF MAP KINASES IN CULTURED VASCULAR SMOOTH MUSCLE CELLS FROM HYPERTENSIVE RATS: PROTEIN KINASE C-DEPENDENT AND -INDEPENDENT MECHANISMS

N. Wilkie, L.L. Ng*, & M.R. Boarder, Department of Cell Physiology and Pharmacology, and *Department of Medicine and Therapeutics, University of Leicester, University Road, Leicester LE1 9HN

Angiotensin II (AII) is a mitogen for vascular smooth muscle derived cells (VSM) from the spontaneously hypertensive rat (SHR) but not from normotensive controls (WKY). This response involves phospholipase D (PLD) (Morton et al, 1995a); this PLD activation is by a tyrosine kinase dependent mechanism (Morton et al, 1995b). Here we investigate a possible role for mitogen activated protein kinase (MAP kinase) in the selective mitogenic response of the SHR derived cells.

Aortic VSM cells were cultured from 12 week old SHR and WKY rats and used between passage 8-14. Following stimulation with 100 nM AII or 100 nM phorbol myristyl acetate (PMA) for 5 min (where not otherwise indicated) cell extracts were assayed for kinase activity using a peptide substrate partially specific for MAP kinases (Clark-Lewis et al, 1991). MAP kinase immunoreactivity was investigated using Western blots.

SHR and WKY derived VSM cells in culture had the same levels of MAP kinase immunoreactivity. When the cell extracts were separated on ion exchange (Resource Q) chromatography the majority of the stimulated enzyme activity eluted with MAP kinase immunoreactivity; this AII and PMA (EC_{50} 4.76 ± 0.32 and 4.98 ± 0.52 nM respectively, mean ± s.e.mean, n=4) stimulated activity was greater for the SHR than for the WKY cells. On time courses the stimulated activity peaked at about 5 min and then formed a plateau. The ratio of stimulated activity for SHR/WKY was 2.90 ± 0.07 for AII and 2.56 ± 0.09 for PMA (n = 3). The protein kinase C inhibitor Ro 31 8220 (10 μM; compound 3 in Davis et al, 1989) reduced the early phase of stimulation of MAP kinase activity by AII , but the later phase was unaffected. With SHR cells, expressed as cpm/μg protein (mean ± s.e.mean, n = 7), with 2 min stimulation: AII alone, 9123 ± 1708; AII + Ro 31 8220, 1863 ± 1085 (significantly different from no inhibitor, P<0.01). With 10 min stimulation, AII alone, 7919 ± 2086; AII + Ro 31 8220, 7713 ± 2075. The tyrosine kinase inhibitor genistein at 100 μM substantially reduced both the early and late phases of stimulation by both AII and PMA.

Thus despite similar levels of MAP kinase enzyme, the enzyme activity in response to AII or PMA was much greater in the SHR cells.The AII stimulation of activity in SHR cells was dependent on tyrosine kinases and partially dependent on protein kinase C. These differences may be important in the proliferative responses of SHR vs WKY vascular tissue.

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42P ANGIOTENSIN IV STIMULATES PROTEIN SYNTHESIS IN PRIMARY CULTURES OF RAT PROXIMAL TUBULAR CELLS

R.P. Weerackody, P.K. Chatterjee, S.K. Mistry, G.M. Hawksworth and J.S. McLay
Departments of Medicine & Therapeutics and Biomedical Sciences, University of Aberdeen, Scotland, U.K.

Angiotensin II (Ang II) has been recognised as an important modulator of cellular growth in a variety of cell types including the proximal tubule. Although Ang II is believed to be the primary mediator, there is increasing evidence that the breakdown fragments of Ang II such as angiotensin IV (Ang IV) may also have unique functions. Ang IV has been reported to exert growth related effects in rabbit cardiac fibroblasts (Wang et al 1995). However, little is known about the functions of Ang IV within the kidney, although specific receptors for Ang IV have been identified in rabbit and opossum proximal tubule fragments (Douglas & Hopfer 1994) and the outer medulla of the rat kidney (Harding et al 1994).

The aim of the present study was to investigate the growth related effects of Ang IV upon primary cultures of rat proximal tubular (RPT) cells.

RPT cells were prepared by collagenase digestion, sieving and Percoll density centrifugation of freshly isolated kidney cortex obtained from Sprague-Dawley rats. Cells were cultured on 35mm collagen-coated plates in DMEM/Ham's F12 media supplemented with 10% FCS for 3 days and made quiescent in DMEM/Ham's F12 containing insulin, hydrocortisone, sodium selenite, transferrin and tri-iodo thyronine for 24hr. Quiescent cells were incubated with Ang IV (10^{-10}, 10^{-8} and 10^{-6} M) for 24 and 72 hours. DNA synthesis was assessed using [^{3}H] thymidine uptake, protein synthesis was determined using the uptake [^{3}H] uridine and [^{3}H] leucine and measurement of total protein content.

Ang IV did not have any significant effect on the DNA synthesis of RPT cell cultures. However, after 72 hours incubation, Ang IV produced a significant increase in protein as summarised in the following table.

Effects of Ang IV upon RPT cell growth

	[^{3}H] thymidine uptake (dpm/plate/hour)	Protein Content (μg/plate)
	24hr incubation	72hr incubation
Control	3584±505	503+24
10^{-10} M Ang IV	3730±533	504+29
10^{-8} M Ang IV	3690±552	579+31*
10^{-6} M Ang IV	3761±565	573+19*
	[^{3}H] uridine uptake (dpm/plate/hour)	[^{3}H] leucine uptake (dpm/plate/hour)
	72hr incubation	72hr incubation
Control	19117±1315	7317+1067
10^{-10} M Ang IV	19435±1215	6949+1153
10^{-8} M Ang IV	24402±1400*	12315+1136*
10^{-6} M Ang IV	25007±1182*	12712+1220*

n=3, *P<0.001 compared with control

This study clearly demonstrates that Ang IV stimulates protein synthesis in RPT cells. Ang IV may therefore be an important modulator of cellular growth, with a significant function within the renin-angiotensin system.

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43P ANGIOTENSIN-(1-7): VASODILATOR OR ANTAGONIST?

C. FORSTER & Y. LE TRAN, Department of Pharmacology, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

It has been suggested that peptides, apart from angiotensin II, are biologically active. Angiotensin-(1-7) is a pathophysiologically active component of the renin-angiotensin system which has little vasoconstrictor activity (Ferrario *et al.*, 1991; Kono *et al.*, 1986). Furthermore, the major production of angiotensin-(1-7) is tissue (particularly endothelium; Santos *et al.*, 1992). Therefore, we investigated whether angiotensin-(1-7) had vasodilator and antagonistic properties.

Male Sprague Dawley rats (N > 6) were used and their thoracic aortae dissected without damaging the endothelial lining. The isolated aortae were cut into 5mm rings and alternate rings were endothelium-denuded. Rings were mounted in organ baths containing Krebs Henseleit with propranolol (10^{-6}M) and indomethacin ($2.8x10^{-6}$M). Following equilibration, rings were precontracted with KCl (20mM) and acetylcholine was added to assess endothelium integrity. Rings were washed, re-equilibrated, and concentration-effect curves were constructed to noradrenaline. When completed, subsequent concentration-effect curves were constructed in the absence and presence of increasing concentrations of angiotensin-(1-7) [10^{-8}-10^{-6}M]. In 4 further intact and denuded rings, angiotensin-(1-7) was evaluated on preconstricted vessels (20mM KCl) to determine whether it exhibited relaxation properties.

None of the denuded preparations exhibited a relaxation in response to acetylcholine confirming successful denudation. Angiotensin-(1-7) produced no contractions (in the concentrations used) in either intact or denuded preparations. Noradrenaline had similar efficacy ($g.mm^{-2}$) in all preparations (14.2 ± 1.2 and 13.1 ± 1.3; intact and denuded, respectively), *but*, the denuded preparations were significantly more sensitive (EC_{50}'s [mean {95% confidence limits}]; [2.5 {0.9-6.0} x 10^{-9}M] and [2.9 {1.2-4.9} x 10^{-11}M] for intact and denuded, respectively). In intact preparations, 10^{-6}M angiotensin-(1-7) significantly lowered the maximum response to noradrenaline (6.6 ± 1.1) and had little effect on the sensitivity ($2.1x10^{-9}$M). In contrast, in denuded preparations, 10^{-6}M angiotensin-(1-7) did not significantly alter the maximum response to noradrenaline (10.2 ± 2.0), but the concentration-effect curves were shifted rightward (EC_{50}; [3.5 {1.0-6.0} $x10^{-10}$M]). Angiotensin-(1-7) produced concentration-dependent relaxations in *only* intact preparations.

These results indicate that angiotensin-(1-7) possesses vasodilator properties *only* if the endothelium is intact. In addition, if the endothelium is denuded, angiotensin-(1-7) has an antagonist effect against noradrenaline. Whether this latter effect is unique to noradrenaline awaits further studies.

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44P CALCITONIN GENE-RELATED PEPTIDE INCREASES GLUCOSE UPTAKE IN L6 SKELETAL MYOCYTES BY CYCLIC AMP

D.R. Poyner, S. Howitt & S. Harkin (J.W.Smith), Pharmaceutical Sciences Institute, Aston University, Birmingham, B4 7ET.

Calcitonin gene-related peptide (CGRP) is reported to inhibit the insulin mediated increase in glycogen synthesis and to activate glycolysis in rat soleus muscle but the biochemical basis for this effect is unclear (Leighton *et al.*, 1988). The rat L6 cell line has the phenotype of skeletal muscle, and possesses CGRP receptors which, at least in some subclones, inhibit insulin mediated glucose uptake (Kreutter *et al.*, 1989). The aim of this study was to investigate the actions of CGRP on glucose uptake in the C8G5 subclone of L6 cells, and to determine its mechanism of action.

L6 were grown to confluence in 24 well plates in 5% foetal calf serum/DMEM, and induced to differentiate by 24h incubation in 0.5% foetal calf serum/DMEM. Cells were pretreated with agonists for 2h before glucose uptake was measured by incubating with 0.2μCi of 0.1mM [^{3}H]-2-deoxyglucose per well, as described elsewhere (Kreutter *et al.*, 1989). Where appropriate H89 (Chijiwa *et al.*, 1990) was added 1h before agonists. Results are expressed as percentages of basal uptake (approximately 5nmol 2-deoxyglucose per 10^6 cells per 10 mins).

In contrast to the findings of Kreutter *et al.*, (1989), CGRP increased glucose uptake (Table 1; EC_{50}=10nM). This was mimicked by 10μM isoprenaline, acting at adenylate cyclase linked β-adrenoceptors in these cells (Poyner *et al.*, 1992), and 1mM dibutyryl cyclic AMP. It was blocked by H89, an inhibitor of protein kinase A. The selectivity of this reagent was confirmed by its failure to block the stimulation of glucose uptake caused by vasopressin, which acts via a V1a receptor linked to phospholipase C (Wakelam *et al.*, 1987).

These results imply that CGRP acts to stimulate glucose transport in the C8G5 clone of L6 cells by increasing cyclic AMP. Further studies are needed to establish if CGRP can influence glycogen metabolism in these cells.

Table 1 Actions of agents on glucose uptake into L6 cells

Agent	%increase glucose uptake
100nM CGRP	135±6 (n=11)*
10μM isoprenaline	139±5 (n=15)*
1mM dibutyryl cyclic AMP	119±2 (n=9)*
1μM vasopressin	137±4 (n=6) *
100nM CGRP + 10μM H89	110±6 (n=4)
10μM isoprenaline + 10μM H89	112±5 (n=4)
1μM vasopressin + 10μM H89	147±9 (n=4)*

Values are means ± s.e.means. * denotes significantly different from basal uptake, P<0.05, Students t-test.

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45P 5-HT RECEPTORS MEDIATING THE INCREASES IN EXTERNAL CAROTID BLOOD FLOW IN VAGOSYMPATHECTOMIZED DOGS PRETREATED WITH GR127935

C.M. Villalón*, A. Sánchez-López & D. Centurión. Department of Pharmacology, CINVESTAV, I.P.N., Apdo. Postal 22026, 14000 México D.F., México.

5-hydroxytryptamine (5-HT) decreases the external carotid blood flow (ECBF) in vagosympathectomized dogs via 5-HT_1-like receptors similar to the 5-HT_{1D} subtype (Villalón *et al.*, 1995a,b). Since this response to 5-HT, but not to sumatriptan, is reverted to vasodilatation after GR127935, a selective 5-HT_{1D} receptor antagonist (Skingle *et al.*, 1993), this study has investigated the receptors involved in 5-HT-induced increase in ECBF in 28 vagosympathectomized dogs under anaesthesia with pentobarbital (30 mg kg^{-1}, i.v.) and pretreated with GR127935 (20 $\mu g\ kg^{-1}$, i.v.). Baseline values of heart rate (HR) and mean blood pressure (MBP) were, respectively, 189±6 beats min^{-1} and 140±7 mmHg. Intracarotid infusions of 5-HT (0.3-30 $\mu g\ min^{-1}$) elicited dose-dependent increases in ECBF (Table 1) without changes in HR or MBP. The increases in ECBF by 5-HT were resistant to antagonists at 5-HT_2, 5-HT_3 and 5-HT_4 receptors, but were blocked by 8-OH-DPAT, lisuride and methiothepin (Table 1). Furthermore, the above effects of 5-HT were potently mimicked by 5-carboxamidotryptamine and 5-methoxytryptamine, the effects of which were similarly antagonized by 8-OH-DPAT, lisuride and methiothepin whithout affecting the increases in ECBF by acetylcholine (not shown).

Table 1. Effect of various compounds on the increases in ECBF (ml min^{-1}) by 5-HT ($\mu g\ min^{-1}$) in GR127935-pretreated dogs

Compound	*Dose*	*0.3 μg*		*1 μg*		*3 μg*		*10 μg*		*30 μg*	
(n=4)	*mg kg^{-1}*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
Saline[a]	-	9±3	10±2	14±2	19±4	34±11	47±9	56±9	79±12	104±15	118±10
Rit. + Graniset.[b]	-	9±4	9±1	34±12	25±9	75±25	54±8	148±45	130±25	222±39	187±19
Cisapride	0.1	5±3	4±2	7±3	6±3	27±7	16±7	58±10	49±9	78±14	76±9
(±)-Pindolol	4.0	7±3	13±3	15±5	15±5	53±18	34±7	100±20	63±8	130±10	78±18
8-OH-DPAT	0.2	9±2	0±0*	18±5	8±3*	26±9	27±7	70±21	58±10	114±21	78±14*
Lisuride	0.1	5±0	1±1	12±3	1±1*	24±4	0±0*	70±10	0±0*	122±26	0±0*
Methiothepin	0.03	9±1	0±0*	25±9	0±0*	54±8	1±1*	130±25	7±4*	187±19	20±9*

*[a], 1 ml/kg. [b], Ritanserin (0.1 mg kg-1) + Granisetron (0.3 mg kg-1). *, P<0.05 vs corresponding value before antagonists.*

It is therefore suggested that 5-HT increases the ECBF in vagosympathectomized dogs pretreated with GR127935 by activation of 5-HT receptors whose pharmacological profile might be similar to the 5-ht_7 subtype (Hoyer *et al.*, 1994).

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46P 5-HT_3 RECEPTORS MEDIATE AN EXCITATORY ACTION OF 5-HT ON DORSAL VAGAL PREGANGLIONIC NEURONES IN ANAESTHETIZED RATS: AN *IN VIVO* IONOPHORETIC STUDY

Yun Wang, *Andrew G. Ramage & David Jordan, Departments of Physiology and *Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF

5-hydroxytryptamine (5-HT) excites dorsal vagal preganglionic neurones (DVPNs) of rats when applied ionophoretically *in vivo* and this could be attenuated by 5-HT_{1A} receptor antagonists (Wang et al., 1995). However 5-HT_{1A} receptor agonists only excited a small population of the DVPNs tested (Wang et al., 1995). The present study investigated whether other 5-HT receptor subtypes are also involved in the excitatory action of 5-HT. Autoradiographic binding studies have demonstrated the presence of 5-HT_3 binding sites in dorsal vagal nuclei (Waeber et al., 1988), and previous studies of our group have shown that 5-HT_3 receptors are involved in vagal bradycardia induced by upper airway stimulation (Dando et al., 1995).

In rats anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) activity was recorded from antidromically identified preganglionic neurones in the dorsal vagal nuclei using 5- or 7-barrel microelectrodes (Wang et al., 1995). 54 of the 66 DVPNs tested were excited by ionophoretically applied phenylbiguanide (PBG, 10mM, pH10.6, 0-40nA). This excitatory action could be either abolished or attenuated by the 5-HT_3 receptor antagonists granisetron (10mM, pH4, 5-20nA) in 8 out of 11 neurones and tropisetron (ICS205-930, 10mM, pH4, 5-20nA) in 2 out of 3 neurones tested. In addition, in 5 out of 6 neurones excited by both PBG and DL-homocysteic acid (DLH, 100mM, pH8.5, 5-15nA), granisetron selectively antagonised the PBG but not the DLH excitations. The PBG excitations were unaffected by the 5-HT_2 receptor antagonist cinanserin (50mM, pH4, 5-20nA) in 2/2 neurones and the 5-HT_{1A} receptor antagonist WAY100802 (10mM, pH4, 5-20nA) (Ramage & Mirtsou-Fidani, 1995) in 6/6 DVPNs tested.

Similarly, the excitatory actions of 5-HT (maleate, 50mM, pH5, 0-10nA) was examined in 16 neurones. This was blocked or attenuated by granisetron (8 of 11) and by tropisetron (5 of 5), but the excitatory effect of DLH was unaffected by these 5-HT_3 antagonists in 5 out of 7 neurones tested.

These results indicate that 1) dorsal vagal preganglionic neurones in rat are excited by activation of 5-HT_3 receptors and 2) the excitation of DVPNs by ionophoretically applied 5-HT is in part mediated by 5-HT_3 receptors.

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47P THE ROLE OF 5-HT AND GABA IN THE REGULATION OF THE 10-Hz RHYTHM IN SYMPATHETIC NERVE DISCHARGE IN ANAESTHETIZED CATS

R.B. McCall & M.E. Clement, introduced by A.G. Ramage, Cardiovascular Pharmacology, The Upjohn Co., Kalamazoo, MI 49001, USA.

The discharge of sympathetic nerves with different cardiovascular targets contain a variable mixture of a 10-Hz rhythm and irregular 2- to 6-Hz oscillations in urethane anaesthetized or decerebrate, baroreceptor-denervated cats. Although both frequency components originate in the brain stem, data support the view that they are generated by different pools of neurons (Barman & Gebber, 1992, 1995). The central neurotransmitter mechanisms responsible for setting the balance between the 10-Hz and 2- to 6-Hz components in sympathetic nerve discharge (SND) and thus the degree of co-ordination of the discharges of nerves with different cardiovascular targets need to be identified. To date there have been no studies dealing with the question whether tonically active serotonergic medullary raphe neurons affect the 10-Hz rhythm in SND. In the present study, we investigated the role of 5-hydroxytryptamine (5-HT), GABA and glycine on the naturally occurring 10-Hz rhythm in SND of urethane (1.2 -1.8 mg kg^{-1}; i.v.) anaesthetized cats. SND was recorded from the inferior cardiac nerve and autospectral analysis was used to characterize the effects of selective receptor agonists and antagonists on the distribution of power in the 10-Hz band of SND and at frequencies ≤ 6Hz (see Orer et al., 1995). The 5-HT_{1A} receptor agonists 8-hydroxy-(di-n-propylamino) tetralin (8-OH DPAT; i.v. 1-30 μg kg^{-1}) and U-93385E (i.v.; 10-300 μg kg^{-1}; McCall et al., 1994) caused a dose dependent decrease in the power in the 10-Hz band of SND reaching a maximum, at the highest doses, of -77 ± 6% and -96 ± 4% (n = 6) respectively, without affecting the power at frequencies ≤ 6 Hz. This effect of these agonists is within the same dose range which will inhibit the firing of serotonergic medullary raphe neurons (McCall et al., 1994). The inhibitory effects of 8-OH DPAT and U-93385E on the power of the 10 Hz band were reversed by the 5-HT_{1A} receptor antagonists (1mg kg^{-1}; n =2) spiperone or WAY 100135 (Fletcher et al., 1993). Microinjection (10 nmol in 100 nl; n = 4) of 8-OH DPAT into medullary raphe nuclei also selectively eliminated the 10-Hz rhythm in SND. The 5-HT_2 receptor antagonist methysergide (i.v.;1 mg kg^{-1};n = 3) blocked the 10-Hz rhythm in SND, whereas the 5-HT_2 receptor agonist 1-(2, 5-dimethoxy-4-iodophenyl)-2-amino-propane (DOI; 0.3 -1 mg kg^{-1}; i.v.) increased peak frequency at the highest dose by 1.9 ± 3.3 Hz and power by 61 ± 9 % in the 10-Hz band of SND (n = 4).

Microinjection of N-methyl -D-aspartic acid (NMDA ; 20 nM in 50 nl; n = 5) into the medullary raphe also enhanced the 10-Hz rhythm in SND. The effects (i.v.) of cumulative doses of the GABA receptor antagonist picrotoxin and the glycine receptor antagonist strychnine were determined on the frequency components in sympathetic activity. Picrotoxin (0.03 - 0.3 mg kg^{-1}; n = 3) increased power at the highest dose by 125% in the 10-Hz component of sympathetic activity and produced a dramatic shift (by 2 to 3 Hz) in the rhythm to higher frequencies. Only small changes in the power (20%) were noted in the 2- to 6-Hz component. Strychnine (0.03 -0.3 mg kg^{-1}; n = 5) produced a small generalized increase in power in both frequency bands in sympathetic activity by 20%. These data support the view that the naturally occurring discharges of serotonergic medullary raphe neurons preferentially enhance the 10-Hz rhythm in SND. In addition, the data suggest that GABA may play an important role in the generation and maintenance of the 10-Hz rhythm in sympathetic activity while glycine is likely to inhibit activity at a site of convergence of the two rhythms in sympathetic activity.

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48P CENTRAL 5-HT_{1A} AND 5-HT_{1D} RECEPTORS HAVE OPPOSING ROLES IN THE REFLEX ACTIVATION OF CARDIAC VAGAL MOTONEURONES BY UPPER AIRWAY STIMULATION IN ANAESTHETIZED RABBITS

S.B.Dando, D. Jordan[1] & A.G.Ramage, Departments of Pharmacology &[1] Physiology, Royal Free Hospital School of Medicine, Rowland Hill Street, Hampstead, London, NW3 2PF

Central 5-HT pathways acting via 5-HT_{1A} receptors have been implicated in increasing the excitability of cardiac vagal motoneurones (CVMs) at rest and to reflex activation in anaesthetized rats (see Wang *et al.*, 1995). Surprisingly the archetypal 5-HT_{1A} agonist 8-OH-DPAT inhibits activation of CVMs by upper-airway stimulation with smoke in anaesthetized rabbits (Futuro-Neto *et al.*, 1993). The present experiments were carried out to investigate the mechanism by which 8-OH-DPAT causes inhibition of this reflex in anaesthetized rabbits using the 5-HT_{1A} receptor antagonist WAY-100635 (Forster *et al.*, 1995), the 5-HT_{1D} receptor antagonist GR127935 (Skingle *et al.*, 1993) and the 5-HT_{1D} receptor agonist sumatriptan.

Male rabbits (1.8-3.0 kg) were anaesthetized with urethane (1.5 g kg^{-1}) and pretreated with atenolol (1 mg kg^{-1}). Smoke was delivered to the upper airways and recordings were made of BP, ECG , renal (RNA) and phrenic nerve activity (PNA) as described by Dando *et al.*(1994). The challenge was repeated every 10 min until a stable bradycardia was observed. 5 min after the stable challenge the test solution, saline (n=5; 20 μl), distilled H_2O (n=5; 20 μl), WAY-100635 (100 μg kg^{-1}; n=5), (+)8-OH-DPAT (n=5; 25 μg kg^{-1} alone or in the presence of GR127935,100 μg kg^{-1}, given i.v. 20 min previously, n=4) or sumatriptan (n=5, 50 μg kg^{-1}, in the presence of WAY-100635, 100 μg kg^{-1}, given i.v. 20 min previously) was administered i.c. and the smoke challenge repeated 5 min later and then at 10 min intervals. Time matched comparisons of drug induced changes with those of vehicle control were made by ANOVA and the least significant difference test was used to compare the means.

WAY-100635 caused a significant ($P < 0.05$) inhibition of the reflex bradycardia as measured by changes in the R-R interval from 158±43 to 91±44 ms 5 min later. The reflex inhibition of PNA (apnoea duration) of 22±2 s and the increase (%) in RNA were unaffected, whereas the pressor response was reduced from 32±6 to 22±7 mmHg. (+)8-OH-DPAT and sumatriptan, the later after 15 min, also significantly inhibited the increase in R-R interval (by 98±23 ms and 59±14 ms, respectively) and the pressor response. Both drugs inhibited the duration of the apnoea after 5 and 25 min, respectively. Only (+)8-OH-DPAT inhibited the reflex increase in RNA. GR127935 potentiated the reflex increase in R-R by 31±19 ms after 5 min which became significant by 25 min (+47±38 ms). Pretreatment with GR127935 i.v. significantly attenuated the (+)8-OH-DPAT inhibition of the reflex increase in R-R interval, apnoea duration, the pressor response and the reflex increase in RNA. Neither WAY-100635 nor GR127935 when given i.v. affected the reflex responses to smoke.

These result show that 5-HT_{1A} receptors have an excitatory role, while 5-HT_{1D} receptors have an inhibitory role in the reflex stimulation of CVMs. The results also suggest that 5-HT_{1D} receptors play a role in the control of central respiratory and sympathetic drives. Further, in the reflex stimulation of CVMs the dominant action of (+)8-OH-DPAT is mediated by 5-HT_{1D} receptors not 5-HT_{1A} receptors. Whether this is due to the location of these receptors in this reflex pathway in relationship to 5-HT_{1A} receptors and/or that the potency of (+)8-OH-DPAT in this system is greater at 5-HT_{1D} than 5-HT_{1A} receptors remains to be determined.

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49P INFLUENCE OF DEXAMETHASONE ON THE REGIONAL HAEMODYNAMIC RESPONSES TO LIPOPOLYSACCHARIDE IN CONSCIOUS RATS: EFFECTS OF THE NON-SELECTIVE ENDOTHELIN ANTAGONIST, SB 209670

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

Recently, we observed that, in the presence of the non-selective endothelin (ET) antagonist, SB 209670, continuous infusion of lipopolysaccharide (LPS) in conscious rats caused progressive hypotension accompanied by peripheral vasodilatation (Gardiner *et al.*, 1995), consistent with ET acting to oppose the vasodilator mechanisms triggered by LPS. Since dexamethasone (DEX) can inhibit the activity of inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2), we tested the hypothesis that pretreatment with DEX and SB 209670 would prevent the haemodynamic effects of LPS.

All experiments were carried out on conscious, male, Long Evans rats (350-450 g), chronically instrumented with pulsed Doppler flow probes and intravascular catheters (all surgery under sodium methohexitone anaesthesia, 40-60 mg kg^{-1}, supplemented as required). Animals were given 23 h co-infusions of saline and LPS (150 $\mu g\ kg^{-1}\ h^{-1}$, Group a, n = 8) , or DEX (12.5 $\mu g\ kg^{-1}\ h^{-1}$) and LPS (Group b, n = 8), or DEX, SB 209670 (600 $\mu g\ kg^{-1}\ h^{-1}$) and LPS (Group c, n = 8).

Table 1 summarises the results. LPS alone caused a slight hypotension, and progressive renal and hindquarters vasodilatation. During co-infusion of DEX and LPS there was a marked, but transient, increase in MAP accompanied by mesenteric and hindquarters vasoconstriction. In the presence of DEX, SB 209670 and LPS, there was a marked hypotension, accompanied by mesenteric and hindquarters vasodilatation, as seen in the presence of SB 209670 and LPS (Gardiner *et al.*, 1995). Thus, the marked peripheral vasodilatation observed in the presence of SB 209670 and LPS involves DEX-sensitive (renal) and DEX-insensitive (mesenteric and hindquarters) mechanisms.

Table 1. Cardiovascular variables before and during infusion in Groups a, b and c. Values are mean ± s.e. mean; *P < 0.05 versus time 0 h (Friedman's test). HR = heart rate (beats min^{-1}); MAP = mean arterial pressure (mm Hg); RVC, MVC, HVC = renal, mesenteric and hindquarters vascular conductance, respectively ([kHz mm Hg^{-1}]10^3).

		0h	5h	8h	23h
HR	a	309 ± 5	349 ± 13*	384 ± 7*	401 ± 12*
	b	330 ± 12	324 ± 10	304 ± 10	324 ± 9
	c	327 ± 8	394 ± 7*	386 ± 9*	353 ± 8
MAP	a	100 ± 1	104 ± 2	98 ± 2	92 ± 3*
	b	105 ± 2	122 ±4*	138 ±3*	108 ± 2
	c	105 ± 2	80 ± 2*	93 ± 2*	96 ± 2*
RVC	a	69 ± 7	110 ± 17*	111 ± 16*	140 ± 17*
	b	59 ± 7	56 ± 5	49 ± 5	60 ± 9
	c	66 ± 8	86 ± 4*	61 ± 6	66 ± 12
MVC	a	75 ± 9	51 ± 6*	56 ± 6*	65 ± 5
	b	57 ± 4	43 ± 5*	32 ± 3*	36 ± 2*
	c	63 ± 6	119 ± 15*	74 ± 9*	69 ± 8
HVC	a	38 ± 4	31 ± 4	43 ± 5	67 ± 6*
	b	41 ± 5	28 ± 3*	22 ± 3*	37 ± 5
	c	37 ± 6	57 ± 5*	60 ± 8*	56 ± 7*

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50P THE ROLE OF ADENOSINE IN HEPATIC ARTERIAL VASODILATATION INDUCED BY PORTAL VENOUS INJECTIONS OF ATP IN THE ISOLATED DUAL-PERFUSED RABBIT LIVER

B Alexander, DJ Browse, RT Mathie, IS Benjamin.
Department of Surgery, The Rayne Institute, King's College School of Medicine & Dentistry, London SE5 9NU

It has previously been established that ATP may play a important role in the regulation of hepatic arterial blood flow (Ralevic *et al.*, 1991). Its precise role in the regulation of the buffer response, the hepatic arterial hyperaemia induced by reductions in portal venous blood flow, remains unclear (Mathie and Alexander, 1990). We have previously established that part of the role of portal venous-derived ATP in inducing hepatic arterial vasodilatation may be attributed to the release of nitric oxide in the hepatic artery following diffusion across the hepatic parenchyma, the so-called "transvascular effect" (Browse *et al.*, 1994). However, ATP may also elicit vasodilatation by activation of purinergic P_1-purinoceptors. This study was conducted to establish whether a proportion of the hepatic arterial vasodilator action of portal venous derived-ATP was due to catabolism to adenosine which could possibly occur during diffusion across the hepatic parenchyma from the portal vein (PV) to the hepatic artery (HA).

12 New Zealand White rabbits (2.2 - 3.1 kg) were anaesthetised with midazolam (12.5 mg kg^{-1} i.v.) and maintained upon a mixture of fentanyl/fluanisone (0.3 ml kg^{-1} i.m.). The HA, PV and bile duct were cannulated according to our previous protocols (Alexander *et al.*, 1992) and the HA and PV perfused at constant flow rates of 25 and 75 ml min^{-1} 100g $liver^{-1}$ respectively with Krebs'-Bülbring buffer. Methoxamine (5.27 ± 0.05 -log M) was added to the perfusate to raise the tone of the preparation to 146 ± 7.7 mm Hg and 3.3 ± 0.8 mm Hg (HA and PV respectively). Vasodilatation was measured as a transient decrease in perfusion pressure in the HA.

The P_1-purinoceptor (adenosine) antagonist 8-sulphonyl theophylline (8-SPT) (10μM), which was added to the shared HA and PV reservoir, significantly attenuated HA-vasodilator dose-related response curves to HA-injected ATP (-log M ED_{50} from 8.7 ± 0.22 to 7.63 ± 0.28, P<0.001*) and PV-injected ATP (-log M ED_{50} from 5.08 ± 0.15 to 4.97 ± 0.12, P<0.05*). PV responses to HA or PV injections of ATP were not significantly altered by 8-SPT . 8-SPT significantly attenuated both HA and PV responses to previously calculated (Ralevic *et al.*, 1991) midrange doses of 10^{-7}moles 100g $liver^{-1}$ HA- and 10^{-8} moles 100g $liver^{-1}$PV-injected adenosine from 50.8 ± 6.2 to 31.6 ± 8.1 mm Hg (P<0.05*) and from 33.3 ± 3.5 to 6.5 ± 3.8 mm Hg (P<0.001*), HA and PV respectively. We have therefore modified our previous conclusions which stated that the relaxant effects of ATP are solely mediated by P_{2y}-purinoceptor activation (Ralevic *et al.*, 1991) and suggest that PV-induced HA vasodilatation may, in fact, be partially mediated through P_1-purinoceptor activation.

*Student's paired t-test. All results quoted are mean ± se.

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51P EFFECTS OF BW A868C ON EXOGENOUS PGD_2-INDUCED NASAL CONGESTION IN ANAESTHETISED DOGS

Y. J. Liu, D. M. Jackson & A. Blackham, Department of Pharmacology, Astra Charnwood, Bakewell Road, Loughborough, Leicestershire, LE11 0RH

Release of PGD_2 in patients suffering from allergic rhinitis is markedly increased, over 2.5 to 11.2 fold higher than basal levels (Prat *et al.*, 1993; Ramis *et al.*, 1991). The present study was intended to investigate the *in vivo* pharmacology of exogenous PGD_2 induced nasal congestion using BW A868C a selective DP receptor antagonist.

Beagle dogs (10 - 16 kg) of either gender were anaesthetised with thiopentone (15 mg kg^{-1}) and chloralose (60 mg kg^{-1}) i.v. and intubated for artificial respiration at 13 ml kg^{-1} and 20 breath min^{-1} with humidified air. A femoral artery and a saphenous vein were cannulated for continuous blood pressure and heart rate recording, blood gas sampling and drug administration. In some experiments, a carotid artery was also cannulated for local arterial infusion of PGD_2. The dog was then placed on its lateral side. The upper nare was carefully intubated and sealed for the recording of nasal resistance through continuous measurement of nasal pressure. The lower nare was used for the collection of nasal fluid by inserting and replacing a pre-weighed absorbent plug at 15 min intervals. Results are expressed as mean ± s.e.m. with n refers to the number of dogs.

Given either topically in the form of aerosol or by local arterial infusion PGD_2 produced dose-dependent nasal congestion as reflected by increases in both nasal resistance and nasal fluid production. With topical administration of PGD_2 the maximal increases were 2.2 ± 0.1 and 19.3 ± 4.1 folds of the basals for nasal resistance and nasal fluid production, respectively (n = 4). Large variations in PGD_2 sensitivity were observed with doses producing maximal responses ranging from 0.1 µmol kg^{-1} to 1.0 mmol kg^{-1} (n = 8). I.v. administration of BW A868C, after recovery from a prior PGD_2 control, was adopted in preference to topical. Similar effects were observed when BW A868C (0.3 µmol kg^{-1}, the highest available) was given before (30 min) and after PGD_2 (a sub-maximal dose). In only 6 out of 14 dogs significant inhibition (over 30% of control) of PGD_2 induced nasal resistance was observed. However, no inhibition of PGD_2 induced nasal fluid production was noted. Oximetazoline an α-adrenoceptor agonist (1.0 nmol kg^{-1}, i.v.) significantly reversed (over 30% of maximum) both PGD_2 induced nasal resistance (n = 11) and nasal fluid production (n = 10).

The present results are not surprising in the light of recent findings that a lower affinity (pK_B=7.3) than that (9.3) reported by Giles et al., (1989) was obtained for BW A868C at DP receptors mediating smooth muscle relaxation in dog nasal vasculature (Liu & Jackson, 1995) and that partial agonist activity was demonstrated with BW A868C at DP receptors mediating Cl- secretion in dog tracheal epithelium (Liu *et al.*, 1995). Possible involvement of BW A868C-insensitive prostanoid receptors in PGD_2 induced nasal congestion could also be a factor. Given the known species variations in DP receptor pharmacology, further *in vitro* investigation using nasal biopsies from patients would be necessary to advance our understanding in this area.

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52P DUAL ACTION OF NITRIC OXIDE ON AIRWAY INFLAMMATION: DIFFERENTIAL EFFECTS AT DIFFERENT AIRWAY LEVELS

M. Bernareggi, *J. A. Mitchell, P. J. Barnes and M. G. Belvisi, Department of Thoracic Medicine and *Department of Applied Pharmacology, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY

Septic shock is usually caused by infection with Gram-negative bacteria which release endotoxin lipopolysaccarides (LPS) into the blood stream and results in hypotension characterised by microvascular leakage and multiple organ failure. Microvascular leakage in various vascular beds including the lung has previously been demonstrated, in a LPS-treated rat model, to be temporally associated with an enhanced formation of nitric oxide (NO) resulting from the induction of nitric oxide synthase (iNOS) (László *et al.*, 1995). However, there is limited information concerning the relationship between NO and microvascular leakage in the airways. Therefore, we have examined the time-dependent plasma exudation in rat airway tissue using a well characterised model of iNOS expression in vivo (Thiemermann, 1994).

Male Wistar rats (200-225g) were anaesthetised with sodium pentobarbitone (60mg/kg, i.p.). The right carotid artery was cannulated for the measurement of mean arterial blood pressure (MAP). Plasma leakage of Evans Blue dye (20mg/kg i.v.), a marker of microvascular permeability, was injected via a jugular vein 5 min prior to endotoxin (E. Coli lipopolysaccarides 0111: B4) (5,10 mg/kg i.v.) or isotonic sterile saline. At 240 min the trachea and bronchi/intrapulmonary airways were removed, blotted dry and weighed. In order to investigate the role of NO, L-NAME (10mg/kg i.v.) was administered 10 min before endotoxin (10 mg/kg i.v.) or vehicle. Evans Blue dye was extracted from tissues according to Evans *et al.*, (1990) and expressed as ng Evans Blue dye/mg of wet weight tissue. Parametric and non parametric tests for significance were performed when appropriate; p values <0.05 were considered significant.

In control animals there was a significant decrease in the MAP over the 240 min period (from 154±5.9 to 130±10.6, n=4). In L-NAME-treated animals there was a significant decrease in MAP (from 139±9.3 to 122±5.6, n=4) but this was not significantly different from that in vehicle-treated animals. Endotoxin (10mg/kg i.v.) resulted in a significant fall in MAP at 240 min (from 132±8.7 to 113±4, n=4), however this was not significantly different from the decrease in MAP in vehicle-treated animals over the same time course. In contrast when endotoxin was co-injected with L-NAME no significant change was seen in MAP at 240min (143±13.8 to 106±4.6, n=4). Endotoxin caused a significant increase in plasma leakage at 240 min in the trachea (control, 13.9±3.5 ng/mg; 5 mg/kg endotoxin, 21.6± 6.6 ng/mg; 10 mg/kg endotoxin, 44.7±5.5 ng/mg; n=4) but not in the bronchi/intrapulmonary airways (control, 54.2±6.1 ng/mg; 5 mg/kg endotoxin, 72.1±4.7 ng/mg; 10 mg/kg endotoxin, 61.3±5.3 ng/mg; n=4). In the trachea of control animals, L-NAME, at 240 min, significantly increased plasma leakage (34.9±4.4 ng/mg; n=4). In contrast, L-NAME significantly inhibited the endotoxin (10 mg/kg)-induced plasma leakage in the trachea (20.6±3.9; n=4). These results suggest that in the trachea but not in the bronchi/intrapulmonary airways, endogenous NO suppresses the normal process of plasma leakage. However, when iNOS is expressed the increased production of NO enhances plasma leakage. The results obtained here in the trachea are consistent with previous observations in the whole lung (László *et al.*, 1995). In contrast to the findings in the trachea, there does not appear to be any influence of NO on plasma leakage in the bronchi/intrapulmonary airways. These findings may have implications for human asthma where NOS is induced (Belvisi *et al.*, 1995) and patients exhale increased amounts of NO (Kharitanov *et al.*, 1994).

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53P EFFECT OF AMINOGUANIDINE ON THE CIRCULATORY FAILURE AND ORGAN INJURY ELICITED BY STAPHYLOCOCCAL LIPOTEICHOIC ACID AND PEPTIDOGLYCAN IN THE ANAESTHETISED RAT

M. Kengatharan, S.J. De Kimpe, C. Thiemermann and J.R. Vane. The William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.

Lipoteichoic acid (LTA) and peptidoglycan (PepG) from the cell wall of *Staphylococcus aureus* (a Gram-positive organism without endotoxin) synergise in inducing nitric oxide synthase (iNOS) *in vivo* and this is associated with multiple organ injury and circulatory failure (De Kimpe *et al*, 1995). Here, we investigated whether inhibition of iNOS activity leads to protection against multiple organ injury and circulatory failure elicited by LTA given together with PepG (LTA+PepG).

Male Wistar rats (250-350g) were anaesthetised with thiopentobarbitone sodium (120mg/kg, i.p.). The trachea was cannulated to facilitate respiration, the carotid artery for measuring mean arterial blood pressure (MAP), heart rate and arterial oxygen pressure (P_aO_2), and the jugular vein for administration of compounds. The iNOS selective inhibitor aminoguanidine (5mg/kg bolus plus 10mg/kg/h infusion) was given 30 min prior to injection of LTA (3mg/kg, bolus)+PepG (10mg/kg, injected over 30-45 min). The pressor response to noradrenaline (NA, 1μg/kg) was assessed prior to and every 60 min after the injection of LTA+PepG. At 360 min, plasma samples were taken for the determination of biochemical markers of organ injury and nitrite+nitrate (total nitrite). The rats were killed and lungs, liver and aorta removed to determine iNOS activity in homogenates via the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline.

Injection of LTA+PepG, resulted in a time-dependent (i) circulatory failure (hypotension and vascular hyporeactivity to NA), (ii) respiratory failure (fall in P_aO_2), (iii) liver injury (increase in plasma levels of alanine aminotransferase, ALT), and (iv) renal failure (increase in plasma urea and creatinine). Additionally, there was a time-dependant increase in the activity of iNOS in lungs, liver and aorta and a rise in total plasma nitrite levels (Table 1). Treatment with aminoguanidine attenuated (i) the hypotension and the vascular hyporeactivity to NA, (ii) the fall in P_aO_2, (iii) the increase in plasma levels of ALT, (iv) the enhanced iNOS activity, and (v) the rise in total plasma nitrite. In contrast, the increase in urea or creatinine was not affected by aminoguanidine.

Thus, enhanced formation of NO due to induction of iNOS caused by LTA+PepG contributes importantly to circulatory failure, respiratory failure and liver injury but not renal failure in anaesthetised rats.

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Table 1. Effect of aminoguanidine on the circulatory failure, iNOS induction and increase in total plasma nitrite elicited by LTA+PepG.

Experimental group	MAP (mmHg)	NA response (% control)	iNOS activity (pmol L-citrulline/30min/mg protein) lung	liver	aorta	Total plasma nitrite (μM)
sham control	113 ± 4	114 ± 10	9 ± 3	5 ± 3	5 ± 2	44 ± 3
LTA+PepG	77 ± 5#	16 ± 2#	660 ± 120#	94 ± 20#	38 ± 2#	365 ± 33#
LTA+PepG+aminoguanidine	106 ± 6*	77 ± 19*	200 ± 40*	22 ± 4*	26 ± 1*	126 ± 17*

Values for 360 min after injection of bacterial components are given as mean ± s.e.mean (n=6-8); # $P<0.05$ compared to sham control and * $P<0.05$ compared to LTA+PepG by ANOVA (Bonferroni's test).

54P EFFECTS OF SIN-1 AND ILOPROST, ALONE AND IN COMBINATION, ON MYOCARDIAL INFARCT SIZE IN ANAESTHETIZED RABBITS

Keri A. Aitchison & Susan J. Coker, Department of Pharmacology & Therapeutics, University of Liverpool, Liverpool L69 3BX.

Nitric oxide (NO) and prostacyclin (PGI_2) have synergistic antiplatelet and antineutrophil activity (Gryglewski *et al.*, 1989) and both of these cell types may have detrimental effects in myocardial infarction. Thus, we investigated whether combined administration of an NO donor (SIN-1) and a PGI_2 analogue (iloprost; Ilo) resulted in a synergistic reduction in infarct size in an anaesthetized rabbit model of myocardial infarction.

Anaesthesia was induced with Hypnorm (0.5ml kg^{-1} i.m.) and maintained with Na pentobarbitone i.v. in male NZW rabbits (2.2-3.2kg) which were then prepared for coronary artery occlusion (CAO) as described previously (Coker, 1989). Rabbits were assigned randomly to one of four groups: control (Ilo vehicle [alkaline saline] + SIN-1 vehicle [saline]; n=10), SIN-1 3μg kg^{-1} + Ilo vehicle (n=10), Ilo 0.03μg kg^{-1} +SIN-1 vehicle (n=9) or both drugs (n=9). All drugs or vehicles (0.05ml min^{-1}) were infused via a dual lumen catheter into the left jugular vein for 10 min prior to CAO and for the duration of the experiment. After 40 min of CAO, the ligature around the coronary artery was released, and the myocardium reperfused for 3h. The heart was then removed and perfused via the aorta with phosphate buffered saline (PBS). The ligature was retied and 5ml of MTT (Thiazolyl blue, 5% w/v) injected into the aorta. Hearts were incubated in PBS (37°C) for 5 min to delineate the area at risk (AAR) then sliced and incubated in triphenyl tetrazolium chloride (1% w/v; 37°C; 15min) to stain viable tissue. Slices were then fixed, photographed and AAR and infarct size (IS) measured by image analysis. Blood was removed from the femoral artery prior to drug infusion (5ml) and after 3h reperfusion (20ml) to study ADP- and collagen-induced platelet responsiveness in whole blood, using electronic impedence aggregometry.

AAR was similar in all groups, however, IS was reduced by Ilo alone and to a greater extent by both drugs. There were no significant differences in heart rate or blood pressure among groups, prior to ischaemia or reperfusion. Myocardial ischaemia/reperfusion reduced platelet responses to collagen in all groups, and to ADP in all groups except SIN-1 (Table 1).

Table 1. Values (mean ± s.e. mean) for AAR and IS (%), for heart rate (HR, beats min^{-1}) and diastolic blood pressure (DBP, mmHg) after 40 min of ischaemia, and platelet aggregation (PA Ω) induced by ADP (3μM) before drug administration (pre) and after reperfusion (post).

	Control	*SIN-1*	*Ilo*	*Both*
AAR	47.6±2.2	49.4±1.9	48.8±1.3	49.2±2.2
IS	33.6±4.7	28.1±1.6	24.6±1.6*	18.8±1.7**#
HR	239±8	252±15	230±6	262±17
DBP	43±2	43±3	46±4	40±3
PA-pre	14.3±0.9	12.9±1.0	11.8±1.1	13.5±1.5
PA-post	7.6±1.5†	11.8±3.4	5.3±0.9†	7.8±1.1†

* $P<0.05$, ** $P<0.001$ compared with Control; # $P<0.05$ compared with SIN-1 and Ilo, Kruskal-Wallis test. † $P<0.05$ compared with pre drug within group, Wilcoxon test.

Although the reduction in IS was greater with both drugs, this does not appear to be a synergistic interaction or to be dependent on effects of the drugs on haemodynamics or platelet aggregation. Further studies are necessary to investigate other possible mechanisms of action.

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55P ENDONEURIAL NITRIC OXIDE DEFICIENCY IN DIABETIC RATS: EFFECTS OF AMINOGUANIDINE TREATMENT

M. Dewhurst, N. Omawari & D.R. Tomlinson, Department of Pharmacology, Queen Mary & Westfield College, Mile End, London

Aminoguanidine treatment (AG) can prevent early conduction defects in experimental diabetic neuropathy, possibly by the prevention of a deficit in nerve blood flow (Kihara *et al.*, 1991). As experimental diabetes may cause endoneurial nitric oxide to be decreased (Omawari *et al.*, 1995), we examined the effects of microinjecting L-NAME and L-arginine into the sciatic nerves of age-matched control and streptozotocin diabetic (55 mg/kg i.p) rats, some of which were treated with AG (50 mg/kg/day i.p. duration 9 weeks). Rats were anaesthetised (halothane 2-3% in O_2) prior to maintenance on Na pentobarbitone (12 mg/kg, i.v.) and diazepam (2 mg/kg, i.v.), administered as required. Sciatic nerve laser Doppler flux (LDF), motor nerve conduction velocity (MNCV) and MAP were monitored as described previously (Karasu *et al.*, 1995). A glass micropipette (10μm tip), positioned a few mm proximal to the LDF probe was used to deliver drugs into the endoneurium. Significance levels were derived either by paired t tests or, for between group comparisons, 2-way ANOVA with Duncans multiple range tests.

MAP, LDF, MNCV and conductance (calculated as LDF/MAP) were significantly decreased in diabetic animals compared to controls ($P<0.01$, except MAP $P<0.05$). AG increased MNCV ($P<0.05$) but not LDF, conductance or MAP. L-NAME (1nM, 1μl), reduced LDF to a greater extent in control than diabetic rats (untreated). The subsequent administration of L-arginine (100nM, 1μl) returned LDF to basal levels for controls, whereas for diabetic rats L-arginine increased LDF to supra-basal levels. The effects of L-NAME and L-arginine were less significant in rats receiving AG, compared to those observed in their untreated equivalents (Table 1). D-NAME failed to affect LDF (n=2-3 all groups) and drug-induced changes were not accompanied by changes in MAP.

These data support the hypothesis that nitric oxide production is reduced in the sciatic nerves of experimentally diabetic animals, possibly due to a deficit in endogenous L-arginine. Although AG increased MNCV, AG had little effect upon LDF, prior to or after L-NAME or L-arginine.

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Table 1. Effect of AG treatment and experimental diabetes on MNCV, MAP and LDF with microinjection of L-NAME followed by L-arginine. All data mean±1S.D, NS=not significant.

			Sciatic nerve laser Doppler flux (Arbitrary units)						
Group	MNCV (m/sec)	MAP (mmHg)	Basal	Post L-NAME	*P*-value	(n)	Post L-arginine	*P*-value	(n)
Control	47.2±4.2	102±3	209±32	120±44	$P<0.005$	6	206±54	$P<0.03$	4
Diabetic	38.2±1.6	95±3	111±29	84±16	$P<0.03$	7	145±32	$P<0.001$	7
Control (AG-treated)	48.8±3.8	102±5	265±91	171±48	$P<0.05$	7	196±107	NS	7
Diabetic (AG-treated)	43.4±5.9	97±6	133±39	106±42	NS	8	127±63	$P<0.02$	8

56P CLENBUTEROL STIMULATES NERVE GROWTH FACTOR EXPRESSION IN CONTROL AND DIABETIC RATS: EFFECTS ON NEUROPEPTIDES

S.S. Riaz and D.R. Tomlinson, Department of Pharmacology, Queen Mary and Westfield College, London, U.K.

Reduced nerve growth factor (NGF) content in peripheral tissues is implicated in the pathogenesis of diabetic neuropathy (Fernyhough *et al.*, 1995). In clinical and experimental diabetes deficits in calcitonin gene related peptide (CGRP) and substance P may be secondary to impaired expression of NGF (Tsigos *et al.*, 1993, Diemel *et al.*,1994). Clenbuterol is reported to induce NGF synthesis *in vitro*, therefore this study investigated the effect of clenbuterol on NGF and neuropeptide levels in control and diabetic rats. Animals were made diabetic with a single injection of streptozotocin (55mg/kg, i.p.). Control and diabetic rats were injected daily with clenbuterol (250μg/kg, s.c.) for the duration of the 8 week study. Untreated control and diabetic animals were maintained for comparison. At the end of the study the sciatic nerve levels of NGF-like immunoreactivity (NGF-LI) (measured by ELISA) were compared to those of substance P-like immunoreactivity (SP-LI) and CGRP-like immunoreactivity (CGRP-LI as measured by RIA; see Fernyhough *et al.*, 1995 for methods). Data (mean ±1SD) are given in the table below.

Reductions in sciatic nerve NGF-LI and SP-LI were concomitant with a 46% decrease in mRNA for the high affinity NGF receptor trkA and a 46% decrease in γ-PPT mRNA (substance P precursor) in the dorsal root ganglia of diabetic animals. In support of the involvement of NGF in the regulation of SP and CGRP gene expression, regression analysis showed a correlation between NGF and substance P, NGF and CGRP (for both $r^2=0.35$, significant at $p<0.05$) and a correlation between substance P and CGRP ($r^2=0.48$ significant at $p<0.01$). The changes demonstrated are consistent with induction of NGF expression in target tissues by clenbuterol.

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Group (n)	NGF-LI (pg/cm nerve)	SP-LI (pg/cm nerve)	CGRP-LI (ng/cm nerve)
Control (10)	$31 \pm 7^{a,}$	$218 \pm 65^{a,}$	$2.3 \pm 0.5^{a,}$
Control treated (10)	46 ± 17^{c}	289 ± 91^{c}	3.3 ± 1.4^{c}
Diabetic (10)	$14 \pm 4^{b,}$	$121 \pm 40^{b,}$	$1.3 \pm 0.3^{b,}$
Diabetic treated (12)	22 ± 6^{d}	176 ± 63^{d}	1.9 ± 0.5^{d}

Level of significance as tested by Two way ANOVA: For a *versus* b, a *versus* c and b *versus* d significant at $p< 0.05$

57P POSITIVE CHRONOTROPIC EFFECT OF BRADYKININ IN PITHED RATS AND IN ISOLATED RAT ATRIA

J. Zhang, J.F. Loro, Q. Li, M. Pfaffendorf & P.A. van Zwieten, Department of Pharmacotherapy, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

Bradykinin (BK) is known to be a potent vasodilator (Sung *et al.*, 1988). Little consideration has been paid so far to its cardiac effects, in particular with respect to heart rate. Recently, BK was found to facilitate the electrically-evoked release of sympathetic catecholamines, thus causing a positive inotropic effect in rat atria (Minshall *et al.*, 1994). A direct positive chronotropic effect mediated by activation of bradykinin B_2 receptors was found to occur in guinea pig atria (Tesfamariam *et al.*, 1995). In the present study we investigated the positive chronotropic effects of BK in pithed rats and in isolated spontaneously beating rat atria.

Pithed rat preparations were set up as described previously (Zhang *et al.*, 1993). The left jugular vein and common carotid artery were cannulated for the administration of drugs and for monitoring the blood pressure, respectively. Heart rate was derived from the blood pressure signal. Bolus injections of BK were applied 20 min after the following treatments (i.v.): saline, propranolol (1 mg kg^{-1}), propranolol (1 mg kg^{-1}) plus prazosin (0.1 mg kg^{-1}), Hoe 140 (0.3 and 1 μmol kg^{-1}) and Lys-[Leu^8]Des-Arg^9-BK (0.1 μmol kg^{-1}), respectively. Isolated rat right atria were mounted in a gassed Tyrode solution at 37°C, at a tension of 0.5 g, and the spontaneous contractile frequency (beats min^{-1}) was recorded. Cumulative concentration-response curves for BK were constructed in the absence and presence (30 min) of the following compounds: propranolol (1 μM), propranolol (1 μM) plus prazosin (1 μM), indomethacin (3 μM), Hoe 140 (1 and 10 nM) and Lys-[Leu^8]Des-Arg^9-BK (1μM), respectively.

In pithed rats BK (3 μg kg^{-1} - 1 mg kg^{-1}) caused a dose-dependent increase in heart rate by maximally 90 ± 8 beats min^{-1}. Propranolol reduced the maximal response to BK in pithed rats by maximally 71.4 ± 4.2% (n = 8; $p < 0.01$). BK-induced tachycardia could not been further depressed by the combination of propranolol and prazosin. In isolated rat right atria preparations, cumulative additions of BK (0.1 nM - 0.1 μM) caused a concentration-dependent increase in frequency by maximally 32 ± 4 beats min^{-1}. Propranolol, the combination of propranolol and prazosin did not influence the concentration-response curves of BK whereas indomethacin virtually abolished the effect of BK (n=8; p< 0.01). Both in pithed rats and in isolated rat right atria the effects of BK were antagonized dose-dependently by Hoe 140, a B_2-receptor antagonist (n=8; p < 0.01), whereas Lys-[Leu^8]Des-Arg^9-BK (B_1-receptor antagonist) did not have any significant effect.

In conclusion, exogenous BK induces a dose-dependent chronotropic effect which is primarily mediated by β_1-receptors and probably in part by BK receptors in pithed rats. BK induces a positive chronotropic effect in isolated rat atria, which is independent of β_1-adrenoceptors. Prostaglandins are involved in BK-induced effect. Both in vivo and in vitro, BK-induced positive chronotropic effects are mediated by B_2-receptors.

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58P ENDOTHELIAL DYSFUNCTION FOLLOWING A BRIEF EXPOSURE TO ENDOTOXIN

K. Bhagat, J. Collier, P. Vallance, Clinical Pharmacology Unit, St. George's Hospital Medical School, London SW17 0RE.

Injection of endotoxin systemically causes characteristic changes within the cardiovascular, haemodynamic and immune systems. In the current study we have developed a model to explore the effects on endothelial function of a brief exposure to endotoxin.

Methods: Subjects lay in a temperature controlled laboratory (28-30°C) with one hand placed on an angled support above the level of the heart. The diameter of a single vein on the hand was recorded by measuring the linear displacement of a light weight probe placed on the skin overlying the summit of the vein when the pressure in a congesting cuff was lowered from 40-0mmHg. For studies of reactivity, drugs or physiological saline were infused continuously (0.25ml/min) through a 23SWG needle placed 5-10mm upstream from the probe.

Study 1: In veins preconstricted with NA to approximately 50% of their resting diameter, dose response curves to bradykinin (BK; 2, 4, 8 pmol/min), arachidonic acid (AA; 200pmol, 2nmol, and 20 nmol/min; each dose for 5 min) and GTN (1, 2, 4 pmol/min; each dose for 5 min) were constructed. The vein was then isolated by means of two wedges and endotoxin (E Coli_Lot EC-5;100 ETX Units) or saline was instilled into the isolated segment. After 1h the contents of the vein were aspirated, the wedges removed. 1h later the vein was constricted with noradrenaline and dose response curves to BK, GTN and AA repeated. BK and AA were assessed in one study (n=5) and in another GTN and BK (n=5).

Study 2: In a separate series of studies, dose response curves to BK, and AA were constructed before and 1h, 24h, 48h and 7 days after endotoxin (n=5).

Study 3: In 5 subjects the dose response curves to BK and AA were constructed and oral hydrocortisone (100mg) was given. 2h later endotoxin was instilled. Dose response curves to BK, and AA were repeated 1h after instillation of endotoxin.

Study 4: Specimens of saphenous vein from patients undergoing by-pass surgery were collected, cut into 3-5mm rings and suspended in an organ bath in Krebs' solution. After preconstriction with phenylephrine 0.9-1μM) endothelial integrity was determined (>40% relaxation to 1μM-BK).In endothelium-intact vesels concentration-response curves to BK (1nM-1μM) and GTN (1nM- 1μM) were contsructed before and 1h after endtotoxin (100EU added to the organ bath).

Study 5: Saphenous vein rings were incubated for 1h in either 10ml of Krebs' solution, or 10ml of Krebs' with endotoxin (100EU); alternatively the rings were added directly to a gluteraldehyde fixative immediately after harvesting. The specimens were coded and then examined by electron microscopy. The microscopist was blinded to the code.

Results were compared using the Students' t Test for paired data or ANOVA as appropriate (*p< 0.05 was considered significant).

Results: Local exposure to endotoxin produced an attenuation in the responses to BK and AA that was still significant 48h later. In contrast, no change in the response to GTN was seen. In control subjects receiving saline instead of endotoxin, there was no change in the dose response curves to BK, GTN or AA.

Study 1: Maximum % Dilatation to BK	Pre ETX	81±6
	1h post ETX	38±5*
Maximum % dilatation to AA	Pre ETX	90±6
	1h post ETX	42±9*
Maximum % dilatation to GTN	Pre ETX	99±1
	1h Post ETX	94±4
Study 2: Maximum % dilatation to BK	Pre ETX	87±6
	1h post ETX	22±15*
	24h post ETX	29±5*
	48h post ETX	34±11*
	7dys post ETX	79±10
Maximum % dilatation to AA	Pre ETX	92±5
	1h post ETX	33±11*
	24h post ETX	41±7*
	48h post ETX	44±7*
	7dys post ETX	90±6
Study 3: Maximum % dilatation to BK	Pre ETX	85±4%
	1h post ETX	70±16%
Maximum % dilatation to AA	Pre ETX	84±10%
	1h post-ETX	91±5%
Study 4: Maximum % dilatation to BK	Pre ETX	70±5%
	1h post-ETX	31±4%*
Maximum dilatation to GTN	Pre ETX	87±4%
	1h post ETX	79±8%

Study 5: An experienced microscopist was unable to distiguish endotoxin-treated vein from vein that was allowed to remain in Krebs' for 1h before adding to fixative or vein that had been added immediately to fixative.

The results demonstrate that a brief local exposure to endotoxin in concentrations similar to those that occur in severe sepsis causes profound endothelial dysfunction that persists for 48h and takes 7 days to recover. The studies with saphenous vein, in vitro, suggest that endothelial denudation is unlikely to account for the changes seen. These findings have implications for understanding the vascular pathophysiology and pharmacology of conditions associated with infection and inflammation. Futhermore, our results suggest that prolonged endothelial dysfunction may occur in patients briefly exposed to endotoxin and this might contribute to the higher cardiovascular mortality and morbidity associated with abdominal surgery

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59P EFFECT OF NITRENDIPINE ON PRESSURE-NATRIURESIS CURVES IN ANAESTHETISED 2K1C GOLDBLATT AND DOCA-SALT HYPERTENSIVE RATS

C. Huang & E.J. Johns, Dept of Physiology, The Medical School, Birmingham. B15 2TT

There is evidence that within the kidney calcium channel blockers cause a greater dilatation at afferent than efferent arterioles (Carmines & Navar, 1989) and blunts renal blood flow autoregulation (Huang & Johns, 1995). We have shown previously that the 2K1C Goldblatt hypertensive rat was relatively insensitive to the haemodynamic actions of the calcium channel blocker. The aim of this study was to investigate the effect of the calcium channel blocker, nitrendipine, on the pressure-natriuresis curves of two models of hypertension, the 2K1C Goldblatt and DOCA-salt hypertensive rats having high and low renin levels.

Four to five weeks prior to use, male Wistar rats (160-190g) were either unilaterally nephrectomised and one week later given saline to drink and twice weekly injections of DOCA (15 $mgKg^{-1}$), or had a silver clip, 0.25 mm diameter, placed on the right renal artery. On the experimental day, anaesthesia was induced (pentobarbitone, 60 $mgkg^{-1}ip$), the kidney exposed via an abdominal incision, its ureter cannulated and a flow proble placed on its renal artery. Renal perfusion pressure (RPP) was adjusted by means of snares around the aorta. Saline was infused at 6 mlh^{-1} and at the end of surgery changed to one containing inulin (1%) and after 1 h adrenal hormones and vasopressin were added (Huang & Johns, 1995). Thirty minutes later, RPP was increased to its highest level and reduced in 20 mmHg steps with two 10 min clearance periods taken at each pressure. Six groups of rats were studied; 2K1C rats given either saline (n=6), or nitrendipine at 0.125 (n=7) or 0.25 $\mu gkg^{-1}min^{-1}$ (n=5); DOCA-salt rats given either saline (n=6) or the low (n=5) or high (n=6) dose of nitrendipine. Data, means ± s.e.mean, were compared between groups using ANOVA with significance taken when $P<0.05$.

Basal blood pressure in the 2K1C rats was 133±12 mmHg. Renal blood flow (RBF), at 3.6±0.4 $mlmin^{-1}gkwt^{-1}$, and glomerular filtration rate (GFR), at 0.64±0.12 $mlmin^{-1}gkwt^{-1}$, were autoregulated effectively over the pressure range 160 to 100 mmHg in the saline group and whereas neither dose of nitrendipine affected autoregulation of RBF, both doses blunted that of GFR (both $P<0.05$). The gradient of the sodium excretion against RPP was 0.065±0.010 $\mu molmin^{-1}gkwt^{-1}mmHg^{-1}$ in the saline group and was increased by the low and high doses of nitrendipine to 0.140±0.012 and 0.118±0.007 $\mu molmin^{-1}gkwt^{-1}mmHg^{-1}$ (both $P<0.05$), respectively. In the DOCA-salt rats, basal blood pressure was 133±7 mmHg, and while RBF, at 1.53±0.13 $mlmin^{-1}gkwt^{-1}$ and GFR, at 0.36±0.03 $mlmin^{-1}gkwt^{-1}$, exhibited low autoregulatory ability over the pressure range 140 to 80 mmHg in the saline group, nitrendipine tended to increase both variables which was only significant in GFR at the high dose ($P<0.05$). The relationship between sodium excretion and RPP in the saline, low and high nitrendipine groups was 0.049±0.09, 0.069±0.15 and 0.108±0.008 $\mu molmin^{-1}gkwt^{-1}$, respectively, with only the latter being significantly ($P<0.05$) higher.

These data show that in the 2K1C rats whereas GFR was relatively responsive to calcium channel blockade, RBF was not; furthermore, the pressure-natriuresis curves were enhanced by the drug. By contrast, the haemodynamic and excretory relationships in the DOCA-salt rats were relatively less affected by the nitrendipine. This may reflect the differing contributions of the renin-angiotensin system to the hypertension in these two different models.

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60P FUNCTIONAL AND MOLECULAR EVIDENCE FOR β_3-ADRENOCEPTORS IN HUMAN AND RAT GASTROINTESTINAL TISSUES

R.J. Summers, S.J. Roberts, M. Papaioannou & B.A. Evans, Department of Pharmacology, University of Melbourne, Victoria 3052, Australia.

In rat ileum and colon there is evidence from functional (Arch & Kaumann, 1993) and binding studies (Roberts *et al*, 1994) for the presence of β_3-adrenoceptors (AR). In human colonic circular smooth muscle spontaneous activity is inhibited by (-)-isoprenaline (ISO), an effect blocked by (-) propranolol (PROP) with a pA_2 value of 7.7 (McLaughlin *et al*, 1988) compared with pA_2 values of 8.6-9.1 at β_1- or β_2-ARs. However this preparation did not relax to the β_3-AR agonist BRL 37344 and responses to (-)-ISO were blocked by the selective β_1-AR antagonist betaxolol (McLaughlin *et al*, 1991). Receptor binding studies in human colon identified low affinity sites for (-) [^{125}I] cyanopindolol (CYP) with the pharmacological characteristics of β_3-AR (Roberts *et al*, 1994).

Relaxation responses were examined in rat ileum, human colon longitudinal muscle or taenia coli pre-contracted with carbachol (0.3-3μM) in modified Kreb's solution (37°C, pH 7.4). Neuronal and extraneuronal uptake of catecholamines and α-ARs were blocked. Rat ileum relaxed to (-)-ISO (pEC_{50} 6.79±0.02; n=4) and biphasically to BRL 37344 (pEC_{50} (1) 7.31±0.04; pEC_{50} (2) 4.39±0.09, n=4-6). Concentration response curves to (-)-ISO were shifted to the right by (-) PROP (pK_B 6.69±0.07; n=4) and (±) CYP (pA_2 7.98±0.07; n=6) but not by CGP 20712A (300nM) or ICI 118551 (300nM). The high but not the low affinity phase of the response to BRL 37344 was antagonised by (-) PROP (pK_B 6.51). In human longitudinal smooth muscle, (-)-ISO relaxation (pEC_{50} 7.27±0.05; n=8) was antagonised by (-) PROP (pK_B 8.46±0.11; n=8) and CGP 20712A (pK_B 9.76±0.21; n=8) but not by ICI 118551. In taenia coli (-)-ISO relaxation (pEC_{50} 6.64±0.02; n=5) was antagonised by (-) PROP, CGP 20712A and ICI 118551 with low affinity (pK_B 7.94±0.25; 7.70±0.08; 7.54±0.48 respectively, n=3-4). The results confirm studies indicating β_3-ARs mediating relaxation in rat ileum and possibly human taenia coli and show that relaxation to (-)-ISO in human colon longitudinal muscle is mediated by β_1-ARs. In molecular studies, RNA was extracted by the acid/guanidinium/phenol/chloroform method, DNase treated and checked for purity by gel electrophoresis. RNA (1μg) was reverse transcribed and PCR (30 cycles) conducted on 1/10 the cDNA using ^{33}P labelled primers. Product was gel electrophoresed, blotted onto Hybond N+ and quantitated by phosphorimaging (Molecular Dynamics SI). In rat, β_3-AR signals were found in ileum=colon smooth muscle>colon submucosa>colon mucosa>ileum submucosa=ileum mucosa. In human, β_3-ARs were present in taenia coli>circular muscle>mucosa. The evidence is consistent with the presence of functional β_3-ARs in rat ileum and human taenia coli.

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61P HOMOMERIC AND HETEROMERIC NMDA RECEPTOR SUBUNIT ASSEMBLIES CAN COEXIST WITHIN THE SAME STABLE CELL LINE

S. Grimwood, B. Le Bourdellès, W. Cockett, J. Atack, P. H. Hutson and P. J. Whiting. Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR.

We have previously used radioligand binding studies to investigate the pharmacological characteristics of human NR1a/NR2A receptors stably co-transfected into L(tk-) cells under the control of a dexamethasone inducible promoter (Grimwood *et al.*, 1995a). Inhibition of [^{3}H]L-689,560 binding to this cell line (NR1a/NR2A-1) by glycine site ligands revealed K_i values similar to those obtained for homomeric NR1a assemblies (ie agonists and partial agonists had lower affinities than at native receptors, whilst antagonists showed similar affinities for native receptors and NR1a assemblies; Grimwood *et al.*, 1995b; Table 1). These results indicated that in addition to heteromeric NR1a/NR2A receptors, the NR1a/NR2A-1 cell line may express homomeric NR1a assemblies. In order to investigate this hypothesis further, we have developed a second stable cell line (NR1a/NR2A-2) by co-transfecting human NR1a and NR2A cDNAs into L(tk-) cells at a ratio of 1:10, and using Northern blot analysis selected a clone expressing higher levels of NR2A mRNA than NR1a mRNA.

We have also developed a stable cell line expressing NR1a/NR2B receptors using similar procedures.

Cell culture techniques and radioligand binding assays were performed as previously described (Grimwood *et al.*, 1995a; 1995b). Affinity values obtained for the inhibition of [^{3}H]L-689,560 binding to NR1a/NR2A-2 and NR1a/NR2B cell membranes by glycine site ligands were compared to those previously obtained for rat cortex/hippocampus, NR1a/NR2A-1 and NR1a membranes (Table 1). Glycine and L-687,414 showed similar affinity values for native, NR1a/NR2A-2 and NR1a/NR2B receptors whilst they were considerably weaker against NR1a and NR1a/NR2A-1 cell lines (Table 1). The antagonists L-689,560 and 7-chlorokynurenic acid displayed similar affinities for all four subunit combinations (Table 1).

These data support the suggestion that homomeric NR1a assemblies were present in the NR1a/NR2A-1 cell line and illustrate that care should be taken when expressing heteromeric receptors, to avoid the simultaneous expression of homomeric subunit assemblies with high-affinity ligand binding sites.

Grimwood *et al.* (1995a) *Br. J. Pharmacol.* 114, 1P.
Grimwood *et al.* (1995b) *J. Neurochem.* 64, 525-530.

Table 1. Inhibition of [^{3}H]L-689,560 binding to NMDA receptor subunit combinations by glycine site ligands.

	Rat brain	NR1a[a]	NR1a/NR2A-1[b]	NR1a/NR2A-2	NR1a/NR2B
Glycine	0.333 (0.281, 0.394)	4.61	3.63	0.601 (0.450, 0.803)	0.215 (0.134, 0.342)
L-687,414	1.90 (1.61, 2.25)	67.8	48.0	1.69 (0.853, 3.35)	2.73 (1.76, 4.23)
L-689,560	0.005 (0.004, 0.006)	0.004	0.004	0.003 (0.002, 0.004)	0.003 (0.003, 0.003)
7-Cl KYNA	0.294 (0.250, 0.347)	0.576	0.618	0.260 (0.219, 0.308)	0.346 (0.295, 0.405)

Data are mean K_i values (μM) from [a]Grimwood *et al.*, 1995b and [b]1995a or geometric mean (-s.e.mean, +s.e.mean) (n=4).

62P DIFFERENTIAL ETHANOL SENSTIVITY OF NATIVE NMDA RECEPTORS STIMULATING NEUROSTRANSMITTER RELEASE: PROBABLE RELATIONSHIP TO SUBUNIT COMPOSITION

M. Göthert & K. Fink, Department of Pharmacology and Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn, Germany

Ethanol at concentrations characteristic for moderate intoxication in humans inhibited the NMDA-evoked [^{3}H]noradrenaline ([^{3}H]NA) release in rat (Göthert & Fink, 1989) and human cerebral cortical slices (Fink *et al.*, 1992), but was less potent in inhibiting the NMDA-evoked [^{3}H]acetylcholine ([^{3}H]ACh) release in rat striatal slices (Göthert & Fink, 1989). The aim of the present study was to examine (1) whether ethanol also inhibits the NMDA-evoked [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT), [^{3}H] dopamine ([^{3}H]DA) and [^{3}H]GABA release, (2) whether differences in ethanol sensitivity are related to the neurone involved or the brain region investigated and (3) whether the differences in sensitivity are dependent on the subunit composition of the NMDA receptor; hints at the subunit assembly can be obtained with ifenprodil, which preferentially inhibits NMDA receptors containing the NR2B subunit (Williams, 1993).

Rat cortical slices preincubated with [^{3}H]NA, [^{3}H]5-HT or [^{3}H]GABA and striatal slices preincubated with [^{3}H]5-HT, [^{3}H]GABA, [^{3}H]DA or [^{3}H]choline were superfused with Mg^{2+}-free Krebs' solution and [^{3}H]transmitter release was induced by 0.3 or 1 mM NMDA. Irrespective of the brain area, ethanol inhibited (in parentheses: IC_{50} in mM; $n \geq 6$ for each point of the concentration-response curves; C, cerebral cortex; S, striatum) the release of [^{3}H]NA (C: 44), [^{3}H]5-HT (C: 51; S: 55) and [^{3}H]GABA (C: 72; S: 74) at clearly higher potency than [^{3}H]ACh (S: 190) and [^{3}H]DA release (S: 234). Basically the same potency order was found for the inhibitory effect of ifenprodil on the NMDA-evoked release of these [^{3}H]transmitters. Accordingly, the inhibitory potencies of ethanol and ifenprodil on NMDA-evoked transmitter release were significantly correlated with each other ($r = 0.96$; $P < 0.001$). After blockade of the highly ifenprodil-sensitive fraction of NMDA receptors (by 100 nM ifenprodil, which inhibited [^{3}H]NA release by about 40 %), the potency of ethanol in inhibiting [^{3}H]NA release (IC_{50}: 274 mM) was decreased to the level characteristic for inhibition of [^{3}H]ACh and [^{3}H]DA release.

In conclusion, (1) the inhibitory potency of ethanol on transmitter release is related to the neurone from which the respective transmitter is released but independent of the brain area, (2) the heteromeric NMDA receptors stimulating NA, 5-HT and GABA release probably contain a higher proportion of the NR2B subunit than the receptors stimulating ACh and DA release and (3) ethanol may be assumed to predominantly inhibit NMDA receptors containing a high proportion of the NR2B subunit.

Acknowledgement. This study was supported by the Deutsche Forschungsgemeinschaft

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63P ω-CONOTOXIN MVIIC REVERSIBLY INHIBITS HIGH-K^+- AND 4-AMINOPYRIDINE-EVOKED GLUTAMATE RELEASE FROM ISOLATED NERVE TERMINALS (SYNAPTOSOMES)

M.S.Perkinton & T.S.Sihra (introduced by A.G.Ramage), Department of Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill Street, Hampstead, London, NW3 2PF.

Glutamate exocytosis, from mammalian CNS nerve terminals, is dependent on localised Ca^{2+}-influx through voltage-sensitive Ca^{2+} channels (Verhage *et al.*, 1991). The Ca^{2+} channels linked to glutamate release remain to be fully characterised. ω-Conotoxin MVIIC (ω-CTx MVIIC), a 26 amino acid peptide synthesised on the basis of a cDNA clone derived from the cone snail, *Conus magus,* has been shown to be a potent blocker of voltage-dependent Ca^{2+}-influx into rat brain synaptosomes (Hillyard *et al.*, 1992). In this study, using rat brain synaptosomes, we investigated the actions of this toxin on evoked glutamate release resulting from the inhibition of Ca^{2+}-channels coupled to exocytosis.

Synaptosomes, from the rat cerebral cortex, were purified on percoll gradients (Dunkley *et al.*, 1986) and evoked glutamate release was assayed by on-line fluorimetry (Nicholls & Sihra, 1986). Ca^{2+}-influx into synaptosomes was assayed by pre-loading synaptosomes with fura-2-AM (5 μM for 20 min at 37°C) and determining cytosolic free Ca^{2+}, ($[Ca^{2+}]_c$) by ratiometric fluorimetry (Grynkiewicz *et al.*, 1985).

Synaptosomes were depolarised using high-K^+ (30mM) or 4-aminopyridine (4AP). Under control conditions, high-K^+, caused the release of 23.0 ± 1.3 nmol glutamate/mg protein over 5 min (n=3), in the presence of 1 mM $CaCl_2$. When ω-CTx MVIIC (1μM) was added 5 min prior to depolarisation, release was reduced to 11.6 ± 0.8 nmol/mg over 5 min.
Glutamate release effected by depolarisation through blockade of K^+-channels using 4AP was reduced from 19.3 ± 0.9 nmol /mg over 5 min (n=3) to 8.3 ± 0.5 nmol/mg over 5 min in the presence of ω-CTx MVIIC. Inhibition of release by ω-CTx MVIIC was dose-dependent and completely reversible upon washing. In parallel experiments using fura-2, from a basal $[Ca^{2+}]_c$ of 100 nM, high-K^+ elevated the non-inactivating component of Ca^{2+}-influx (shown to be coupled to glutamate release) to 200 nM. Addition of ω-CTx MVIIC (1μM) 5 min prior to depolarisation reduced this component of influx by some 65%. When 4AP was used as the depolarising agent, ω-CTx MVIIC caused a 55% decrease in the evoked Ca^{2+}-influx.

Our results demonstrate that ω-CTx MVIIC is a potent and reversible inhibitor of the major proportion of voltage-dependent Ca^{2+}-channels coupled to glutamate release from isolated nerve terminals. As such, the toxin represents a valuable tool for the elucidation of Ca^{2+}-dependent processes modulating glutamate exocytosis.

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64P ANTAGONISM OF THE PRESYNAPTIC ACTION OF L-AP4 ON GABAergic INHIBITORY TRANSMISSION BY (±)-α-METHYL-4-PHOSPHONOPHENYLGLYCINE (MPPG) IN THE RAT VENTROBASAL THALAMUS

T.E. Salt & J.P. Turner, Institute of Ophthalmology, University College London, 11-43 Bath Street, LONDON EC1V 9EL.

We have shown previously that the Group II and Group III metabotropic glutamate receptor (mGluR) agonists (2S,3S,4S)-α-(carboxycyclopropyl)-glycine (CCG-I) and L-2-amino-4-phosphonobutyrate (L-AP4) can have presynaptic actions at the terminals of GABAergic nucleus reticularis thalami (nrt) neurones which project into the ventrobasal thalamus (VB), thus reducing synaptic inhibition onto VB relay neurones (Salt & Eaton, 1995). The objective of the present work was to test the novel antagonist MPPG (Thomas *et al.*, 1995) against these responses in order to characterise the receptors and to evaluate MPPG as a pharmacological tool in VB.

Extracellular recordings were made from single VB relay neurones in male Wistar rats (270-450g: anaesthetised with urethane 1.2g/kg, I.P.) with seven-barrel iontophoretic pipettes (Salt & Eaton, 1995). Sensory synaptic stimulation was provided by 10ms air jets directed at the vibrissal receptive field of VB neurones, and a condition-test paradigm was used to reveal inhibitory input from nrt onto VB neurones (Salt & Eaton, 1995). Iontophoretic applications of either L-AP4 (69±7.6nA, 9 neurones, mean ± s.e.m.) or CCG-I (10±2.2nA, 8 of the same 9 neurones), both from 25mM in water, pH8.5 solutions, were made during regular cycles of sensory stimulation.

L-AP4 and CCG-I reduced the synaptic inhibition by 40±5.6% and 56±9.1% respectively. When MPPG was applied alone (129±11.8nA, n=9, 50mM in water, pH8.5), it had little direct effect on synaptic inhibition (3±1.6% increase). However, during MPPG ejection, L-AP4 reduced synaptic inhibition by only 6±3.5% (significantly different from values during L-AP4 alone: $P<0.01$, Wilcoxon Rank Sum Test). In contrast, CCG-I was still able to reduce synaptic inhibition by 44±8.8% during MPPG ejection ($P<0.01$ compared with control values; not significantly different from CCG-I alone values).

These findings indicate that MPPG is a selective antagonist of L-AP4 compared to CCG-I in the rat VB, and this is in agreement with previous work in the isolated spinal cord (Thomas *et al.*, 1995). This provides further evidence in favour of a location of both Group II (CCG-I-sensitive) and Group III (L-AP4-sensitive) mGluRs on GABAergic terminals in VB. Furthermore, it is likely that these receptors modulate synaptic inhibition and thus regulate sensory processing (Salt & Eaton, 1995), although the physiological conditions under which they may be activated remain to be elucidated.

The support of the Wellcome Trust is gratefully acknowledged.

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65P PHARMACOLOGICAL CHARACTERISATION OF METABOTROPIC GLUTAMATE RECEPTOR-INDUCED INCREASES IN INTRACELLULAR [Ca^{2+}] IN CULTURED RAT CORTICAL NEURONES AND GLIAL CELLS

A. Mathie, B.J. Amos & C.D. Richards[1]. Departments of Pharmacology & Physiology[1], Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF.

Activation of the G-protein linked glutamate receptors (mGluRs) of rat cortical neurones and glial cells results in sustained or oscillatory changes in intracellular calcium [Ca^{2+}] and pH (Amos *et al.* 1995). The object of this study was to characterise the subtype(s) and the sensitivity of the mGluR(s) which mediate the changes in [Ca^{2+}] in these cells using the general mGluR receptor agonist 1S,3R, ACPD and the more selective agonists (S)-3HPG (for mGluR1 and perhaps mGluR5, Hayashi *et al.* 1994) and L-AP4 (for mGluRs 4,6 & 7, Nakanishi 1994).

Cortical neurones and glial cells were isolated from 3 day old rats and maintained in culture for 6-9 days. [Ca^{2+}] was imaged ratiometrically using the fluorescent dye Fura-2 and cells were excited at 340 and 380 nm with emission signals collected at wavelengths above 490 nm.

1S,3R, ACPD (0.25-250 μM) and (S)-3HPG (10-1000 μM) induced increases in [Ca^{2+}] in both neurones and glial cells (see Fig. 1). Both the number of neurones responding and the magnitude of the change in [Ca^{2+}] increased with agonist concentration. Thus 3 out of 22 neurones (11%) responded to 1 μM, 16 out of 20 (80%) to 100 μM, and 30 out of 34 (84%) to 250 μM 1S,3R ACPD. Similarly, 3 out of 34 neurones (9%) responded to 10 μM, 17 out of 41 (41%) to 100 μM and 32 out of 39 (82%) to 1 mM (S)-3HPG. In contrast only 1 out of 38 neurones (3%) responded to 100 μM L-AP4. A similar agonist profile was found for cortical glial cell responses (see Fig. 1).

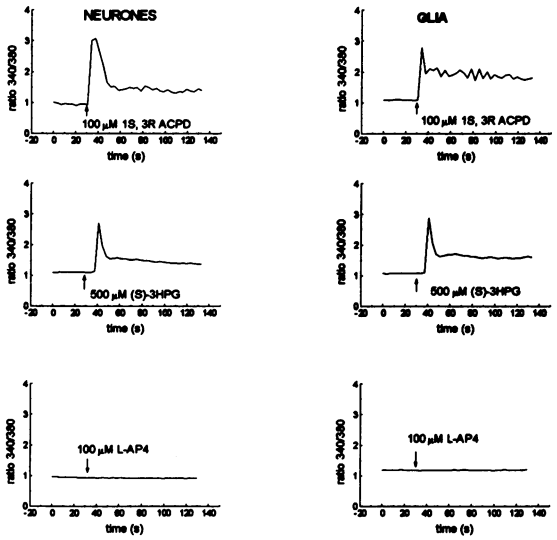

FIG. 1

Since (S)-3HPG mimics the actions of 1S,3R ACPD and L-AP4 does not, it is concluded that it is activation of the mGluR1 or mGluR5 receptor subtype which leads to an increase in [Ca^{2+}] in both cortical neurones and glial cells.

Thanks to the BBSRC and the Wellcome Trust for support

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66P PHARMACOLOGICAL AND KINETIC PROPERTIES OF CALCIUM TRANSIENTS INDUCED BY mGluR AGONISTS IN VARIOUS REGIONS OF RAT HIPPOCAMPUS *IN VITRO*

H.W.G.M. Boddeke, Preclinical Research, Sandoz Pharma Ltd., CH 4002 Basel, Switzerland (Introduced by D. Hoyer)

It is well known that the metabotropic glutamate receptor splice forms mGluR1a,b and c as well as mGluR5a and b mediate an increase in intracellular calcium. Since these receptor subtypes are expressed differentially in various cell layers of the hippocampus we have measured calcium responses after administration of mGluR agonists in pyramidal cells and in interneurons of the stratum oriens and stratum moleculare in rat hippocampus in vitro.

Rat hippocampal slices (250 μm thick) were loaded for 45 min with the calcium indicator Fura-2 acetylmethoxyester (5 μM) in buffer of the following composition (in mM; NaCl 124, KCl 5, NaH_2PO_4 1.3, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 2) at 32°C at pH 7.4. The slices were subsequently transferred to a submersion perfusion chamber mounted on an inverted microscope and perfused with the same buffer at 37°C. Changes in intracellular calcium were measured at the excitation wavelength ratio of 340 and 380 nm and an emission wavelength of 510 nm. The calcium responses were quantified using a Fuji HR-1700 Deltaron mos-sensor camera and calculated using standard methods (Grynkiewicz et al., 1985). The increase in fluorescence, induced by mGluR agonists, was determined in the presence of the AMPA receptor antagonist CNQX (10 μM) and the NMDA receptor antagonist D-AP-5 (50μ M). After application of mGluR agonists a strong transient calcium response was observed in cells of the pyramidal layer and in the stratum oriens, whereas the response in the stratum moleculare was much weaker. In the pyramidal layer the response to 100 μM (1S,3R)-ACPD had a fast rise-time of 312 ± 24 ms (n = 16), while a slower response with a rise-time of 864 ± 19 ms (n = 14) was recorded in the stratum oriens. Very slow responses with a time to peak value of 2210 ± 198 msec (n = 19) were recorded in the stratum moleculare after appliction of (1S,3R)-ACPD. The rank order of potencies of the mGluR agonists (quisqualate > glutamate > ibotenate > (1S,3R)-ACPD) was similar in the three hippocampal cell layers.

The difference in the kinetics of the calcium responses and the similarity of the pharmacology in the various hippocampal cell layers may reflect activation of the different splice forms of the mGluR1 and 5 subtypes which have the same binding domain but show difference in their G-protein coupling domains.

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Table 1: The potencies and efficacies of mGluR agonists

	quisqualate		glutamate		ibotenate		(1S,3R)-ACPD	
	Potency	Efficacy	Potency	Efficacy	Potency	Efficacy	Potency	Efficacy
Str. Pyr.	6.32 ± 0.41	100 %	5.94 ± 0.38	96 ± 9.2 %	4.22 ± 0.32	89 ± 7.2 %	3.69 ± 0.28	73 ± 6.2 %
Str. Mol.	6.64 ± 0.28	100 %	5.73 ± 0.47	88 ± 8.4 %	4.06 ± 0.27	82 ± 6.9 %	3.61 ± 0.31	64 ± 8.4 %
Str. Or.	6.82 ± 0.46	100 %	5.62 ± 0.24	91 ± 7.4 %	4.54 ± 0.17	79 ± 4.8 %	3.53 ± 0.28	67 ± 5.3 %

The pEC_{50}'s and efficacies (expressed as a percentage of the response to quisqualate) in three hippocampal layers. The data represent mean values ± s.e.mean (n =8-11).

67P ORIGIN OF DEPOLARIZATIONS EVOKED IN THE RAT STRIATUM BY COMPETITIVE INHIBITION OF GLUTAMATE UPTAKE WITH L-*TRANS*-PYRROLIDINE-2,4-DICARBOXYLATE (L-*TRANS*-PDC)

J. Urenjak[1], E. Zilkha & T.P. Obrenovitch, Dept of Neurological Surgery, Institute of Neurology, Queen Square, London WC1N 3BG and [1]Dept of Discovery Biology, Pfizer Central Research, Sandwich CT13 9NJ

Inhibition of glutamate uptake by application of L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC) using microdialysis markedly increases brain extracellular concentrations of glutamate, and evokes small depolarizations of the extracellular field (d.c.) potential (Urenjak *et al.*, 1995). Here, we examined whether ionotropic glutamate receptors contribute to these depolarizations, by using dizocilpine (MK-801) and 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX) to block *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA)/kainate receptors, respectively.

Adult male Sprague-Dawley rats (250-350 g) were anaesthetized with halothane in N_2O:O_2 (1:1) throughout. Microdialysis probes incorporating an electrode were implanted in the striatum, and perfused at 1 μl min^{-1} with artificial CSF (ACSF) (Urenjak *et al.*, 1995). The d.c. potential was recorded between the microdialysis electrode and a reference electrode. In all animals, 10 mM L-*trans*-PDC was applied through the probe during 3 separate 20-min periods, each followed by 30 or 45 min of perfusion with ACSF alone. In one group, animals received MK-801 (2 mg kg^{-1} i.v.) 15 min after the first L-*trans*-PDC application, and NBQX (30 mg kg^{-1} i.p.) 15 min after the second. This order was reversed in another group. Control animals did not receive any inhibitor of ionotropic glutamate receptors. This experimental design allowed us to examine the effect of MK-801 and NBQX, either alone or in combination, on L-*trans*-PDC evoked depolarizations.

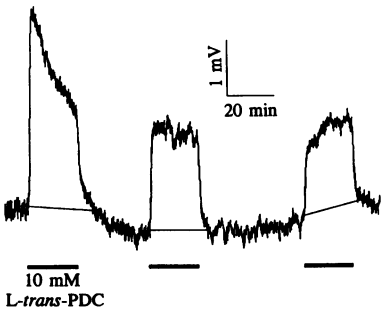


Figure 1. Typical depolarizations evoked by 3 applications of L-*trans*-PDC (horizontal solid bars) in control experiments. Identical changes were obtained when animals were treated with antagonists of ionotropic glutamate receptors.

Repeated applications of L-*trans*-PDC produced consistent responses, except for the first depolarization which rose initially to a much higher level, before gradually decreasing as L-*trans*-PDC was still applied (Figure 1). In controls, the areas of the 3 consecutive responses were: 44.9 ± 2.4, 35.6 ± 3.4 and 31.6 ± 3.4 mV.min (mean ± s.e.mean, n = 6). The magnitude of the 2nd and 3rd depolarization relative to the first (i.e. around 78 and 70 %, respectively) was not significantly altered by MK-801 and/or NBQX. In addition, the rapid onset of depolarization (Figure 1) contrasted with the progressive increase of dialysate glutamate levels (Urenjak *et al.*, 1995).

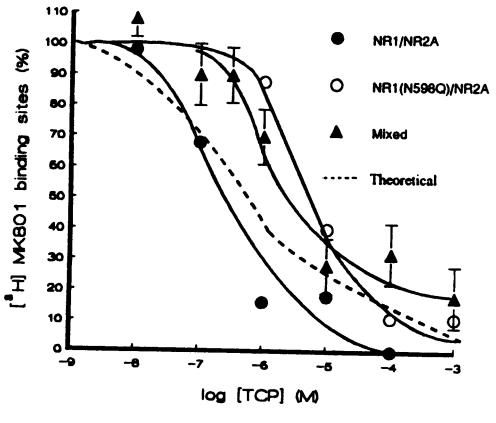
These data do not support the hypothesis that L-*trans*-PDC induced depolarizations result from increased extracellular glutamate acting on ionotropic glutamate receptors. These depolarizations may rather reflect effective, electrogenic uptake of L-*trans*-PDC.

Urenjak, J., Obrenovitch T.P. & Zilkha, E. (1995) *Br J Pharmacol* 115(Suppl.), 18P.

68P EVIDENCE FOR AT LEAST TWO NR1 SUBUNITS PER NMDA RECEPTOR AS DEDUCED FROM THE RADIOLIGAND BINDING PROPERTIES OF WILD-TYPE AND MUTANT NR1/NR2A RECEPTORS

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

N-Methyl-D-aspartate (NMDA) receptors are fast-acting glutamate-gated cation channels with a high permeability for Ca^{2+}. Several cDNAs encoding NMDA receptor subunits, NR1 and NR2A-D, have been identified and it is thought that different combinations coassemble to form heteromeric, functional receptors. The quaternary structures of these are unknown. We have approached this problem by the characterisation of [^{3}H]MK801 radioligand binding to wild-type and mutant NR1/NR2A heteromeric receptors expressed transiently in human embryonic kidney (HEK) 293 cells. The Inhibitory Constants, K_is, for MK801, tenocyclidine (TCP) and ketamine binding to the wild-type NR1/NR2A were 7 ± 2 nM, 64 ± 32 nM and 2600 ± 500 respectively for at least n = 3. The mutant, NR1(N598Q)/NR2A, where the point mutation is in the putative membrane domain, M2, has been shown to reduce Ca^{2+} permeability and receptor-mediated cytoxicity compared to wild-type NR1/NR2A. The K_is for the binding to NR1(N598Q)/NR2A for MK801, TCP and ketamine were 38 ± 12 nM, 3050 ± 770 nM and 70800 ± 20500 nM respectively for at least n = 3. The affinity constants for Zn^{2+} were unchanged. Values were 0.58 ± 0.3 mM (wild-type) and 0.59 ± 0.27 mM (mutant) (n = 3). Cotransfections were carried out using both wild-type NR1, mutant NR1(N598Q) and NR2A with DNA ratios 0.5:0.5:3. Cells were harvested and competition binding curves using [^{3}H]MK801 (20nM) and TCP carried out (Chazot et al., 1994). The experimental curve for displacement by TCP differed from that predicted for 1 NR1 subunit per receptor assuming no effect on receptor assembly between the two NR1 subunits thus suggesting that this NMDA receptor subtype must have at least two NR1 subunits (Figure). It is in agreement with functional studies where two glutamate and glycine binding sites per NMDA receptor are shown. We have determined a molecular size for the NR1/NR2A receptor of 700000 - 850000 daltons. Furthermore, we have shown the coexistence of at least two NR2 subunits per receptor (Chazot et al., 1994). Therefore, the results herein are consistent with a pentameric structure with a proposed subunit ratio of $(NR1)_2(NR2A)_3$.



Chazot PL, Coleman SK, Cik M & Stephenson FA (1994) J.Biol.Chem. 39, 24403-24409.

69P POTENTIATION OF AMPA-INDUCED EXCITATION BY SELECTIVE METABOTROPIC GLUTAMATE RECEPTOR AGONISTS ON RAT SPINAL NEURONES *IN VIVO*

David Lodge, Ann Bond, John Goldsworthy, S. Richard Baker, Darryle D. Schoepp+ & James A. Monn+, Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey, GU20 6PH, UK, and +Lilly Research Laboratory, Indianapolis, IN46285, USA.

Metabotropic glutamate receptors (mGluRs) are divided into Group 1 (PI-coupled) and Group 2 and 3 (cyclase-coupled) receptors. We have previously reported that metabotropic glutamate receptor (mGluR) agonists potentiate the actions of excitatory and inhibitory amino acids on rat spinal neurones (Bond and Lodge, 1994). Most of the compounds used, eg. 1S,3R-aminocyclopentane dicarboxylate (1S,3R-ACPD), were mixed mGluR Group 1 and 2 agonists. Two compounds, 3,5-dihydroxy-phenylglycine (3,5-DHPG) and 2R,4R-4-aminopyrrolidone-2,4-dicarboxylate (aza-ACPD) show good selectivity for PI-coupled and cyclase-coupled receptors respectively. Thus on adult rat hippocampal slices, 3,5-DHPG enhanced PI hydrolysis with an EC50 of 27 ± 2 μM but was inactive at 1mM on forskolin stimulated cAMP levels. Aza-ACPD, by contrast, was inactive on PI hydrolysis but inhibited forskolin stimulated cAMP levels with an EC50 of 13 ± 3 μM. 1S,3R-ACPD had EC50s of 47 ± 8 and 6 ± 2 μM in these two assays. In the present study, using the technique of microelectrophoresis, we have compared the actions 3,5-DHPG and aza-ACPD with that of 1S,3R-ACPD on excitation of spinal neurones by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) in pentobarbitone anaesthetised rats.

Compared on 7 neurones, DHPG (ejected at 5-20 nA from a 50mM solution in 150mM NaCl), increased responses to AMPA by 42 ± 7 % whereas 1S,3R-ACPD similarly ejected produced an increase of 60 ± 14 %. On a further 6 neurones, aza-ACPD (25-40nA; 25mM in 175mM NaCl) increased responses to AMPA by 47 ± 4 % whereas 1S,3R-ACPD (5-25nA; 25mM in 175mM NaCl) increased them by 75 ± 14 %.

Since DHPG and aza-ACPD are approximately twice and half as potent as 1S,3R-ACPD on phosphoinositide hydrolysis and inhibition of adenylcyclase respectively but proportionately considerably less active as potentiators of AMPA responses, it seems likely that activation of Group 1 and Group 2 mGluRs act synergistically to enhance responses to ionotropic agonists.

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70P (S)-α-ETHYL-GLUTAMIC ACID AND (RS)-α-CYCLOPROPYL-4-PHOSPHONOPHENYLGLYCINE AS ANTAGONISTS OF L-AP4- AND (1S,3S)-ACPD-INDUCED DEPRESSION OF MONOSYNAPTIC EXCITATION OF NEONATAL RAT MOTONEURONES

N.K. Thomas, D.E. Jane, H.-W. Tse and J. C. Watkins, Pharmacology Department , Bristol University, Bristol, BS8 1TD.

Activation of presynaptic metabotropic glutamate receptors (mGluRs) on primary afferent terminals of neonatal rat motoneurones results in depression of the monosynaptic component of the dorsal root evoked ventral potential (DR-VRP). L-2-amino-4-phosphonobutyrate (L-AP4) and (1S,3S)-1-aminocyclopentane-1,3-dicarboxylate (ACPD) are capable of eliciting a decrease in the DR-VRP by acting at different presynaptic mGluRs (Jane *et al.*, 1994). We describe the potency and selectivity of two novel antagonists synthesized in our own laboratories, (S)-α-ethyl-glutamic acid (EGLU) and (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), on L-AP4- and (1S,3S)-ACPD-induced depressions of the monosynaptic excitation of neonatal rat motoneurones.

All experiments were performed on isolated hemisected spinal cords of 1-5 day old Wistar rats of either sex (Evans *et al.*, 1982). Recordings were taken from the ventral root following stimulation of the corresponding dorsal root (30V, 2 pulses min^{-1}). To isolate the non-N-methyl-D-aspartate (non-NMDA) component of the response, DR-VRPs were recorded in the presence of 2mM $MgSO_4$ and 50μM D-2-amino-5-phosphonopentanoate (D-AP5). To determine the selectivity of EGLU and CPPG for presynaptic receptors, the effect of 1mM antagonist was investigated against depolarisations which were directly produced in the motoneurones by NMDA, (S)-α-amino-3-hydroxy-5-methylisoxazole (AMPA) and (1S,3R)-ACPD. For these experiments, tetrodotoxin (10^{-5} for 2min, then 10^{-7}M continuously) was included in the standard Ringer solution, but in the absence of $MgSO_4$ / D-AP5.

CPPG (50 and 200μM) potently antagonised L-AP4- and (1S,3S)-ACPD-induced depressions of the DR-VRP with apparent K_D values of 2.5 ± 0.3μM and 51 ± 3μM (n=6), respectively. EGLU (50 and 200μM) exhibited selective antagonism for the (1S,3S)-ACPD-sensitive mGluR, mean apparent K_D of 63 ± 4μM (n=6), displaying no antagonism at the L-AP4-sensitive receptor at 200μM.

At 1mM, CPPG resulted in 93 ± 8%, 70 ± 12% and 128 ± 3% recovery of control responses respectively for AMPA, (1S,3R)-ACPD and NMDA (n=3, for each agonist), with EGLU at the same concentration resulting in 94 ± 12%, 43 ± 4% and 116 ± 3% recovery respectively.

CPPG is more than 3 times more potent and more specific than the previously reported selective L-AP4-receptor antagonist MPPG (Jane et al., 1995), while EGLU is twice as potent as MCCG, the previously reported specific (1S,3S)-ACPD receptor antagonist (Jane et al., 1994). CPPG and EGLU should therefore be considered as the antagonists of choice to block L-AP4 and (1S,3S)-ACPD-induced activity in the primary afferent endings on motoneurones of the neonatal rat spinal cord.

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71P MODULATION OF LONG-TERM POTENTIATION IN CA1 REGION OF HIPPOCAMPAL BRAIN SLICES BY BENZODIAZEPINES

Guy R. Seabrook, G. Dawson, & Barbara J. Bowery. Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR.

The induction of long term potentiation (LTP) and memory formation in mammals is reduced by enhancement of $GABA_A$ receptor function with benzodiazepine agonists like diazepam (Del Cerro *et al.*, 1992). To further investigate this phenomenon the ability of the inverse agonist DMCM (methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) to modulate LTP-induction in hippocampal slices from mouse brain was compared to that of the high efficacy agonist flunitrazepam.

Conventional extracellular microelectrode recordings were made from the CA1 region of brain slices from male C57 mice. Excitatory postsynaptic potentials (e.p.s.p.s) were elicited by stimulation of the Schaffer collateral pathway with bipolar stimulating electrodes (0.033 Hz). The effects of drugs on the induction of LTP by threshold stimulus frequencies (*threshold stimulus*; 10 events at 100 Hz) was compared with that induced by bursts of high frequency stimuli (*burst stimuli*; 4 x 10 events at 100 hz every 20 sec). Under control conditions the threshold stimulus potentiated e.p.s.p. rate of rise by 238 ± 22% (at 0-5 mins following stimulus; n = 12), and e.p.s.p.s remained significantly potentiated after 30 mins at 202 ± 23% ($P < 0.01$ paired *t*-test). The LTP induced by burst stimuli (291 ± 28% at 30 mins) was greater than that caused by threshold stimuli. Previous studies in rat brain have shown that LTP formation is critically dependent upon activation of postsynaptic NMDA receptors (e.g. Zalutsky & Nicoll 1990). Consistent with this, LTP formation in mouse hippocampus was attenuated by the non-competitive NMDA receptor antagonist MK-801 (1 μM, n = 8) and was abolished ($p < 0.01$, ANOVA) by the NMDA receptor glycine site antagonist L-701,324 (1 μM, n = 7; Kulagowski *et al.*,1995). Flunitrazepam (1 μM) alone caused a slight but significant reduction in e.p.s.p. rate of rise (to 83 ± 8%, n = 9). This was associated with the complete inhibition of LTP induction by threshold stimuli (122 ± 31% *c.f.* control = 202%) and attenuation of LTP induced by burst stimuli (169 ± 43%, n = 4; *c.f.* control = 291%). The inhibition by flunitrazepam was overcome by sustained high-frequency stimulation (100 events at 100 Hz, n = 6). In contrast, the inverse agonist DMCM (100 nM) potentiated LTP formed by both threshold (to 251 ± 39%) and burst stimuli (to 598 ± 66%, n = 6), and this was associated with the appearance of paroxysmal burst discharges.

In conclusion, modulation of $GABA_A$ receptor function with benzodiazepine agonists and inverse agonists can inhibit and potentiate LTP formation respectively, in the CA1 region of mouse hippocampus.

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72P THE EFFECT OF K+-DEPLETED MEDIUM ON ILOPROST- AND FORSKOLIN-MEDIATED DOWN-REGULATION OF IP PROSTANOID RECEPTORS IN NG108-15 CELLS

M Keen & A Krane, Department of Pharmacology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK.

Pretreatment of NG108-15 cells with either IP receptor agonists, such as iloprost, or agents which increase intracellular cAMP, such as forskolin, produces down-regulation of IP prostanoid receptors (Kelly et al., 1990; Keen et al., 1992). However, the mechanisms by which iloprost and forskolin produce receptor down-regulation do not appear to be the same (Krane et al., 1994). We have previously reported preliminary evidence that iloprost-mediated down-regulation may be inhibited by the use of K^+-depleted medium (Krane and Keen, 1995), which is reported to inhibit receptor internalization via inhibition of the formation of clathrin coated pits (Cantau et al., 1988). In this study we have examined the effect of K^+-depleted medium on iloprost-mediated IP receptor down-regulation in more detail, and have also determined the effect of K^+-depleted medium on the down-regulation of IP receptors produced by forskolin.

Confluent NG108-15 cells (passage 19-26) were pretreated for 17h in the absence or presence of 10μM iloprost or 10μM forskolin. Parallel inculations were carried out in Dulbecco's modified Eagle's medium (DMEM) and a version of DMEM prepared by Gibco in which K^+ ions had been replaced with equilmolar Na^+ (K^+-depleted). Cells were subsequently harvested and washed, and the binding of [^{3}H]-iloprost measured as previously described (Krane et al., 1994). Data were analyzed using a paired *t*-test; $P<0.05$ was considered significant.

There was no effect of K^+-depleted medium on the binding of [^{3}H]-iloprost to homogenates of vehicle treated cells. The specific binding of 1nM and 10nM [^{3}H]-iloprost was 34.78±5.84 and 129.45±18.22 fmol.mg protein^{-1}, respectively in DMEM and 32.78±5.36 and 117.90±15.40 fmol.mg protein^{-1}, respectively in K^+-depleted medium (all values mean±s.e.mean, n=8).

Similarly, there was no significant effect of K^+-depletion on the coupling of IP receptors to G_s. The non-hydrolyzable GTP analogue, 100μM guanylylimidodiphosphate, reduced the binding of 1nM and 10nM [^{3}H]-iloprost by 67.00±4.75% and 57.45±2.86%, respectively in DMEM and by 61.59±5.91% and 54.51±4.25%, respectively in K^+-depleted medium (all values mean ±s.e.mean, n=8). In support of our previous findings, the loss of IP receptors produced by pretreatment with iloprost was significantly reduced in K^+-depleted medium; iloprost pretreatment significantly reduced the binding of 1nM and 10nM [^{3}H]-iloprost by 80.38±1.66% and 74.80±6.41%, respectively in DMEM, but by only 45.79±15.67% and 52.88±8.66%, respectively in K^+-depleted medium (all values mean ±s.e.mean, n=4). However, K^+-depleted medium was without significant effect on IP receptor down-regulation produced by forskolin; forskolin pretreatment significantly reduced the binding of 1nM and 10nM [^{3}H]-iloprost by 18.48±4.06% and 19.04±2.45%, respectively in DMEM and by 17.75±4.60% and 13.45±5.75%, respectively in K^+-depleted medium (all values mean ±s.e.mean, n=4).

Thus the use of K+-depleted medium significantly reduced the loss of IP receptors produced by iloprost pretreatment, suggesting that receptor internalization may be involved in this process. However, K+-depletion was without effect on forskolin-mediated IP receptor down-regulation in these cells.

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73P DIFFERENTIAL EFFECTS OF PROTEIN KINASE C INHIBITION ON PAF- AND C5a-INDUCED EOSINOPHIL ACTIVATION

M.M. Teixeira & P.G. Hellewell, Department of Applied Pharmacology, National Heart and Lung Institute, Imperial College, Dovehouse Street, London SW3 6LY

We have previously shown that drugs which elevate cyclic AMP (e.g. prostaglandin E_2) inhibited more potently and more effectively eosinophil aggregation induced by PAF than that induced by C5a (Teixeira et al, 1995a). This differential inhibition suggests these stimuli may differ in the way they induce eosinophils to aggregate. In this study, we investigated the effects of the protein kinase (PK) C inhibitors, Ro 31-8220 (Ro) and staurosporine, on eosinophil aggregation induced by PAF and C5a. The effects of these agents on LTB_4-induced aggregation was also assessed for comparison.

Eosinophils, elicited in the peritoneal cavity of horse serum-treated guinea pigs, were purified on a discontinuous Percoll gradient. Cells which were over 95% pure were diluted (5 x 10^6 eosinophils/ml) in PBS and 300 μl aliquots placed in aggregation cuvettes at 700 rpm, 37°C for 3 min. The activating stimuli (PAF 0.01μM, C5a 0.1μM, PMA 0.1μM or LTB_4 0.01μM) were added and aggregation assessed as changes in light transmission using a platelet aggregometer (Teixeira et al, 1995b). Eosinophil aggregation is presented as % maximal aggregation, which is defined as the aggregation response to 1μM PMA. Intracellular calcium levels after activation with PAF, C5a or LTB_4 were measured using FURA-2 as previously described (Jose et al, 1994). Cells were pretreated with the inhibitors for 3 min prior to addition of the various stimuli.

Ro (10 and 30μM) completely inhibited eosinophil aggregation induced by 0.1μM PMA demonstrating the efficacy of the drug as a PKC inhibitor. Whereas Ro significantly potentiated eosinophil aggregation induced by C5a and LTB_4, PAF-induced responses were significantly inhibited (eg. C5a, 22.2 ± 3.9%; C5a + Ro 30μM, 51.3 ± 3.4%; LTB_4, 24.8 ± 2.6%; LTB_4 + Ro, 40.9 ± 6.0%; PAF, 16.9 ± 4.6%; PAF + Ro 30μM, 2.8 ± 1.6% maximal aggregation; n= 4-8). Similarly, 30μM Ro enhanced the duration and peak of calcium transients after activation with C5a and LTB_4, but inhibited PAF-induced calcium responses. Staurosporin at a concentration (0.1μM) which inhibited PMA-induced responses by 80%, potentiated not only eosinophil aggregation induced by C5a and LTB_4, but also aggregation induced by PAF (eg. LTB_4, 18.7 ± 4.1%; LTB_4 + staurosporine, 58.3 ± 5.8%; PAF, 12.1 ± 3.3%; PAF + staurosporine, 58.6 ± 4.2% maximal aggregation, n= 5-6). Because staurosporine may also inhibit PKA in addition to its inhibitory effects on PKC, we assessed the effects of the PKA inhibitor H89 on eosinophil aggregation. At the concentration of 10 μM, H89 significantly potentiated eosinophil aggregation induced by C5a, LTB_4 and PAF by 69%, 72% and 61%, respectively (n= 4).

Our results suggest opposing roles for PKC on PAF- versus C5a/LTB_4-induced eosinophil aggregation. Wherease PKC appears to be directly involved in the process of aggregation induced by PAF, this enzyme appears to down-regulate the responses induced by C5a and LTB_4. The potentiating effects of staurosporine on PAF-induced responses may not be related to its ability to inhibit PKC, but possibly to the ability of this drug to inhibit other enzymes in the cell such as PKA.

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74P EFFECT OF L-NAME AND L-ARGININE ON CARDIAC STUNNING (RECOVERY OF SYSTOLIC AND DIASTOLIC CONTRACTILE FUNCTION DURING REPERFUSION FOLLOWING NORMOTHERMIC GLOBAL ISCHAEMIA) IN RAT ISOLATED HEART

R. Pabla & M.J. Curtis, VBRC, Pharmacology Group, King's College London, Manresa Road, London SW3 6LX.

Endogenous nitric oxide (NO) protects against reperfusion-induced arrhythmias following 60 min ischaemia in the rat (Pabla & Curtis, 1995). We examined whether NO plays a similar role in protecting against stunning (reversible impairment of recovery of contractile function during reperfusion; Bolli *et al,* 1990).

Hearts were excised from male Wistar rats (240-260g) anaesthetised with pentobarbitone (60 mg kg^{-1}, i.p.) plus 250 iu sodium heparin (i.p.) then perfused (Langendorff mode) with Krebs' solution modified to contain (in mM): KCl 4.0 and $CaCl_2$ 1.4. Hearts were randomised to control perfusion (drug-free), 100 µM L-NAME, 10 mM L-arginine (L-arg) or 100 µM L-NAME plus 10 mM L-arg (n=6/group). These concentrations significantly reduce (L-NAME) or increase (L-arg) coronary effluent NO content in this preparation (Pabla & Curtis, 1995). Hearts were paced via the right atrium to maintain heart rate constant (330 beats min^{-1}). After 20 min of drug-free perfusion, isochoric ventricular pressure was measured using a non-elastic fluid filled balloon and a Starling curve was constructed with 0.02 ml volume increments to the balloon. Hearts were subjected to 60 min normothermic global ischaemia followed by 60 min reperfusion and Starling curves were re-evaluated. Time-matched drug-free controls were not subjected to ischaemia/reperfusion.

Data are mean±s.e.mean and were analysed by ANOVA plus Dunnett's test; *P<0.05 versus control.

Drug-free control developed pressure was initially impaired during reperfusion (Table 1) but was not different from the time-matched value (112±10 mmHg) by 30 min after the start of reperfusion and thereafter, indicating recovery from systolic stunning. L-NAME worsened systolic stunning, an effect prevented by L-arg (Table 1). However, recovery of coronary flow was prevented (L-arg-reversible) by L-NAME at 5 and 15 min after the start of reperfusion (P<0.05). Recovery from diastolic stunning was incomplete in all groups even after 60 min of reperfusion (Table 1), time-matched diastolic pressure being 12±2 mmHg; there was no effect of L-NAME.

In conclusion, an apparent role for NO in protecting against systolic stunning is likely to be spurious since the primary action of L-NAME was to delay recovery of coronary flow in an L-arginine-preventable manner. Diastolic stunning is not limited by the action of endogenous NO.

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Table 1.

	Developed Pressure (mmHg)					Diastolic Pressure (mmHg)				
	5min	15min	30min	45min	60min	5min	15min	30min	45min	60min
Control	42±2	60±5	106±18	104±16	90±20	112±27	93±16	73±13	76±15	73±19
L-NAME	0±0*	0±0*	78±18	90±15	95±10	110±12	95±15	75±15	65±15	63±18
L-NAME + L-arg	30±6	56±13	90±16	95±10	105±16	106±10	95±10	73±18	61±10	58±13
L-arg	35±16	86±13	101±10	100±10	108±18	108±8	85±9	66±7	61±7	55±5

75P NITRIC OXIDE SYNTHASE INHIBITORS POTENTIATE CYTOKINE-INDUCED DAMAGE OF MURINE LUNG EPITHELIAL CELLS

A.C. Burke-Gaffney & P.G. Hellewell, Applied Pharmacology, National Heart and Lung Institute, Imperial College, Dovehouse Street, London SW3 6LY, U.K.

Lung epithelial cells express inducible nitric oxide synthase (iNOS) following cytokine treatment (Barnes & Liew, 1995), however, the consequences of this are not well understood. Recent studies on lung epithelial cells suggest cytoprotective and cytotoxic roles for nitric oxide (Heiss *et al.*, 1994; Su *et al.*, 1995). In the present study we have investigated the effects of the NO synthase inhibitors, aminoguanidine and N^Gmonomethyl-l-arginine (L-NMMA) on cytokine-induced lung epithelial cell damage.

The murine lung epithelial cell line, LA-4, was maintained in HAMs F12 medium supplemented with foetal calf serum (15%), non-essential amino acids (1%) and antibiotics. Confluent monolayers of LA-4 cells grown on 96-well plates were incubated for 24h with 'cytomix', a mixture of human recombinant tumour necrosis factorα (hrTNFα; 10ngml^{-1}), hr interleukin-1β (IL-1β; 10ngml^{-1}) and murine recombinant interferonγ (IFNγ, 200 Uml^{-1}), in combination with increasing concentrations of aminoguanidine (3-1000μM), L-NMMA (10-1000-μM) or D-NMMA (10-1000 μM). The Griess reaction was used to measure nitrite accumulation in the culture medium. Epithelial cell damage was assessed using ethidium homodimer-1, a high-affinity red fluorescent DNA dye that is internalized only through compromised cell membranes (Levesque *et al.*, 1995). Cytotoxicty was calculated from fluorescence for cytomix $\pm$ inhibitor-treated cells expressed as a percent of fluorescence of cells treated with detergent (0.1% saponin).

Cytomix had a cytotoxic effect on LA-4 cells at 24h which varied between experiments. The range over nine separate experiments was 3.7-21.7% (mean $\pm$ s.e. mean was 14.0 $\pm$ 1.7%). Nitrite accumulation measured at 24h time was 79.0 $\pm$ 0.13 nmols nitrite/ 10^6 cells (n=9 experiments). In a concentration-dependent manner, aminoguanidine decreased nitrite production and, in parallel, increased cytomix-induced cytotoxicity at 24h. For example aminoguanidine (1000 μM) inhibited nitrite accumulation by 96.0 $\pm$ 0.2% and increased cytotoxicity of cytomix-treated cells from 16.4 $\pm$ 1.7% (n= 16 determinations) to 30.3 $\pm$ 1.9% (n=16 determinations). Similar effects were seen with L-NMMA (77.7 $\pm$ 0.2% inhibition of nitrite accumulation and an increase in cytotoxicity from 9.7 $\pm$ 0.9 % to 19.6 $\pm$ 0.9 % with 1000μM L-NMMA) and were stereospecific since D-NMMA did not alter cytotoxicity under the conditions used in this study.

These results are consistent with the idea that endogenous NO, induced by cytokines, limits cytokine-induced lung epithelial cell damage. NO production by airway epithelial cells following exposure to cytokines may, in part, be a mechanism whereby damage to these cells during inflammation is minimized.

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76P REGULATION OF NO SYNTHESIS BY DIMETHYLARGININE DIMETHYLAMINOHYDROLASE

Raymond J. MacAllister[1], Guy St.J. Whitley[2], Heather Parry[1,2], Masumi Kimoto[3], Tadashi Ogawa[3], Harold Hodson[4], Salvador Moncada[4] and Patrick Vallance[1].

[1]Departments of Pharmacology and Clinical Pharmacology, and [2]Cellular and Molecular Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K. [3]Department of Nutrition, School of Medicine, University of Tokushima, Tokushima 770, Japan. [4]Wellcome Research Laboratories, Beckenham, Kent, U.K.

N^GN^Gdimethyl-L-arginine (ADMA) and N^Gmonomethyl-L-arginine (L-NMMA) are guanidino-substituted arginine analogues that inhibit nitric oxide (NO) synthesis. They have been identified in biological fluids and tissues and are synthesised by endothelial cells in culture (Vallance *et al.*, 1992; MacAllister *et al.*, 1994). Intracellular methylarginines could regulate the L-arginine:NO pathway by local inhibition of NO synthases. Methylarginines (L-NMMA and ADMA, but not the inactive stereoisomer N^GN^G'dimethyl-L-arginine (SDMA)) are metabolised to citrulline within NO-generating cells by a calcium-independent enzyme, dimethylarginine dimethylaminohydrolase (DDAH). Using a monoclonal antibody, DDAH was identified in rat and human tissue homogenates and in human endothelial cells in culture. The activity of DDAH was assessed by measuring the conversion of [^{14}C]L-NMMA to [^{14}C]citrulline and data are expressed as mean$\pm$s.e.mean. Human umbilical vein endothelial cells in culture (SGHEC-7 cell line), homogenates of rat heart, brain, liver, lung and kidney, and DDAH purified from rat kidney, metabolised L-NMMA to citrulline. We identified a compound, 2-amino-4(3-methylguanidino)butanoic acid (4142W), that inhibited the metabolism of [^{14}C]-L-NMMA to [^{14}C]-citrulline by rat tissue homogenates, human endothelial cells in culture (EC_{50} 103.9$\pm$23μM; n=9) and isolated purified DDAH. Addition of 4124W to cultures of SGHEC-7 cells for 72h increased the concentration of ADMA in the culture supernatant by approximately 70% from 3.1$\pm$0.3 to 5.0$\pm$0.7 μM (n=15; $p<0.005$) and altered the ratio of ADMA:SDMA from 4.7$\pm$0.4 to 9.2$\pm$0.7. In the presence of 30μM L-arginine, 4124W (1μM-1mM) did not inhibit human placental or neuronal NO synthase. However 4124W (1mM) increased the tone of submaximally pre-constricted rat aorta by 69.9$\pm$14.7% (n=5; $P<0.05$), and reversed endothelium-dependent relaxation (bradykinin 1μM) of preconstricted human saphenous vein by 37.9$\pm$15.3% (n=4; $P<0.05$); the effects of 4124W on isolated blood vessels were reversed by L- but not D-arginine. Decreased activity of DDAH may provide an alternative mechanism for inhibition of NO generation in tissues.

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77P ROLE OF NITRIC OXIDE IN REPOLARIZATION AND RELAXATION TO ACETYLCHOLINE IN THE RABBIT ISOLATED CAROTID ARTERY

F. Plane, S. Najibi[1], R. A. Cohen[1] & C.J. Garland, Dept. of Pharmacology, University of Bristol, Bristol, BS8 1TD and [1] Vascular Biology Unit, Boston University Medical Center, 88E Newton St., Boston 02118, USA.

Endothelium-derived nitric oxide (NO) activates calcium-dependent potassium channels (KCa) in smooth muscle cells from large arteries (Bolotina *et al.*, 1994). However, the contribution this action makes to relaxation is unclear. In small resistance vessels, hyperpolarization makes a major contribution to the endothelium-dependent relaxation but is not apparently mediated by NO (Garland *et al.*, 1995). In this study, the contribution of NO to acetylcholine (ACh)-evoked repolarization and the importance of this response for relaxation was studied in the rabbit carotid artery, by simultaneous recording of tension and membrane potential.

New Zealand White rabbits of either sex were anaesthetised (sodium pentobarbitone, 60 mg kg^{-1}) and killed by rapid exsanguination. Segments of the carotid artery (2 mm in length) were mounted in a myograph under a normalised tension (Mulvany and Halpern, 1977), and superfused at 7-8 ml min^{-1} with oxygenated Krebs buffer at 37°C, containing indomethacin (1 μM). All data is expressed as mean ± s.e. mean. Differences between mean values was calculated using the Students t-test.

The resting membrane potential of the smooth muscle cells was -57.1 ± 5.3 mV (n=50 cells from 17 tissues). Phenylephrine (PE; 1 μM) evoked depolarization and contraction of the arterial segments which was concentration-dependently reversed by ACh (0.03-10 μM). The maximum changes in membrane potential and tension to ACh (10 μM) were 98.5 ± 2.6 % and 98.8 ± 1.3 % (n=4), respectively, and the pD_2 values for the two components of the response were 6.85 ± 0.04 and 6.71 ± 0.02, respectively (n=4). Exposure to the NO-synthase (NOS) inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME; 30 μM; 20 mins), inhibited the repolarization and relaxation to a similar extent reducing the maximum responses to 63.8 ± 4.7 % and 70.8 ± 8.1 %, respectively (n=5; $p<0.01$). Additional exposure to N^G-Nitro-L-arginine (L-NOARG; 300 μM; 30 mins), further reduced the maximum changes in membrane potential and tension to 18.4 ± 2.0 % and 29.3 ± 5.0 %, respectively (n=4; $p<0.01$). Pre-incubation with charybdotoxin (50 nM; 10 mins), an inhibitor of KCa attenuated repolarization and relaxation to low concentrations of ACh, but the maximum responses were not significantly reduced (n=4; $p>0.05$). However, exposure to charybdotoxin and L-NAME together abolished both repolarization and relaxation to ACh (n=4; $p<0.01$). Parallel measurements of ACh-stimulated nitrite release by chemiluminescence under control conditions, with L-NAME or with additional L-NOARG were 165 ± 35, 99 ± 16 and 55 ± 14 $pmol/cm^2$ endothelial surface area, respectively. Arterial cyclic GMP contents following ACh stimulation were 19.0 ± 3.0, 0.7 ± 0.2, and 0.2 ± 0.1 fmol/μg protein, respectively.

The very close correlation between repolarization and relaxation to ACh, and the sensitivity of both components of the response to NOS inhibitors, suggests that both are mediated by NO in the carotid artery. This is confirmed by measurements of NO release which correlated well with both repolarization and relaxation. In the presence of the NOS inhibitors, relaxations occurred without a rise in cyclic GMP, conditions under which NO can relax by activating KCa directly and hyperpolarizing the smooth muscle cell membrane.

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78P COMPARISON OF THE ABILITY OF HUMAN, RAT AND RABBIT VESSELS TO PRODUCE NITRIC OXIDE AND PROSTANOIDS IN RESPONSE TO LPS

David Bishop-Bailey, Simon W. Larkin, *John R. Pepper, *Magdi Yacoub, Timothy J. Williams and Jane A. Mitchell
*Department of Applied Pharmacology, The National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, *Department of Surgery, The Royal Brompton NHL Hospital, Sydney Street, London.*

The release of nitric oxide (NO) and prostacyclin (PGI_2) is regulated by constitutive isoforms of NO synthase (NOS) and cyclo-oxygenase (COX; COX-1) respectively. However, certain cytokines, growth factors or bacterial lipopolysaccharide (LPS) can also induce novel isoforms of both NOS (iNOS) and COX (COX-2), (see Mitchell et al., 1995). An altered production of these mediators is associated with vascular diseases. Using isolated segments of rat and rabbit aorta, human saphenous vein (SV), and internal mammary artery (IMA) obtained from patients undergoing bypass surgery we have investigated the effect of LPS on the release of NO and prostanoids.

Rats or rabbits were killed by overdose of sodium pentobarbitone, and their aortas immediately removed. Vessels were collected, and under sterile conditions washed and dissected under sterile phosphate buffered saline (PBS) containing penicillin (1000 IU.ml^{-1}) and streptomycin (1 mg.ml^{-1}; Pen-Strep). Organ culture methods were as previously described (Bishop-Bailey et al., 1995). Briefly, tissue rings of approximately equal weight (2-5 mm wide) were placed in separate wells of a 48 well culture plate containing 500 μl of DMEM supplemented with 2 mM glutamine, Pen-Strep, and 10% foetal calf serum (37°C; 5% CO_2; 95% air). After 1h the medium was replaced and LPS (10μg.ml^{-1}) added. At 48h the medium was removed. NO was measured as total nitrite concentration (NOx), by the Greiss reaction after conversion of all nitrate to nitrite (Schmidt et al. 1992). Prostanoid concentration was measured by radioimmunoassay (Mitchell et al., 1993). At the end of the experiment tissues were blotted dry and weighed. LPS caused an increase in the release of NO by rat aorta, however NOx was undetectable (ND) in incubations of rabbit aorta, SV, or IMA. LPS did not increase prostanoid release from segments of rat aorta in this study, but did when smaller pieces were used (Bishop-Bailey et al., this meeting). However in incubations of SV, IMA or rabbit aorta LPS induced the release of 6-keto $PGF_{1\alpha}$ and in the SV and IMA, PGE_2 and TXB_2 (Table1).

Here we show differential induction of NO and prostanoid release by LPS in rat, rabbit aorta, and human SV and IMA. These increases in NO or prostanoid production are probably due to an induction of iNOS and COX-2/ phospholipase A_2 enzymes. The induction of iNOS in IMA (Mitchell et al., 1995) is clearly less than seen in rat aorta, and too low to be detected by the Greiss reaction. The use of rat models to study iNOS may misrepresent the relative contribution of NO to pathologies when compared to human or other species.

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Table1	Rat Aorta		Rabbit	Aorta	Saphenous	Vein	Internal Mammary	Artery
Protocol	Vehicle	LPS	Vehicle	LPS	Vehicle	LPS	Vehicle	
NOx	0.9 ± 0.6	4.4 ± 0.6 *	ND	ND	ND	ND	ND	ND
6-keto $PGF_{1\alpha}$	4.2 ± 0.6	6.3 ± 1.4	9.4 ± 3.3	25.5 ± 8.6 *	11.9 ± 1.5	46.1 ± 12.8 *	6.4 ± 1.5	18.7 ± 4.2 *
PGE_2	26.8 ± 5.7	36.9 ± 9.6	15.8 ± 5.2	26.4 ± 5.9	11.3 ± 2.8	57.6 ± 13.5 *	8.3 ± 2.4	18.2 ± 4.8 *
TXB_2	0.6 ± 0.2	1.0 ± 0.0	1.0 ± 0.3	1.5 ± 0.5	2.4 ± 1.1	5.8 ± 2.3 *	0.8 ± 2.1	2.1 ± 0.7 *

*Table 1: Release (mean ± s.e.mean) of NOx (nmols.ml-1.mg-1; 48h) and prostanoids (ng.ml-1.mg-1;48 h) from rat aorta (n=6; 8.1±0.6mg), rabbit aorta (n=6-7; 11±0.6mg), SV (n=7-8; 11±0.6mg), and IMA (n=8; 12.8±0.8mg). *=p<0.05 Wilcoxon between vehicle and LPS.*

79P INDUCIBLE ISOFORMS OF NOS AND COX CONTRIBUTE TO THE SUSTAINED RELEASE OF NO AND PGE_2 BY SEGMENTS OF RAT AORTA EXPOSED TO LPS IN ORGAN CULTURE

David Bishop-Bailey, Simon W. Larkin, Timothy J. Williams and Jane A. Mitchell
Department of Applied Pharmacology, The National Heart and Lung Institute, Dovehouse Street, London SW3 6LY.

Certain cytokines, growth factors or bacterial lipopolysaccharide (LPS) can induce inflammatory isoforms of nitric oxide (NO) synthase NOS (iNOS), and cyclo-oxygenase (COX; COX-2) (see Mitchell et al., 1995) in vascular smooth muscle cells (Inoue et al., 1993). Using an organ culture system we have characterised this co-induction of iNOS and COX-2 in segments of rat aorta.

Male Sprague-Drawley rats (250-300g) were killed by overdose of sodium pentobarbitone (350mg.kg-1; i.p.), and the aortas immediately removed. Under sterile conditions, vessels were washed and dissected in PBS containing penicillin (1000 $IU.ml^{-1}$) and streptomycin (1 $mg.ml^{-1}$; Pen-Strep). Ring segments of approximately 2-3 mg wet weight were incubated in 500 µl DMEM containing 2mM glutamine, Pen-Strep, and 10% foetal calf serum (37°C; 5% CO_2; 95% air). After 1h the medium was replaced, and LPS ($10\mu g.ml^{-1}$) or drugs added. Every 24h the medium was removed and fresh medium and drugs were added. At days 1,2,3 and 8 NO was measured as total nitrite concentration (NOx), determined by the Greiss reaction after conversion of all nitrate to nitrite (Schmidt et al. 1992). Prostanoid concentrations were measured by radioimmunoassay (Mitchell *et al.*, 1993). At the end of the experiment tissues were blotted dry and weighed.

LPS caused a co-release of NO and 6-keto prostaglandin $(PG)F_{1\alpha}$ (6k), and PGE_2, but not thromboxane $(TX)B_2$, peaking over 1-2 days (Table 1). Over this initial peak (day2) NOx or 6-keto $PGF_{1\alpha}$ release was significantly ($p<0.05$) reduced by 63±8% for NOx, and 80±4% for 6k by dexamethasone (1µM), and 100% for NOx and 85±3% for 6k by cycloheximide (1µM). Similarly NOx was inhibited by 55±9% with N^G-nitro-L-arginine methyl ester (L-NAME 1mM; 6k increased by 10±23%). Furthermore, 6k but not NOx was inhibited by 80±12% with indomethacin (30µM; NOx 13±16%) or by 88±1% with the selective COX-2 inhibitor NS-398 (Futaki et al.,1994; 30µM; NOx 23±19%).

Table 1		DAY 1	2	3	8
NOx	veh	1.3±0.4	4.4±1.3	2.7±1.2	2.2±0.6
	LPS	4.5±0.8*	9.2±1.5*	5.5±1.1*	5.8±1.4*
6k	veh	22.5±4.3	25.5±3.5	9.3±2.8	5.3±1.1
	LPS	47.1±9.1*	55.8±12.5*	10.1±1.1	12.5±0.6*
PGE_2	veh	23.9±7.3	50.0±9.3	20.6±6.8	28.1±5.7
	LPS	55.6±11.1*	60.5±5.8	14.5±2.1	47.3±3.0*
TXB_2	veh	1.8±0.3	6.1±0.7	3.6±0.4	1.7±0.1
	LPS	2.6±0.3	6.1±0.3	3.1±0.3	1.3±0.1

*Table 1: Release (mean ± s.e.mean) of NOx ($pmols.ml^{-1}.mg^{-1}$; 24h) and prostanoids ($ng.ml^{-1}.mg^{-1}$; 24h) from rat aorta (n=8). *=$p<0.05$ between vehicle and LPS (unpaired t-Test).*

Here we show that LPS induces the iNOS and COX-2 pathways in isolated segments of rat aorta in organ culture. In contrast to some preparations (see Mitchell et al., 1995), at a time of peak mediator release, we found little evidence for interactions between NO and prostanoid pathways. Furthermore, unlike observations in isolated cells (Inoue et al., 1993), NOx and PGE_2 release remained elevated for at least 8 days. In contrast to both NOx and PGE_2, the release of 6k peaked on day 2, suggesting an exhaustion or inhibition of PGI_2 synthetase. We suggest that the use of intact vessels in organ culture, rather than isolated cells, represents a more physiological system, and may better reflect changes occuring *in vivo* in diseases such as septic shock or atherosclerosis.

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80P COMPARISON OF THE EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AS INHIBITORS OF COX-2 METABOLITES DERIVED FROM ENDOGENOUS VERSUS EXOGENOUS ARACHIDONIC ACID

M.A Saunders, M.G. Belvisi, P.J. Barnes *T.J. Williams and *J.A. Mitchell. Department of Thoracic Medicine and *Applied Pharmacology, National Heart & Lung Institute, Dovehouse Street, London SW3 6LY

Arachidonic acid (AA) is liberated from membrane phopholipids by phospholipase A_2 (PLA_2) and is subsequently metabolised by the enzyme cyclooxygenase (COX) to form prostanoids. The inducible isoform of COX, COX-2, is thought to be responsible for the production of prostanoids at the site of inflammation (see Mitchell *et al.*, 1995). Acetyl salicylic acid (ASA; aspirin) directly inhibits COX-2 activity *in vitro* by acetylation of the essential 530 serine residue (Mitchell *et al.*,1995). ASA is rapidly deacetylated to salicylic acid (SA), which is thought to be its active metabolite *in vivo*. However, SA is a much weaker inhibitor than ASA of COX activity *in vitro* (Vane, 1971; Mitchell *et al.*, 1993). In an attempt to understand the mechanism of action of SA on prostaglandin (PG) release we have used the human epithelial cell line A549 which is known to express COX-2 in response to IL-1β (Mitchell *et al.*, 1994).

Protocols were designed to determine the relative contribution of PLA_2/COX (endogenous AA) or COX alone (exogenous AA) in the release of PG in response to IL-1β. A549 cells were cultured in 96 well plates in medium containing 2mM calcium, unless otherwise stated, according to Mitchell *et al.*, (1993). The effect of drugs on PGE_2 release was measured (by radioimmunoassay; Mitchell *et al.*,1993) in three protocols. Protocol I, cells were exposed to NSAIDs and IL-1β for 24 hours before the measurement of PGE_2; protocol II, in the same cells, fresh medium containing 30µM AA was added for 15 min and PGE_2 measured. Protocol III, IL-1β (10ng/ml) was added to cells for 24 hours, fresh medium containing NSAIDs was then added for 30 minutes followed by the addition of 30µM AA for 15 minutes. Using protocol I, the effect of different concentrations of exogenous calcium (0.2-2 mM) on IL-1β-induced PGE_2 release was determined. In separate experiments cells were cultured on 6 well plates and treated as described in protocol I, cells were extracted and western blot analysis performed using a specific COX-2 antibody (Mitchell *et al.*,1994). ASA, 5-amino salicylic acid (5ASA), indomethacin and flurbiprofen inhibited the release of PGE_2 in all three protocols (table 1). In contrast SA had a much weaker direct effect on COX-2 activity (protocol III) or on PGE_2 formed from exogenous AA after 24 hour exposure to the drug (protocol II). However, SA completely inhibited PGE_2 formed from endogenous AA (protocol I), (table 1). Western blots indicated that none of the NSAIDs tested decreased COX-2 protein levels at concentrations that maximally inhibit PGE_2. IL-1β-induced PGE_2 release was comparable in the presence of 0.2mM calcium (33.39 ±2.63), 1mM calcium (40.12±0.78) or 2mM calcium (47.01±17.46)(n=3) suggesting the involvement of cytosolic PLA_2 and not secretory PLA_2.

NSAIDs	Protocol 1	Protocol 2	Protocol 3
ASA (aspirin)	0.072	0.142	1.67
5ASA	28.99	146.16	49.42
Indomethacin	0.0044	0.0035	0.027
Flurbiprofen	0.017	1.145	0.220
SA	5.80	>1000	*1000

*Table 1. ED_{50} values (ug/ml) (n=10-22 individual wells from 4-5 experimental days), * denotes ED_{48} value*

All NSAIDs tested were potent inhibitors of PGE_2 release from endogenous stores of AA (protocol I), when both COX-2 and PLA_2 are involved. When exogenous AA was added, by-passing the PLA_2 step, all drugs tested, except indomethacin, were less potent. Moreover, SA was inactive in the presence of AA in protocol II and produced only a maximum inhibition of 48% in protocol III, suggesting that SA inhibits COX-2 metabolites by interfering with the availability of AA, possibly by inhibiting cytosolic PLA_2 activity or induction. Alternatively SA maybe more active against the relatively low levels of endogenous substrate, compared with that added exogenously in this study.

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