302P α_1 ADRENOCEPTOR PROFILE IN HUMAN SKJELETAL MUSCLE RESISTANCE ARTERIES IN CRITICAL LIMB ISCHAEMIA

Y.P.R. Jarajapu, J.C. McGrath*, A. MacDonald & C. Hillier. Vascular Assessment Group, School of Biological & Biomedi-cal Sciences, Glasgow Caledonian University, 70 Cowcaddens Rd., Glasgow G4 0BA. *Autonomic Physiology Unit, Glasgow University.

Recently, we reported hyper-responsiveness of human skeletal muscle resistance arteries (SkMRAs) to noradrenaline (NA) in critical limb ischaemia (CLI) (Jarajapu *et al.*, 2001a). In this study the α_1-adrenoceptor profile was characterised using the antagonists prazosin (α_1-selective), 5-methyl-urapidil (5MU, α_{1A}-selective) and BMY7378 (α_{1D}-selective) and the preferential α_{1B}- alkylating agent, chloroethyl-clonidine (CEC) (Hancock, 1996).

SkMRAs (normalised diameter $L_{0.9}$ = 312±10 μm, n=43/19 no. of segments/no. of patients) were isolated from ischaemic areas of limbs amputated for CLI. Arterial segments were mounted on a small vessel wire myograph in physiological saline solution at 37°C continuously bubbled with carbogen and normalised according to Mulvany & Halpern (1977). Propranolol (1 μM), corticosterone (3 μM), cocaine (3 μM) and RS79948 (0.1 μM) (α_2- selective antagonist) were present throughout the experimental protocol. Arterial segments were activated by 123 mM KCl twice and 10 μM NA. Cumulative concentration response curves (CRCs) to the agonists NA and A-61603 (α_{1A}- selective, Knepper *et al.*, 1995) were obtained in the absence and presence of the antagonists. Arterial segments were washed for 60 min after 30 min incubation with CEC (Hancock, 1996).

Prazosin (0.1 μM) and 5MU (0.3 μM) competitively antagonised the responses to NA giving pK_B values of 9.56 (n=4) and 8.35 (n=8), respectively. CEC (10 μM) decreased the maximum responses to NA and A-61603 to 57% ($p<0.05$, n=5) and 72% ($p<0.05$, n=3) of the control, respectively. The sensitivity of NA-mediated responses to CEC was significantly higher than that of A-61603 ($p<0.05$). The potency of NA was poorly shifted by 1 μM BMY7378, giving a pK_B value of 7.05±0.3 (n=4). Baseline tension was not affected by 5MU and CEC during the incubation period of 30 min. The results obtained with the antagonists were almost similar to those obtained in non-ischaemic arteries (Jarajapu *et al.*, 2001b).

Our earlier studies in the non-ischaemic arteries showed that the α_{1A}-adrenoceptor subtype predominantly mediates contractile responses to NA. Results from the present study suggest that the α_1-adrenoceptor profile in human SkMRAs is preserved in ischaemic conditions. A functional contribution of the α_{1B}-adrenoceptor subtype to NA-mediated responses in the ischaemic arteries cannot be ruled out.

Hancock, A.A. (1996). *Drug Develop. Res.* 39, 54-107.

Jarajapu, Y.P.R., Coats, P., McGrath, J.C. *et al.* (2001a). *Cardiovasc.Res.* 49, 218-225.

Jarajapu, Y.P.R., Coats, P., McGrath, J.C. *et al.* (2001b). *Br. J. Pharmacol.* 133, 679-686.

Knepper, S.M., Buckner, S.A., Brune, M.E. et al. (1995). *J. Pharmacol. Exp. Ther.* 274, 97-103.

Mulvany, M.J. & Halpern, W. (1977). *Circ. Res.* 41, 19-26.

303P QUANTITATIVE IMAGING OF QAPB-ASSOCIATED FLUORESCENCE IN SMOOTH MUSCLE CELLS FROM HUMAN SKELETAL MUSCLE RESISTANCE ARTERIES

Y.P.R. Jarajapu, A. MacDonald, C. Hillier, J.C. McGrath* J.F. Mackenzie* & C. Daly*. Vascular Assessment Unit, School of Biological & Biomedical Sciences, Glasgow Caledonian University, 70 Cowcaddens Rd., Glasgow G4 0BA. *Autonomic Physiology Unit, Glasgow University.

In this study the quantitative imaging of quinazolinyl piperazino borate-dipyrromethene (QAPB) -associated fluorescence in freshly dissociated smooth muscle cells (SMCs) from human skeletal muscle resistance arteries (SkMRAs) was performed using confocal laser scanning microscopy. The technique was validated by determining the affinity of QAPB to α_1-adrenoceptors and the sensitivity of fluorescence binding to inhibition by 5-methyl-urapidil (α_{1A}-selective antagonist, 5MU).

SkMRAs were isolated from the non-ischaemic areas of limbs amputated for critical limb ischaemia (CLI). SMCs were dissociated according to Kamishima and McCarron (1997) and plated on a cover slip. A Noran Odyssey real-time laser scanning confocal microscope with a Nikon Diaphot (inverted) microscope was used for fluorescence imaging of the cells and the fluorescence was measured over time using MetaMorph software (Universal Imaging). Initially baseline fluorescence was obtained followed by cumulative addition (0.4 – 10 nM) of the QAPB. Non-specific binding was defined as fluorescence remaining in the presence of 10 μM phentolamine. In the experiments with 5MU the SMCs on the cover slips were treated with the antagonist for 20 min before imaging and the antagonist was present throughout the imaging process.

No background fluorescence was observed during incubation with QAPB (0.4 - 20 nM). QAPB-associated fluorescence binding was concentration-dependent (Fig. 1) and saturable with an affinity (pFK_D, pK_D of a fluorescent ligand) of 8.4±0.8 (n=5). This is comparable with the pA_2 value of 8.0±0.3 (n=3) obtained from functional studies and also with the affinity values observed in rat fibroblasts expressing α_1-adrenoceptor subtypes (Mackenzie *et al.*, 2000). In the presence of 10 μM phentolamine, fluorescence binding in the working concentration range of QAPB was almost completely abolished showing the specificity of the binding. 5MU (3 nM) antagonised the fluorescence binding and significantly decreased the area under the curve of concentration-fluorescence curves (control: 422±77; 5MU: 127±70 sq units, $p<0.05$, n=4).

These results show the feasibility of characterising α_1-adrenoceptors in freshly dissociated, live SMCs from human SkMRAs.

Kamishima, T. & McCarron, J.G. (1997). *J. Physiol.* 501, 497-508.

Mackenzie, J.F., Daly, C.J., Pediani, J.D. *et al. J. Pharmacol. Exp. Ther.* (2000). 294, 434-443.

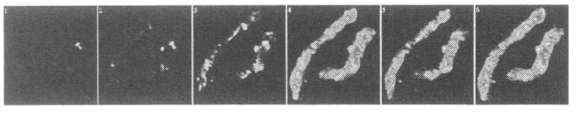


Fig. 1. QAPB (0, 0.4, 1, 2, 5 and 10 nM)-associated fluorescence binding in SMCs from human SkMRAs

Acknowledgements: VASCAN project, EC.

304P TAIL ARTERIES FROM D79N MICE SHOW REDUCED RESPONSES TO UK14304 COMPARED WITH WILD TYPE

M. McBride, C.J. Daly & J.C. McGrath, Division of Neuroscience & Biomedical Systems, IBLS, University of Glasgow, Glasgow, G12 8QQ

The aim was to analyse the putative vasoconstriction by the α_2-adrenoceptor-selective agonist UK14304 in mouse tail artery *in vitro*. In previous studies of isolated blood vessels, α_2-adrenoceptor-mediated contraction often required the presence of a synergist or suppression of nitric oxide synthesis (Templeton et al, 1991; Gordon et al, 1992). The present study investigated whether these factors also applied in mouse tail artery. This allowed the application of transgenic technology to the study of post-junctional α_2-adrenoceptors, in this instance using the D79N mutant of the $\alpha_{2A/D}$-adrenoceptor.

Tail arteries from four month old wild type and D79N mice were cut into 2mm rings. These were mounted in Krebs at 37°C on a Mulvany/Halpern myograph. Cumulative concentration response curves to UK14304 were constructed (10^{-9}-3 x 10^{-5}M) with or without L-NAME, an NO synthase inhibitor (10^{-4}M)(30 min) and/or pre-constriction with U46619 (U19).

Concentration-related contractions to UK14304 had similar EC_{50} in both strains but an increased maximum response in wild type. At a concentration of U46619 eliciting contraction comparable to 50% of the noradrenaline maximum, responses to UK14304 in both strains were substantially potentiated in both pEC_{50} and maximum. However the maximum responses in wild type remained approximately double those in D79N. At a lower concentration of 3 x 10^{-9}M, U46619 produced potentiation of pEC_{50} in both strains and a significant increase in maximum only in wild type.

L-NAME (10^{-4}M) produced an increase in maximum and EC_{50} in both strains. Combining U46619 and L-NAME produced no further potentiation than did L-NAME alone.

Elevation of vascular tone or inhibition of NO production potentiates contractile responses to UK14304 to an extent allowing pharmacological analysis *in vitro*. Arteries from wild type mice consistently exhibited greater responses than did those from D79N, suggesting that α_{2A}-adrenoceptors contribute to UK14304-induced contractions in the mouse tail artery.

	Wild Type		D79N	
	Max (g)	pEC_{50}	Max (g)	pEC_{50}
Controls	0.11† ±0.05	5.9 ±1.03	0.04† ± 0.02	5.7 ± 0.01
U19(50% of NA)	0.39*†±0.08	8.2*±0.17	0.21*† ± 0.04	8.1*± 0.22
U19($3x10^{-9}$M) +L-NAME	0.20* ±0.02	6.6*±0.10	0.12 ± 0.02	7.2*± 0.23

* P<0.05; significant difference between drug treatments.
† P<0.05; significant difference between each strain.

Table 1: Effects of preconstriction or NOS inhibition on contractions to UK14304.

Templeton, A.G.B., McGrath, J.C. & Whittle, M.J. (1991) *Br. J. Pharmacol.* 103 1079-1084.

Gordon, J.F., Baird, M., Daly, C.J. & McGrath, J.C. (1992) *J. Cardiovasc. Pharmacol.* 20 S68-S71.

Supported by BHF. The group is a member of the EC FP5 project 'Vascan' (QLG1-CT-1999-00084)

305P EFFECTS OF GW7845, A POTENT, SELECTIVE PPAR (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR)-GAMMA AGONIST, ON BARRIER FUNCTION OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

K.W. Buchan, C.J. Campbell, P. Houston and V.J. Ludbrook. GlaxoSmithKline, Gunnels Wood Road, Stevenage, Herts, UK

PPARγ agonists increase oedema in some patients. This may result from an effect on endothelial barrier function (EBF). We tested this *in vitro*, using GW7845, a selective PPARγ agonist (Willson *et al.*, 2000). EBF was measured using transfer of FITC-dextran (70 kDa ;F-D) (2h) or trans-endothelial electrical resistance (TEER), using HUVEC cultured in transwells. TNF (26h) or thrombin (THR, 2h) each enhanced transfer of F-D, relative to control (Table 1).

Table 1. % FITC-dextran transfer (control =100%); n =4-5

GW7845	Agonist	Agonist + GW7845
Control 100	TNF 1ng/ml 222±44*	TNF 1ng/ml 210±68*
1nM 98.9±12.6	TNF 10ng/ml 306±62*	TNF 10ng/ml 296±54*
10nM 68.2±6.1*	THR 0.01U/ml 223±35**	THR 0.01U/ml 240±34**
100nM 63.0±5.1**	THR 0.1U/ml 256±50*	THR 0.1U/ml 297±54**
1μM 72.5±5.9*		

GW7845 (26h; 1nM-1μM) inhibited the transfer of F-D (Table 1), but had no significant effect (at1μM) on TNF (26h) or THR (2h)-induced F-D transfer (p>0.05).

Table 2:Change in TEER(Ω)relative to vehicle response;n=4-5

GW7845 (24h)	VEGF (24h)	VEGF+GW7845 (1μM) (24h)
1nM -0.38±0.99	1pM 0.42±0.62	1pM 1.43±0.72
10nM -0.63±0.72	10pM -0.22±0.68	10pM 0.08±0.11
100nM 0.29±0.49	100pM -3.9±1.08*	100pM -4.13±0.60***
1μM -0.67±1.11	1nM -7.65 ±0.74***	Not determined

Resting TEER was 29.9 ± 2.7Ω (n=5). GW7845 (24h) had no effect (p>0.05) on EBF, as measured by TEER (Table 2). In contrast, hVEGF(165) (24h) induced a concentration–dependent fall in TEER (Table 2). However, co-addition of GW7845 (24h;1μM) with VEGF had no significant effect on the VEGF response (p>0.05)(Table 2). We conclude that activation of PPARγ does not reduce EBF in HUVEC, or modulate the effects of the tested inflammatory agents. In contrast, GW7845 inhibits the transfer of a high molecular weight marker, under certain conditions, suggesting the potential to enhance the EBF of some vascular beds.

Statistical analysis: *, **, *** indicates p<0.05, 0.01 or 0.001, relative to control (paired t-tests). Data are mean ± sem

Willson, T.M., Brown, P.J., Sternbach, D.D. & Henke, B. (2000) J.Med Chem., 43(4), 527-550.

306P THE EFFECTS OF LIPOPOLYSACCHARIDE ON LEVCROMAKALIM- AND FORSKOLIN- INDUCED RELAXATION IN ENDOTHELIUM-DENUDED RAT AORTA

Andrew J. Wilson[1], Jonathan Alis[1,2], Mervyn Singer[2] & Lucie H. Clapp[1]. [1]Centre for Clinical Pharmacology, & [2]Bloomsbury Institute for Intensive Care Medicine, UCL, London, UK.

The targets of nitric oxide produced by iNOS have not been fully characterised, although there is convincing evidence that vascular smooth muscle ATP-sensitive K^+ (K_{ATP}) channels are involved (Clapp & Tinker, 1998). In the present study, we investigated whether NO generated from iNOS modulates K_{ATP} channel pharmacology in endothelium-denuded rat aorta.

Male Sprague-Dawley rats (250-300g) were humanely killed by cervical dislocation and the thoracic aorta removed and dissected. Aortic rings were mounted under 1g isometric tension in a 25ml organ bath containing physiological saline solution, and incubated in the absence and presence of *S. typhosa* lipopolysaccharide (LPS; 1 μg ml^{-1}; 4 hrs). Rings were contracted with phenylephrine (PE, 1 μM), and preincubated for 15 mins with an inhibitor of K_{ATP} channels. Data are expressed as mean±s.e.m. Differences were calculated using ANOVA with Bonferroni correction.

Application of levcromakalim (10^{-9}–10^{-5} M) produced a concentration-dependent relaxation of PE-induced tone, with a pD_{50} value of 6.61±0.03 and a maximal response (E_{max}) of 47.4±4%. Preincubation with LPS caused a significant leftward shift in the pD_{50} to 7.05±0.07 and potentiated the E_{max} to 84.3±3% ($P<0.005$, n=12). The magnitude of the PE-induced contractions were not significantly affected by LPS, being 1.97±0.1g and 2.38±0.2g in the presence and absence of LPS respectively (n=13). Moreover, potentiation was reversed by application of the selective iNOS inhibitor, 1400W (10μM) which shifted the pD_{50} value for levcromakalim from 7.18 ± 0.05 to 6.70±0.03 and E_{max} from 89.3±2.8% to 67.2±3%, which were not significantly different from those observed in control tissues (pD_{50} and E_{max} being 6.71±0.05 and 60.1±2%, respectively; n=5). Under control conditions, the K_{ATP} channel inhibitors, glibenclamide (10 μM), and PNU-37883A (1 μM) virtually abolished relaxation to levcromakalim, reducing E_{max} to 15.3±8% and 7.1±2% respectively (n=5). However, in LPS-treated tissues, these drugs appeared less effective, with the E_{max} increasing to 52.7±13% and 31.6±7% for glibenclamide and PNU-37883A respectively (n=5). Preincubation of tissues with 1400W (10 μM) however, completely restored the effect of glibenclamide (E_{max} 13.3±0.7%, n=3).

Relaxation induced by forskolin (10^{-10}-10^{-5} M) was similarly potentiated by LPS, with the pD_{50} shifting from 6.89±0.2 to 7.93±0.1 in the absence and presence of LPS respectively ($P<0.001$, n=6). Under control conditions, neither glibenclamide, nor PNU-37883A had any significant effect on the forskolin responses. However, following incubation with LPS, PNU-37883A did significantly inhibit relaxation, shifting the pD_{50} to 7.23±0.1, ($P<0.05$, n=6), which was not significantly different from the pD_{50} value obtained under control conditions ($P=0.08$). Glibenclamide was without effect.

These results suggest that LPS can modulate the pharmacology of K_{ATP} channels *via* production of NO. Whether this effect is related to altered affinity of the drugs for the sulphonylurea receptor and/or the pore remains to be determined.

Clapp, L. & Tinker, A. (1998). *Curr. Opin. Nephrol. Hypertens.*,7:91-8.

Supported by the Medical Research Council UK.

307P IDENTIFICATION OF TWIN-PORE POTASSIUM CHANNELS IN RAT MESENTERIC ARTERIES

M.J. Gardener, M.P. Burnham, K. E. Gilling, I.T. Johnson, G. Edwards & A.H. Weston. School of Biological Sciences, University of Manchester, G.38 Stopford Building, Oxford Road, Manchester, M13 9PT, UK

Two-pore potassium channels functionally exist as dimers with each subunit containing four transmembrane domains and two pore-forming regions which are thought to combine to form the pores. To date seventeen gene products (KCNK1-17) relating to eleven functional channels are known and these are divided into family groups according to their electro-physiological and pharmacological properties. Reports of these channels in the vasculature are limited to the presence of TWIK-1 in mouse aorta (Arrighi *et al.*, 1998) and TASK-4 in human aorta (Decher *et al.*, 2001). Here we report for the first time the presence of two-pore K^+ channels in resistance arteries from the rat.

Male Sprague-Dawley rats (150-200g) were killed by stunning and cervical dislocation. *RT-PCR*:- Rat second and third order mesenteric arteries were homogenised and total RNA isolated using the RNeasy mini kit (Qiagen). Samples were subsequently DNase-treated and reverse transcribed. Gene-specific PCR was carried out using primers designed against known sequences of the two-pore K^+ channels. *Immunohistochemistry*:- Rat mesenteric arteries were fixed in a periodate-lysine-paraformaldehyde solution and embedded in OCT® compound. Sections (4μm) were subsequently stained using commercially-available anti-two-pore K^+ channel antibodies (Alomone) and visualised using Texas Red-conjugated secondary antibodies; nuclei were labelled blue with DAPI.

Amplicons relating to the two-pore channels TASK-1, TASK-2, TREK-1, TWIK-1 and THIK-1 were present in mesenteric artery samples (N=2). Negative results using primers for TASK-3, TREK-2 and TRAAK were obtained in mesenteric artery cDNA samples although amplicons were seen using these primers with cDNA isolated from rat brain.

Immunohistochemical staining of mesenteric artery sections showed TASK-1, TASK-2, TWIK-2 & TREK-1 were present N=3). TASK-1 and TWIK-2 were abundant in both the smooth muscle and the endothelium, with TASK-2 predominantly localised to the smooth muscle. Immunolabelling of TREK-1 showed that the channel was associated with the nuclei of both the smooth muscle and endothelial cells.

The functional role of these channels in the cardiovascular system remains unclear. However, there is electrophysiological and pharmacological evidence that they may be cardioprotective in ischaemia (Aimond *et al.*, 2000) and may also function as mechano- (Maingret *et al.*, 1999) and O_2-sensors (Buckler *et al.*, 2000). Pharmacologically TASK-1 is modulated by vasoactive compounds such as anandamide (Maingret *et al.*, 2001) and angiotensin II (Czirjak *et al.*, 2001). These channels may thus represent novel targets for the pharmacological modulation of vessel tone.

Aimond, F. *et al.* (2000) *J. Biol. Chem.* **275**, 39110-39116.
Arrighi I. *et al.* (1998) *FEBS Lett.* **425**, 310-316.
Buckler, K.J. *et al.* (2000) *J. Physiol.* **525**, 135-142.
Czirjak, G. *et al.* (2001) *Mol. Endocrinol.* **14**, 863-874.
Decher, N. *et al.* (2001) *FEBS. Lett.* **492**, 84-89.
Maingret, F. *et al.* (1999) *J. Biol. Chem.* **274**, 26691-26696.

308P ANTIOXIDANTS ATTENUATE EDHF-MEDIATED VASODILATATION IN THE BOVINE ISOLATED PERFUSED EYE

A.J. McNeish, W.S. Wilson & W. Martin. Institute of Biomedical and Life Sciences, West Medical Building, University of Glasgow, Glasgow, G12 8QQ.

Acetylcholine (ACh)-induced vasodilatation in the ciliary vascular bed of the bovine eye is mediated by endothelium-derived hyperpolarizing factor (EDHF) and is powerfully inhibited by ascorbate (McNeish *et al.*, 2001). In this study we have sought to determine if other antioxidants share the ability of ascorbate to inhibit EDHF-mediated vasodilatation.

Bovine eyes, obtained from a local abattoir, were perfused according to the constant flow method of Wilson *et al.* (1993). Briefly, eyes were cannulated through a long posterior ciliary artery and perfused at 2.5 ml min^{-1} at 37°C with Krebs solution gassed with O_2 containing 5% CO_2. Perfusion pressure was raised (to ~120 mmHg) with the thromboxane A_2-mimetic, U46619 (300 nM), and vasodilator responses to bolus doses of ACh (10 nmol), injected immediately proximal to the cannulae, were elicited every 15 min. Once vasodilator responses to ACh had stabilised, a number of antioxidants were infused continuously and their effects monitored in the ensuing 120 min. Vasodilator responses are given as % reduction of U46619-induced perfusion pressure and vasoconstrictor responses are given in mmHg. Data are expressed as mean ± s.e. mean, n≥6. Differences were determined using an unpaired *t*-test.

ACh (10 nmol) induced powerful vasodilator responses (Table 1), which remained constant for 120 min. Treatment with ascorbate (50 μM) produced a time-dependent fall in ACh-induced vasodilatation during the 120 min period and uncovered a normally masked vasoconstrictor response. This action of ascorbate was not mimicked by dehydroascorbate (50 μM), superoxide dismutase (SOD, 250 units ml^{-1}), catalase (1250 units ml^{-1}), or glutathione (1 mM). In contrast, dithiothreitol (DTT, 100 μM) and N-acetyl-L-cysteine (NAC, 1 mM), produced time-dependent falls in ACh-induced vasodilatation and uncovered a vasoconstrictor response.

Table 1. Responses to ACh (10 nmol) following 120 min treatment with a range of antioxidants (*P<0.05, **P<0.01 and ***P<0.001 indicate significant differences from control).

Drug	Vasodilatation (%)	Vasoconstriction (mmHg)
None (control)	59.5 ± 4.5	0.0 ± 0.0
Ascorbate	5.8 ± 3.0***	88.1 ± 13.7***
Dehydroascorbate	71.4 ± 8.3	0.0 ± 0.0
SOD	59.6 ± 5.6	0.0 ± 0.0
Catalase	63.6 ± 5.4	0.0 ± 0.0
Glutathione	51.2 ± 3.9	0.0 ± 0.0
Dithiothreitol	5.1 ± 3.3***	32.5 ± 8.1***
N-acetyl-L-cysteine	32.5 ± 5.8**	23.2 ± 9.5*

It is likely that ascorbate inhibits ACh-induced, EDHF-mediated vasodilatation by an antioxidant action, since inhibition was not mimicked by dehydroascorbate. The effects of ascorbate are, however, unlikely to involve scavenging of superoxide anion or hydrogen peroxide, since they were not mimicked by SOD or catalase, respectively. Although glutathione was inactive, two more powerful thiol reducing agents, DTT and NAC, did mimic the actions of ascorbate. The possibility that ascorbate inhibits EDHF-mediated relaxation by reducing a critical disulphide group requires further investigation.

McNeish A.J., Wilson W.S., Martin W (2001) *Br. J. Pharmacol.* (Abstract, this meeting).
Wilson, W.S., Shahidullah, M., Millar, C. (1993) *Curr. Eye Res.* 12, 609-620.

309P INHIBITION OF SMOOTH MUSCLE CELL DNA SYNTHESIS BY C_2-CERAMIDE AND INHIBITORS OF SPHINGOSINE-1-KINASE IN PRIMARY PORCINE CORONARY ARTERY SMOOTH MUSCLE CELLS.

R. Mcdonald, N. Pyne, S. Pyne, R.M. Wadsworth & C.L. Wainwright. Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Taylor Street, Glasgow, UK.

The pathogenesis of post-angioplasty restenosis is characterised by an abnormal accumulation of vascular smooth muscle cells (VSMCs) within the intimal space. This is possibly through changes in the balance between the molecular mediators and genes which modulate cell survival and the induction of apoptosis. It is well established that ceramide and sphingosine can induce apoptosis and cell cycle arrest in a variety of cell lines (Dbaibo *et al* 1995; Ohta *et al* 1994). Thus the present study utilized exogenous cell permeable analogues of ceramide and sphingosine to determine their effects on VSMC proliferation. Sphingosine can be phosphorylated by sphingosine kinase to sphingosine-1-phosphate, which is a proliferative agent in smooth muscle cells, and therefore would counter the anti-proliferative effects of sphingosine. Therefore, we evaluated the effect of two sphingosine kinase inhibitors D-*erythro*-N, N-dimethyl-sphingosine and DL-*threo*-dihydrosphingosine on DNA synthesis.

For measurements of ^{3}H-thymidine uptake, porcine coronary SMC were cultured to 75% confluence (14-18 days), quiesced for 24hrs and stimulated with serum (10%) ± drug for a further 25hrs. [^{3}H]-thymidine (9.25kBq/well) was added for the last 5hrs and uptake quantified by liquid scintillation counting. Serum stimulation resulted in a 24-fold increase in [^{3}H]-thymidine incorporation into DNA (basal 420 ± 123dpms and serum stimulated 10558 ± 836dpms; n=5). Mitogen-activated protein kinase (MAPK) activation was determined in smooth muscle cells quiesced for 48hrs prior to stimulation with serum (10%) ± drug, by Western blotting with anti-phospho p44/p42 MAPK antibody. Serum stimulation resulted in a marked increase in p44/p42 MAPK phosphorylation.

Pretreatment with C_2-ceramide (25-100μM) produced a marked inhibition of [^{3}H]-thymidine incorporation (81 ± 5 % at the highest concentration) (n=4) and a 67±5% reduction in p44/p42 MAPK activation (n = 3) compared to serum stimulated controls. In contrast, C_2-dihydroceramide (0.1-100μM), the inactive analogue of C_2-ceramide did not inhibit either [^{3}H]-thymidine incorporation or MAPK activation.

Sphingosine (10-100μM) also induced an inhibition of both [^{3}H]-thymidine incorporation (78±4% at the highest concentration) and p42/p44 MAPK (89% at 10μM; n=2) activation. The two inhibitors of sphingosine kinase, N, N-*dimethyl*sphingosine and D, L-*threo*-dihydroceramide (10-100μM; n=5) reduced [^{3}H]-thymidine incorporation by 81±10% and 76±9%, respectively, at the highest concentration tested. Preliminary studies also suggest that they induce a comparable inhibition on p42/p44 MAPK activation.

This study demonstrates that these sphingolipids can induce anti-proliferative effects on coronary smooth muscle cells and that this inhibition may be mediated, at least in part through blockade of serum-stimulated p42/p44 MAPK activation.

Dbaibo, G., Pushkareva, M.Y., Jayadev, S., *et al* (1995) Proc. Natl. Acad. Sci. USA 92:1347-1351.

Ohta, H., Sweeny, A., Masamune, A., *et al* (1995) cancer research 55:691-697.

310P EFFECT OF ANTIPROLIFERATIVE AGENTS ON SMOOTH MUSCLE GROWTH, VASCULAR CONTRACTION AND ENDOTHELIUM-DEPENDENT RELAXATION IN THE PORCINE CORONARY ARTERY

S. Kennedy, B. Clarke, R.M. Wadsworth & C.L. Wainwright. S.I.B.S., University of Strathclyde, Taylor Street, Glasgow, UK.

The microtubule stabilising agent paclitaxel reduces restenosis in animal models (Herdeg *et al* 2000) while local delivery of farnesyl protein transferase inhibitor III (FPT III) reduces restenosis in pigs (Work *et al* 2001), possibly by limiting smooth muscle cell (SMC) proliferation. Two other antiproliferative agents, perillyl alcohol (PA; an inhibitor of geranylgeranyl transferase) and Van10/4 (a Strathclyde University patented fucopyranoside isolated from *Calendula officinalis)* may also be useful to reduce restenosis. PA reduces SMC proliferation *in vitro* (Unlu *et al* 2000) but the effect of Van 10/4 on SMC proliferation is unknown. In view of the importance of maintaining normal vascular function after angioplasty, we examined the effects of these compounds on vascular contraction and endothelium-dependent relaxation in porcine coronary artery rings. We also examined their effects on ^{3}H-thymidine uptake in cultured porcine coronary artery SMCs.

Rings (3-4mm) from the LAD coronary artery of porcine hearts obtained from an abbatoir were set-up in 10ml baths (37°C) containing gassed Krebs solution (95%0_2/5%CO_2) for measurement of isometric contraction and relaxation. Paclitaxel (10 and 50μM), FPT III (10 and 25μM), PA (1 and 2mM) and Van10/4 (10 and 25 μM) were incubated with rings for 30 minutes or overnight (20hrs at 4°C). In the presence of drug, rings were contracted with 1μM 5-HT (30min incubation) or 0.5μM U46619 (20hr incubation) and relaxation to cumulative addition of calcimycin (1nM-1μM) was measured. For ^{3}H-thymidine uptake, porcine coronary SMC were cultured to 75% confluence (14-18days), quiesced for 24hrs and stimulated with PDGF (10μg/ml)±drug for a further 24hrs. ^{3}H-thymidine (9.25kBq/well) was added for 5hrs and uptake determined by scintillation counting.

For all drugs, 30 minutes incubation had no effect on endothelium-dependent relaxation to calcimycin. Van10/4 and paclitaxel also had no effect on vascular contraction to 5-HT (Table 1). FPT III (25μM) caused a non-significant attenuation of the contractile response, whereas arteries could not be contracted in the presence of 1 or 2mM PA, which was only partially restored by washing (Table 1).

	FPT III	PA	Van10/4	Paclitaxel
Control	5.2±1.7	4.8±0.8	4.0±0.8	1.5±0.3
+Drug	1.9±1.0	1.0±0.8*	2.4±0.6	1.6±0.4

Table 1: Values are mean±SEM of contractions (g), induced by 1μM 5-HT in paired artery rings, in the absence or presence of the highest concentrations of drugs tested (n=6 per group). Contraction in the presence of PA was that obtained following prolonged washing. *P<0.05 compared to control using student's paired t-test.

Overnight incubation of paclitaxel, FPT III and Van10/4 at 10μM had no effect on calcimycin-induced relaxation whereas the higher concentration of all three significantly attenuated relaxation. Both Van10/4 and FPT III caused a concentration-dependent decrease in thymidine uptake. The IC_{50} values were 1.7±0.8μM (Van10/4; n=3) and 1.3±0.1μM (FPT III; n=3). PA had no effect on thymidine uptake.

This study demonstrates that the antiproliferative drugs Van10/4, paclitaxel and FPT III have no detrimental effects on endothelium-dependent relaxation or vascular contraction in the porcine coronary artery when applied for short periods or at low concentrations. This suggests that use of effective antiproliferative concentrations *in vivo* should not induce short-term vascular dysfunction. In contrast, PA had a marked inhibitory effect on contraction yet failed to demonstrate an effect on cell proliferation, suggesting its potential use in the prevention of restenosis is limited.

Herdeg, C., Oberhoff, M., Baumbach, A. *et al* (2000) *J. Am. Coll. Cardiol.* **35**:1969-1976.

Work, L.M., McPhaden, A.R., Pyne, N.J. et al (2001) *Circulation.* **104**, 1538-1543

Unlu, S., Demoliou Mason, C., Schachter, M. et al (2000) *J. Cardiovasc. Pharmacol.* **35**, 341-344.

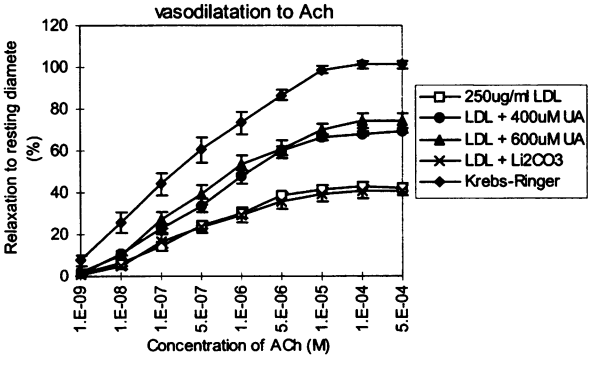
311P URATE PREVENTS NATIVE LOW DENSITY LIPOPROTEIN-INDUCED INHIBITION OF ENDOTHELIUM-DEPENDENT VASODILATATION

C. Marshall, D.J. Webb & S.R.J. Maxwell Clinical Pharmacology Unit, The University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU

The exposure of endothelial cells to native-low density lipoprotein (n-LDL) induces increased O_2^- production (Vergnani *et al*, 2000). NO and O_2^- react to produce peroxynitrite ($ONOO^-$), a reactive nitrogen species that causes oxidative damage in the vascular wall. Urate (UA) is a chain-breaking antioxidant which interacts with $ONOO^-$ to prevent $ONOO^-$-induced oxidative damage, and also forms a stable nitrate derivative which acts as an NO donor (Skinner *et al,* 1998). Therefore, UA may prevent n-LDL induced endothelial dysfunction by scavenging peroxynitrite and restoring NO-mediated vasodilatation. We aimed to investigate the hypothesis that UA can prevent n-LDL induced endothelial dysfunction in healthy blood vessels.

Sections of third order mesenteric artery (200-300μm diameter) were dissected from male Wistar Kyoto rats (250-300g) and cannulated in an arteriograph chamber containing oxygenated Krebs-Ringer solution at 37°C ± 0.5 and pH 7.4. The lumen was perfused with Krebs-Ringer buffer, 250μg/ml LDL solution or LDL solution supplemented with 400μM UA, 600μM UA or vehicle Li_2CO_3 solution, at an intraluminal pressure of 60mmHg, then kept in a no-flow state during the addition of the vasoactive drugs. The arteries were contracted with 125mM KCl then washed out. Cumulative dose-response curves were performed to phenylephrine (PE), and after washout and preconstriction, ACh and SNP. Results shown are mean values ± SEM of 8 experiments, and were analysed using an ANOVA with Bonferroni correction.

Figure 1: The effect of urate supplementation on vasodilatation to Ach



Both concentrations of UA significantly prevented n-LDL inhibition of endothelium-dependent vasodilatation to ACh (Fig.1), but had no effect on the responses to PE or SNP.

UA partially restores NO-mediated vasodilatation following acute exposure of isolated blood vessels to n-LDL. UA may be scavenging ONOO-, thus preventing the deleterious effect of LDL on NO bioavailability and increasing functional NO levels.

Skinner K.A. *et al, J. Biol. Chem.* 1998;273(38):24491-24497

Vergnani L. *et al, Circulation* 2000;101:1261-1266

312P INHIBITION OF RHO-KINASE IMPAIRS CHEMOTAXIS AND MITOGENESIS AND INDUCES A CHANGE IN MORPHOLOGY IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

GF Clunn, MK Patel, PS Sever & AD Hughes. National Heart and Lung Institute, Imperial College School of Medicine, London, United Kingdom.

Rho-kinase (ROCK) is a direct downstream effector of the monomeric GTPase rho. ROCK has been demonstrated to play important roles in the vasculature, particularly in neointimal formation in response to vessel injury in a rat model. A specific inhibitor of ROCK, Y27632, inhibited neointimal formation in the aforementioned study (Shibata *et al.*, 2001). Key to neointimal formation is the migration and proliferation of vascular smooth muscle cells (SMC). We therefore examined the potential functional involvement of ROCK in SMC chemotactic and mitogenic pathways using Y27632.

The study used cultured human saphenous vein SMC derived from patients undergoing bypass surgery. Cells were obtained using an explant technique and used for experiments at passage 2. DNA synthesis was measured by [^{3}H]-thymidine incorporation, chemotaxis was quantified using blind well chambers, morphology was assessed using time-lapse light microscopy and F-actin was labelled using FITC-labelled phalloidin and detected using fluorescence microscopy.

Treatment of cells with Y27632 in the range 1μM30μM (Shingo *et al.*, 2001) dose-dependently inhibited DNA synthesis in response to platelet-derived growth factor-BB (PDGF-BB, 10ng/ml) and foetal calf serum (FCS, 15% vol/vol) as measured by [^{3}H]-thymidine incorporation, although there were Y27632 insensitive components of 31±7% and 42±5% respectively at the highest concentration (mean±s.e.m. from 4 individuals). In contrast, peak stimulated chemotaxis in response to PDGF-BB (2ng/ml, n=4) and unstimulated chemokinesis were completely abolished at 10μM Y27632. When cells were seeded sparsely (<1000/cm^2) to allow full spreading, treatment with Y27632 (10μM30μM) resulted in dramatic alterations in cell morphology, particularly the development of neuron-like protrusions within 1-4 hours. The length of the protrusions ranged from approximately 0.1 to 4-5 times the original length of the cell body. Phalloidin staining revealed these features contained elongated F-actin filaments, indicating uncontrolled actin polymerisation.

Thus, ROCK plays multiple roles in human vascular SMC. Entry into S phase appears to proceed via ROCK dependent and independent pathways, but chemotaxis and maintenance of morphology are critically dependent on ROCK activity. It is suggested that highly localised variations in ROCK activity (hence actin organisation) could underlie the coordinated sequence of morphological rearrangements required for induction of cell polarity in directed cell motility. These results could therefore explain, at least in part, the outcome of the effects observed in the *in vivo* situation.

Shibata, R. *et al.*, (2001). *Circulation* **103**, 284-289.
Shingo, A. *et al.*, (2001). *Atherosclerosis* **155**, 321-327.

313P THE ANTIMITOGENIC ACTION OF THE SULPHATED POLYSACCHARIDE FUCOIDAN DIFFERS FROM HEPARIN IN HUMAN VASCULAR SMOOTH MUSCLE CELLS.

M. Patel, B. Mulloy[1], K. Gallagher, L. O'Brien & A.D. Hughes. Clinical Pharmacology, NHLI, Imperial College, London W2 1NY and [1]National Institute for Biological Standards and Control, South Mimms, Herts. EN6 3QG, UK.

The sulphated polysaccharides fucoidan and heparin affect a wide range of processes, such as cell proliferation, chemotaxis, cell-cell and cell-matrix interactions. In this study we compared their actions on DNA synthesis, [^{3}H]-heparin binding and ERK1/ERK2 activation in human vascular smooth muscle cells (hVSMC) cultured from saphenous vein.

The effect of the polysaccharides (1nM-10μM) on DNA synthesis was assessed by measuring [^{3}H]-thymidine incorporation stimulated by 15% foetal calf serum (FCS), platelet-derived growth factor (PDGF-BB, 10ng/ml), or the matricellular protein, thrombospondin-1 (TSP-1, 5μg/ml). The ability of the polysaccharides to displace specific binding of [^{3}H]-heparin (100nM) was measured as previously described (Patel *et al.*, 1999). The effect of fucoidan (1μM) and heparin (100μM) on 10ng/ml PDGF-induced ERK1 and ERK2 activation was assessed in hVSMC lysates by SDS PAGE and Western blotting using an antibody that specifically recognised dual phosphorylated ERK1 and ERK2. Data are means±s.e.means, statistical comparisons were made using a Student's t-test and $p<0.05$ was considered significant.

Fucoidan potently inhibited DNA synthesis stimulated by FCS, PDGF-BB and TSP-1 (Table 1). By contrast, heparin inhibited the mitogenic action of FCS and TSP-1, but failed to inhibit PDGF-BB-induced DNA synthesis. In parallel studies, fucoidan inhibited ERK1 and ERK2 activation by 60±7% and 74±8% (n=6, $p<0.0005$) respectively following PDGF-BB stimulation, whereas heparin had no significant effect. Both fucoidan (K_i = 16±8nM, n = 4) and heparin (K_i = 28±10nM, n = 4) displaced [^{3}H]-heparin binding to hVSMC with similar affinity.

Table 1. pIC_{50} values (M) for inhibition of DNA synthesis by fucoidan and heparin using different mitogens.

	Fucoidan (n = 6)	Heparin (n = 6)
FCS	7.90±0.13	4.56±0.45
PDGF	6.17±0.18	>4
TSP-1	5.92±0.19	4.03±0.17

In summary, the polysaccharides, heparin and fucoidan have clearly distinguishable effects on DNA synthesis and ERK1/ERK2 activation suggesting that they act *via* different mechanisms. The ability of both polysaccharides to bind to the cell surface with similar affinity suggests that this site may not be directly responsible to the anti-proliferative effects of the polysaccharides. The potent antimitogenic action of fucoidan emphasises the need to further investigate its mechanism of action in hVSMC and suggests that this agent may have therapeutic potential.

Patel, M.K. *et al.*, (1999). *Br. J. Pharmacol.* **127**, 361-8.

314P A COMPARISON OF THE BINDING AFFINITIES OF ENDOTHELIN (ET) RECEPTOR ANTAGONISTS AT RECOMBINANT AND NATIVE ET_A RECEPTORS

Declan A. Flynn, Anthony J. Chuck, Stephen Ballard & Michael A. Tones. Discovery Biology, Pfizer Global Research & Development, Sandwich, Kent UK CT13 9NJ

The use of recombinant technology has greatly enhanced screening capabilities in drug discovery by providing the means to express at high levels a defined receptor population in a host cell. However, potential differences in alternative splicing, post translational modification and level of expression raise the possibility that the pharmacological or structural properties of a native receptor may not always be reflected by those of the recombinant receptor. Radioligand binding studies by Davenport (1994) have shown the ET_A selective antagonist [^{125}I] PD-151242 to have a K_D of 500-1700 pM in a variety of native human vessels. This contrasted with the K_D obtained in-house using a recombinant human ET_A receptor expressed in CHO cells (61.0 ± 9.1 pM, n=3).

To investigate these differences further, a comparison was made between binding properties at recombinant ET_A and a native human ET_A receptor expressed in SK-N-MC cells, a human neuroblastoma cell line (Wilkes et al., 1991). Membrane preparations of SK-N-MC cells or CHO-ET_A cells (obtained from Dr Masaki, Kyoto University) were produced by homogenisation of cell suspensions and differential centrifugation. Binding of radio-ligand to the receptors was determined using a scintillation proximity assay. K_D values for ^{125}I PD-151242 ligand binding to recombinant and native receptor were similar (61.0 ± 9.1 and 57.3 ± 7.4 pM respectively) in both assays. IC_{50} values for inhibition of [^{125}I] PD-151242 (60 pM) binding by a range of ET receptor antagonists (Douglas, 1997) were determined and Ki values calculated using the Cheng-Prussoff equation (Table 1).

There was good agreement between the Ki values for native and recombinant receptors (r= 0.89), with the notable exception of sitaxsentan, which was 9-fold more potent with the native receptor (* $p< 0.01$ using unpaired students t-test)

Table 1: Comparison of binding affinity in recombinant and native ET_A receptors for a range of ET receptor antagonists

Compound	CHO pKi	SK-N-MC pKi	CHO/SK-N-MC Ratio
PD 156707	9.83 ± 0.08	10.05 ± 0.38	1.66
A-127722	9.90 ± 0.10	9.53 ± 0.11	0.43
SB 209670	9.38 ± 0.13	9.52 ± 0.12	1.38
TA-0201	9.85 ± 0.08	9.44 ± 0.12	0.39
BMS 193884	9.25 ± 0.03	9.32 ± 0.08	1.16
Sitaxsentan	7.81 ± 0.06	8.61 ± 0.12*	8.85
Darusentan	8.24 ± 0.09	8.67 ± 0.21	2.74
YM-598	8.53 ± 0.06	8.60 ± 0.09	1.15
Bosentan	8.25 ± 0.03	8.50 ± 0.14	1.78

These data indicate that binding affinity at the recombinant ET_A receptor expressed in CHO cells is generally predictive of binding affinity at the native receptor although the anomaly seen with sitaxsentan requires further investigation.

Davenport, A., Kuc, R.E., Fitzgerald, F. et al. (1994) *Brit. J Pharmacol.*, 111 (1) p4-6
Douglas, S. (1997) *Trends Pharm. Sci.* 18 (11) p408-13
Wilkes, L.C., Boarder, M.R. (1991) *Brit. J. Pharmacol.* 103 (4) p750-4

315P DIFFERENTIAL EFFECTS OF ENDOTHELIN ET_A AND ET_B RECEPTORS STIMULATION ON CARDIAC ELECTROPHYSIOLOGY OF ISOLATED WORKING RABBIT HEARTS.

C. McCabe, M.N. Hicks*, K.A. Kane & C.L. Wainwright. Department of Physiology & Pharmacology, University of Strathclyde & *Department of Medical Cardiology, Royal Infirmary, Glasgow, U.K.

Endothelin (ET-1) has been reported to cause ventricular arrhythmias associated with a decline in coronary blood flow. However, evidence of a direct arrhythmogenic effect that is not attributable to myocardial ischaemia has also been proposed (Toth *et al.*, 1995). The precise mechanisms of this direct arrhythmogenic effect are not known but regional heterogeneity and increased action potential duration dispersion have been postulated. The aim of this study was to determine the effects of ET-1 (an ET_A/ET_B agonist) and S6c (an ET_B agonist) on endocardial and epicardial action potentials in an isolated working rabbit heart preparation in which effects on coronary blood flow could also be measured.

Hearts were excised from male NZW rabbits (2.5-3.5kg) and perfused with Tyrodes buffer in the working heart mode and paced at 3.3Hz. Monophasic action potentials were recorded continuously from the apical endocardium and epicardium. Hearts were perfused with solution containing either vehicle (n=6), ET-1 (10^{-12}M-10^{-9}M, n=6) or S6c (10^{-12}M-10^{-8}M, n=6) in a cumulative manner with 15 min exposure at each concentration. Monophasic action potential duration was measured at 90% of repolarisation ($MAPD_{90}$), together with aortic, coronary flow and left ventricular pressure. Statistical comparisons within groups were carried out using a repeated measure ANOVA and a Dunnett's post test with $P<0.05$ taken as statistically significant.

Electrophysiological and haemodynamic variables were constant throughout the experimental protocol in vehicle treated hearts. ET-1 at concentrations of 10^{-10}M and above significantly reduced epicardial $MAPD_{90}$ (from 127 ± 3 to 109 ± 5 ms, mean ± S.E.M), while concentrations of $3x10^{-10}$M and above also significantly reduced endocardial $MAPD_{90}$ (from 129 ± 5 to 97 ± 6 ms). This shortening of $MAPD_{90}$ was associated with a reduction in coronary blood flow (from 74 ± 7 to 67 ± 7 ml/min), cardiac output (from 269 ± 15 to 191 ± 34 ml/min), myocardial contractility, dP/dt_{max}, (from 804 ± 42 to 647 ± 71 mmHg/sec) and induction of VF in 66% of hearts. In contrast to ET-1, S6c significantly increased endocardial but not epicardial $MAPD_{90}$ at a concentration of $3x10^{-9}$M and above (from 122 ± 4 to 132 ± 4 ms) and did not exhibit a pro-arrhythmic effect. S6c, at concentrations of 10^{-10}M and above, also significantly reduced coronary blood flow (from 72 ± 6 to 63 ± 6 ml/min) and cardiac output (from 245 ± 16 to 203 ± 21 ml/min) but had no effect on myocardial contractility.

In conclusion, the pro-arrhythmic effect observed with combined ET_A and ET_B receptor stimulation with ET-1 was associated with a reduction in coronary blood flow and $MAPD_{90}$. ET_B receptor stimulation with S6c caused a similar reduction in coronary blood flow to that of ET-1 but a prolongation in endocardial $MAPD_{90}$. These results suggest that ET_B receptor stimulation has a direct electrophysiological effect on the endocardium which opposes a pro-arrhythmic effect of ET_A stimulation.

Toth, M., Solti, F., Merkeley, B. et al. (1995). *J. Cardiovasc. Pharmacol.*, 26(Suppl 3), S153-S155.

C McCabe is supported by the British Heart Foundation.

316P ADRENOMEDULLIN PROTECTS AGAINST ISCHAEMIA-INDUCED ARRHYTHMIAS AND INJURY IN ANAESTHETISED RATS

Y.H. Looi, C.L. Wainwright, K.A. Kane, Department of Physiology & Pharmacology, University of Strathclyde, SIBS, 27 Taylor St., Glasgow G4 0NR.

Adrenomedullin (AM), a novel 52-amino acid hypotensive peptide discovered in 1993, possesses multifunctional biological properties, of which its effect in the regulation of cardiorenal homeostasis is the most pronounced. AM is implicated in the pathophysiology of congestive heart diseases, hypertension and myocardial infarction (Eto *et al.*, 1999). Experimental studies performed in rats have shown that ventricular AM peptide and mRNA levels were significantly increased 6 hours post infarction (Nagaya *et al.*, 2000). The aim of this study is to determine if AM has a cardioprotective effect during myocardial ischaemia and to investigate the role, if any, of leukocyte generated reactive oxygen species in any observed effect.

Male Sprague-Dawley rats (250-400g) were anaesthetised with pentobarbitone (60 mg/kg) intraperitoneally. The right carotid artery was cannulated for measurement of mean arterial blood pressure (MABP) and withdrawal of blood, and the left jugular vein for administration of drug/vehicle. Animals were subjected to 30 min of ischaemia induced by occlusion of left main coronary artery and 120 min of reperfusion. The number and incidence of cardiac arrhythmias, MABP and heart rate were recorded. *Ex vivo* reactive oxygen species generation, in response to Zymosan A (10^6 particles) was determined in whole blood by luminol chemiluminescence (CL). Hearts were stained with Evan's blue and triphenyltetrazolium chloride at the end of experiment for area at risk and infarct size measurements respectively. Animals were administered a bolus i.v dose of AM (1nmol/kg) (n=13) 5 min before occlusion while control animals received saline (n=16). Blood was withdrawn for *ex vivo* studies 15 and 1 min pre-occlusion and 15 and 30 min post-occlusion occlusion. CL data was expressed as percentage of -15 min samples. Data (mean±s.e.mean) was compared using ANOVA with Dunnett's post-hoc test. The percentage incidence of ventricular fibrillation (VF) was compared using Fischer's exact test. Statistical significance was taken if $P<0.05$.

AM significantly reduced both the number of ventricular ectopic beats that occurred during the 30 min of ischaemia from 1362±128 to 520±74 ($P<0.0001$) and the incidence of VF (65% to 26%, $P<0.05$). Infarct size was also significantly reduced by AM (38±3% to 16±2% of area at risk, $P<0.001$). AM caused a significant decrease in MABP 4 min post administration from 115±4mmHg to 107±5mmHg ($P<0.01$). 1 min after occlusion, the percentage decrease in MABP in AM treated animals was significantly greater than in controls (59±3% vs. 47±4%, $P<0.05$) but, thereafter, there were no significant differences in MABP between the groups. AM treatment also caused a significant increase in CL generation 1 min (135±15%, $P<0.05$) pre-occlusion and subsequently at 15 min (134±16%, $P<0.05$) post-occlusion compared to controls.

This study has demonstrated that AM exhibits cardioprotective effects against ischaemia-induced arrhythmias and injury. This is in agreement with the hypothesis that AM may play a compensatory role acting against deterioration of cardiovascular pathological conditions (Eto *et al.*, 1999). The vasodilator effect of AM and production of reactive oxygen species from leukocytes may contribute to the cardioprotective effects of AM.

Eto T., Kitamura K., Kato J. (1999) Clin. Exp. Pharmacol. Physiol. 26, 371-380.
Nagaya N., Nishikimi T., Yoshihara F. *et al.* (2000) Am. J. Physiol. Integr. Comp. Physiol. 278, R1019-R1026.

317P DIAZEPAM POTENTIATES THE POSITIVE INOTROPIC EFFECT OF DOPAMINE AND DOBUTAMINE IN RAT VENTRICULAR MYOCARDIUM

M.J. Juan, M.L. Vargas, J.S. Serrano[1] & J. Hernández, Department of Pharmacology, Universidad de Murcia and [1]Department of Pharmacology, Universidad de Sevilla, Spain

We recently demonstrated that diazepam exerts an inhibitory activity on type 4, cyclic AMP-specific phosphodiesterase (PDE4) and enhances the effect of some cyclic AMP-dependent positive inotropic agents (Collado *et al.*, 1998; Hara *et al.*, 1998). Dobutamine and dopamine are commonly used drugs which increase cardiac contractility through a β-adrenergic receptor (β-AR) mediated mechanism, and diazepam would be expected to potentiate this effect. To examine this hypothesis, we have studied whether diazepam modifies the inotropic responses to dobutamine and dopamine in the electrically driven rat right ventricle. The type of β-AR involved was also investigated.

Sprague-Dawley rats of either sex (250-300g) were killed by stunning and cervical dislocation. The procedures were performed in Tyrode solution at 34°C saturated with 95% O_2-5% CO_2. Right ventricular strips were set up longitudinally between two platinum electrodes under 1 g tension and electrically paced (1 Hz, 1 ms, threshold voltage +25%). All tissues were exposed to 5 μM phenoxybenzamine (to block α–AR). Isometric tension was recorded and after equilibration, cumulative concentration-response curves (CRC) were constructed for dopamine and dobutamine alone or in the presence of diazepam or diazepam-related agents. Experiments were terminated by raising the Ca^{2+} concentration to 9mM.

Dobutamine(0.1-100 μM) and dopamine(1-100 μM) increased the contractility of this preparation, producing a maximal effect of 50% (for dobutamine) and 58% (for dopamine), with respect to Ca^{2+}. Diazepam (10 μM), but not solvent, produced a leftward shift in the dobutamine and dopamine CRCs. The –log EC_{50} values in the absence or presence of diazepam were respectively, 5.86±0.11 (mean ± s.e.mean) and 6.47±0.08 for dobutamine ($p<0.05$, ANOVA with Tukey's test, n=6) and 5.31±0.05 and 5.67±0.03 for dopamine ($p<0.05$, n=6). The antagonists of benzodiazepine receptors of the central or peripheral type, flumazenil and 1- (2 –chlorophenyl) – N – methyl – N - (1 – methylpropyl) - 3 –isoquinolinecarboxamide (PK 11195) respectively, at 5 μM, did not alter the diazepam induced potentiation of either, dobutamine or dopamine. The –log EC_{50} of dobutamine-diazepam (6.47±0.08) did not differ ($p>0.05$, n=6), when co-incubated with flumazenil (6.43±0.05) or PK 11195 (6.35±0.04). Similarly, the –log EC_{50} of dopamine-diazepam 5.67±0.03 was not significantly different in the presence of flumazenil 5.62±0.06 or PK 11195 5.65±0.08 ($p>0.05$, n=6). The antagonist of β_1-AR, CGP 20712A (0.3 μM), which by itself is devoid of effect, virtually abolished the effects of dobutamine and dopamine, alone and in the presence of diazepam (n=5 for each combination). However, the antagonist of β_2-AR, ICI 118551 (50 nM) had no effect and failed to alter the effect of dobutamine and dopamine alone or in the presence of diazepam (n=5 for each combination).

Thus dopamine and dobutamine produce a β_1-AR mediated inotropic response in rat ventricular myocardium, which is potentiated by diazepam, not through a central or peripheral benzodiazepine-related mechanism, but presumably due to PDE4 inhibition.

Collado, M., *et al.* (1998) *Br. J. Pharmacol* 123, 1047-1054.
Hara, Y., *et al.* (1998) *J. Vet. Pharmacol. Ther.* 21, 375-379.

318P CYCLIC NUCLEOTIDE PHOSPHODIESTERASE TYPE 2 REGULATES THE CONTRACTILE EFFECT MEDIATED BY β_2- ADRENOCEPTORS, BUT NOT THOSE MEDIATED BY β_1- ADRENOCEPTORS IN HUMAN RIGHT ATRIA.

V.Arocas, M. Martínez, M. Real, M.D. Carceles & J. Hernández (introduced by A.J. Kaumann), Hospital U.C.S.V. Arrixaca, Murcia, Spain.

Agonists of β_1- and β_2-adrenoceptors (AR) increase cardiac contractility by enhancing cyclic AMP production which is hydrolized by the family of enzymes, cyclic nucleotide phosphodiesterases (PDEs) (Steinberg, 1999). Several PDEs, including types 1-4, are present in human heart (Wallis et al. 1999), but their contribution to inotropic responses mediated by β_1- and β_2-AR is not clearly established. The aim of the present study has been to investigate how selective inhibition of one PDE subtype modulates β_1- and β_2-AR mediated inotropy in human myocardium.

All protocols for obtained human cardiac tissue were approved by the ethics committee of our Hospital. Right atrial trabecula, obtained from patients undergoing open heart surgery who were not treated with β-AR antagonists, were suspended in an organ bath, superfused with Tyrode's solution and paced by field stimulation at 1 Hz. Tissues were in contact with phenoxybenzamine (5 μM), to block α-AR as well as the neuronal and extraneuronal uptakes. Concentration-response curves (CRC) of noradrenaline, in the presence of 50 nM of the β_2-AR blocking agent ICI 118551, and adrenaline, in the presence of 0.3 μM of the β_1-AR blocking agent CGP 20712A, were performed in the absence and in the presence of 1 μM of the following selective inhibitors of PDEs types 1-4: 8-methoxymethyl-3-isobutyl-1-methylxantine (MIMX) for PDE1, erythro-9-(2-Hydroxy-3- nonyl) adenine (EHNA) for PDE2, milrinone for PDE3 and rolipram for PDE4. Results were expressed as percentages of the Emax obtained by rising Ca^{+2} concentration to 9 mM.

Noradrenaline (0.1-100 μM) and adrenaline (0.01-100 μM), increased the contractility of this preparation, producing a similar maximal effect which amounted to 83±5%. Milrinone enhanced contractility by 23±7% and produced a leftward shift in the CRCs of noradrenaline and adrenaline. The other PDE inhibitors were devoid of inotropic effect alone and only EHNA shifted to the left the CRC of adrenaline and signify-cantly increased its –log EC_{50} (Table 1).

Table 1. Inotropic potency of noradrenaline and adrenaline alone and in combination with PDEs 1-4 inhibitors.

	Noradrenaline		Adrenaline	
	n	-log EC_{50}	n	-log EC_{50}
Control+	25	6.18±0.13	22	6.78±0.06
MIMX	6	6.27±0.05	5	6.79±0.05
EHNA	5	6.17±0.08	6	7.11±0.06*
Milrinone	8	6.54±0.07*	5	7.52±0.10*
Rolipram	6	6.05±0.16	6	6.81±0.02

*P<0.05 when compared with their respective controls. ANOVA followed by Tukey's method. Values given as mean ± s.e.mean.

Thus, PDE2 seems to be involved only in the regulation of β_2-AR but PDE3 may control both β_1- and β_2-AR mediated inotropy in the failing human heart.

This work was supported by grant BFI2000,1013 (MEC,Spain)

Steinberg S.F. (1999) *Cir. Res.* 85, 1101-1111
Wallis R.M., *et al.* (1999) *Am. J. Cardiol.* 83, 3C-12C

319P EFFECTS OF THE ANTIPSYCHOTIC RISPERIDONE ON NORMAL AND HYPERTROPHIED GUINEA-PIG CARDIAC TISSUE

A. Botchway, M. Maginn, T. Mow & J. Matz. Department of Safety Pharmacology. H.Lundbeck A/S. Ottiliavej 9. Copenhagen. Denmark DK2500.

Risperidone (Risperdal®) is an antipsychotic prescribed for the treatment of acute and chronic psychoses. Previous studies have shown adverse effects such as cardiac death following initiation of risperidone therapy, which was related to prolongation of ventricular repolarisation (Ravin and Levenson, 1997). It is possible that sudden cardiac death is due to drug induced prolongation of action potential duration (APD) leading to arrhythmias and sudden death. Furthermore, Left ventricular hypertrophy (LVH) also increases APD (Botchway *et al* 2000) and the risk of arrhythmias leading to sudden cardiac death (Levy *et al* 1987). In this study we investigated drug induced changes in APD in combination with a known predisposing factor of cardiac hypertrophy.

Male Dunkin Hartley guinea-pigs (600-800g) were anaesthetised by a 3% halothane and O_2 and LVH was induced by aortic constriction with a high density plastic clip (internal diameter 1.99 mm, 1.5 mm thick as previously described (Turner *et al* 2000). Naive unoperated animals served as controls. After 49 ± 2 days, hearts were removed after stunning and cervical dislocation. Papillary muscles were superfused in Tyrode solution at 37 °C. Transmembrane action potentials were recorded using standard microelectrode techniques at a stimulation frequency of 1.0 Hz. After a stabilisation period, risperidone at 3 μM was superfused and studied for 30 minutes at both 0.2 and 1.0 Hz. Data presented as mean ± s.e.m (n), and analysed by Student's t-test.

Aortic constriction increased LV weight to body weight ratio from 1.6±0.02(5) to 2.06±0.08(5), $p<0.05$. Baseline APD_{90} (ms) increased from 174.6±7.1(7) to 193.0±3.4 (9), $p<0.05$ in LVH hearts. At 1.0 Hz stimulation frequency risperidone increased APD_{90} from 174.6±7.1(7) to 221.7±6.6 (7), $p<0.05$ in control hearts and from 193.0±3.4(9) to 246.0±8.4 (9), $p<0.05$ in LVH hearts. Low frequency pacing (0.2 Hz) had no further effect on APD_{90} after risperidone induced prolongation [LVH; 246.0±8.4 (9) vs 247.0 ±6.7 (9), control; 221.7±6.6 (7) vs 219.3±7.2 (7), p= NS] 1.0 and 0.2 Hz respectively.

The data from this study show that changes in action potential characteristics in the guinea-pig papillary muscle correlates to the clinical findings that non-cardiac drugs such as risperidone and LVH prolong APD. Furthermore LVH potentiates drug induced proarrhythmic potential which could increase mortality.

Botchway, A.N.S., Turner, M.A., Sheridan, D.J., et al (2000). J Physiol 526P, 82

Levy, D.,Anderson, K.M.,Savage, D.D.,et al (1987). Am. J. Cardiol .60,560 –565.

Ravin, D.S and Levenson, J.W. (1997). Ann Pharmacother. 31: 867-870.

Turner, M.A., Botchway, A.N.S., Flores, N.A., et al (2000). J Physiol 525P,6

320P EFFECTS OF GW274150, A NOVEL AND POTENT INHIBITOR OF THE ACTIVITY OF INDUCIBLE NITRIC OXIDE SYNTHASE ON THE MULTIPLE ORGAN INJURY IN HAEMORRHAGIC SHOCK IN THE ANAESTHETISED RAT

M. Izumi, M.C. McDonald & C. Thiemermann. Dept Experimental Medicine & Nephrology, The William Harvey Research Institute, St. Bartholomew's and The Royal London School of Medicine and Dentistry, Charterhouse Square, London, ECIM 6BQ, U.K.

GW274150 is a novel inhibitor of iNOS activity, which has a very high degree of selectivity for iNOS versus both eNOS (>250-fold) and nNOS (>80-fold). GW274150 is a sulphur-substituted acetamine amino acid, which causes a NADPH-dependent inhibition of iNOS activity, which is slow in onset, long-lasting and reversible with L-arginine. The half-life of GW274150 in the rat is 5 h and a single injection of this iNOS inhibitor (3 mg kg^{-1}) abolishes the increase in nitrite and nitrate caused by an injection of endotoxin (Alderton *et al.*, 2001). Here we investigate the effects of GW274150 on the circulatory failure and the organ injury/dysfunction caused by severe haemorrhagic shock (HS) in the anaesthetised rat.

Thirty-Five male Wistar rats (220 g–300 g) were anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.). The femoral artery was cannulated to measure mean arterial blood pressure (MAP) and heart rate (HR), the jugular vein for the intravenous administration of drugs, and the carotid artery for blood withdrawal. The animals were pre-treated with the iNOS-inhibitor GW274150 (5 mg kg^{-1} i.v.) or saline (1 ml kg^{-1}) and 30 min later subjected to haemorrhage to lower MAP to 45 mmHg for 90 min and subsequently resuscitated with the shed blood. Four hours after the onset of resuscitation, blood samples were taken for the measurement of biochemical markers of organ injury.

When compared to sham-operated rats (S), HS caused a delayed fall in MAP ($P<0.05$). In addition, HS caused significant rises in the serum levels of (i) urea and creatinine (renal dysfunction), and (ii) aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (liver injury) (Table 1). GW274150 significantly attenuated the rise in the serum levels of creatinine and the liver injury (n=9, $P<0.05$), but not the rise in the serum levels of urea caused by HS (n=9, $P>0.05$). (Table 1).

The effects of the iNOS inhibitor, GW274150 on the renal dysfunction and liver injury caused by haemorrhagic shock

Group	n	Urea (mmol L^{-1})	Creatinine (μmol L^{-1})	AST (iu L^{-1})	ALT iu L^{-1})
S	7	7±1*	42±3*	173±36*	75±5*
S-GW	4	7±2*	57±4*	176±33*	81±7*
HS	12	16±1	107±12	719±149	343±81
HS-GW	12	13±1	67±7*	394±53*	168±22*

Table. 1 Data are expressed as mean±s.e.mean, *$P<0.05$ vs. HS, analysed using one-way ANOVA followed by Dunnett's post hoc test.

Thus, GW274150 attenuates the renal dysfunction and the hepatocellular injury caused by severe haemorrhage and resuscitation.

Alderton W.K., Cooper C.E. & Knowles, R.G (2001). *Biochem. J.* **357**, 593-615.

321P THE EFFECT OF NO-ASPIRIN ON ARRHYTHMIAS AND INFARCT SIZE IN ISOLATED SPONTANEOUSLY HYPERTENSIVE RAT HEARTS.

S.G. Burke[1], B.L. Furman[1], C.L. Wainwright[1], P. Del Soldato[2], I. Vojnovic[3] & T. Warner[3]. [1]Department of Physiology & Pharmacology, University of Strathclyde, 27 Taylor Street, Glasgow, G4 0NR. [2]Nicox S.A. Milan, Italy. [3]William Harvey Research Inst. London

The use of aspirin in the secondary prevention of acute myocardial infarction is limited by its gastric side effects. NCX4106 is a member of a new class of NO-NSAIDs that release nitric oxide (NO) and has reduced gastric side effects, whilst maintaining the pharmacological profile of aspirin (Del Soldato *et al.* 1999). The release of NO may also improve the cardioprotective effect of aspirin. The aim of this study was to assess the effect of chronic NO-aspirin (NCX4016), on arrhythmias and infarct size resulting from coronary artery occlusion in hearts isolated from spontaneously hypertensive rats (SHR).

Male SHRs (300-380g, 12-14 weeks) were anaesthetised with pentobarbitone (60mg kg^{-1}, i.p.). The right carotid artery was cannulated for preliminary measurement of mean arterial blood pressure (MABP), and ECG was recorded via standard (lead II) limb electrodes. A 6/0 suture was then placed around the main left coronary artery (MLCA). The heart was then excised and perfused with modified Krebs (pH 7.38, 37°c,Langendorff mode, 12ml min^{-1}). Hearts were subjected to 30 min regional ischaemia (MLCA occlusion), and 120 min reperfusion. The coronary perfusion pressure (CPP), HR and the number and incidence of arrhythmias (VPB) were recorded via an epicardial ECG. Hearts were then stained to determine area at risk and infarct size. Animals were pre-treated once daily orally for 5 days with either (i) PEG_{400} (1ml kg^{-1}, n=12), (ii) NCX4016 (120mg kg^{-1}, n=12), or (iii) aspirin (65.2mg kg^{-1}, n=9). TxB_2 levels were measured in blood withdrawn from the carotid artery by RIA. Data (mean ± s.e.mean) was compared using ANOVA with Tukey's post-test. The percentage incidence of VT and VF was compared using Fischer's exact test.

Neither drug significantly modified MABP or HR from the control values of 160±7 mmHg and 361±9 bpm respectively. Initial CPP was significantly higher in the aspirin group (78±4 vs. 69±1 mmHg $P<0.05$), but was not modified by NCX4016. However, in controls, CPP rose to 203±17 mmHg by the end of reperfusion. This was significantly lower in the NCX4016 treated hearts (157±23 $p<0.05$) but not altered by aspirin (209±27). *Ex vivo* HR did not differ at any point. Neither drug treatment altered the incidence of VF or VT but NCX4016 tended to reduce the VPB count (180±88 vs. 545±305 in controls) whilst aspirin tended to increase the VPB count (805±654). Most importantly NCX4016 significantly reduced infarct size (47%±3 to 27%±5 of area at risk, $P<0.05$), aspirin had no effect (45%±3). Plasma salicylate levels one hour after the last dose for the NCX4016 and aspirin groups were 35±8 and 63±6 μg/ml respectively. Thromboxane B_2 (TxB_2) levels in activated plasma were not significantly different between controls and NCX4016 treated animals (8.8±0.9 vs. 8.1±0.9), but were significantly reduced by aspirin (1.6±0.3 $p<0.05$).

The reduced CPP in the NCX4016 treated group is a marker of improved myocardial function and is consistent with previous studies in rabbit hearts (Rossoni *et al.* 2000). The reduction in infarct size also demonstrates that the reduction in infarcts size seen in a previous study in normotensive rats (Rossoni *et al* 2001) is also evident in hypertensive hearts. While recent preliminary data on human subjects suggests that when given twice daily NCX4016 has an anti-platelet effect (Unpublished observations from Nicox S.A.), the present results indicate that the cardioprotective mechanism of NCX4016 is not related to cyclooxygenase inhibition.

Del Soldato *et al.* (1999). *TIPS.* 20, 319-323.

Rossoni *et al.* (2000). *Ital Heart J.* 1(2), 146-155.

Rossoni *et al* (2001). *J Pharmacol Exp Ther.* 297(1), 380-387.

SGB is supported by a studentship from NICOX S.A.

322P INHIBITION OF NUCLEAR FACTOR KAPPA-B DOES NOT MODIFY EARLY MYOCARDIAL REPERFUSION INJURY

A Kis, D M Yellon & G F Baxter. The Hatter Institute, UCL Hospitals and Medical School, London, WC1E 6DB

Nuclear factor kappa-B (NFκB) is a ubiquitously expressed transcription factor, located in the cytosol in inactive form. Its activation and translocation to the nucleus following various physiological and pathological stimuli has been associated with regulation of cell survival and cell death pathways. The aims of the present study were to examine if myocardial ischaemia-reperfusion activates and translocates NFκB and if such activation plays any role in myocardial reperfusion injury.

To examine if NFκB is activated by reperfusion, male New-Zealand White rabbits (2.0-3.0 kg) were anaesthetised with sodium pentobarbitone (30 mg kg^{-1}, iv) and ventilated. After thoracotomy, they were subjected to either 30 min left coronary artery occlusion (I), or to ischaemia followed by 10 min of reperfusion (I+R). Sham operated controls underwent surgery had no coronary occlusion (con). The non-specific NFκB inhibitor diethyldithiocarbamate (DDTC, 200 mg kg^{-1}) was given i.v. 5 min prior to reperfusion. Hearts were excised, the risk and non-risk area separated, and rapidly frozen in liquid nitrogen. NFκB activity in the risk zone was measured by electromobility shift assay (Xuan, 1999). We observed that ischaemia alone did not translocate NFκB whereas ischaemia plus 10 min reperfusion rapidly translocated NFκB into the nucleus. Translocation was inhibited by DDTC (see figure 1).

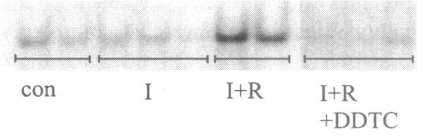


Figure 1. Representative electromobility shift assay blot

We next examined if NFκB activation at reperfusion contributes to myocardial injury *in vivo*. Anaesthetised rabbits underwent 30 min left coronary artery occlusion followed by 180 min reperfusion as described above. At the end of reperfusion the risk area was determined by Evans blue and the infarct was measured by triphenyltetrazolium staining and expressed as a percentage of the risk zone. Control infarct size was 51.5±3.8% (mean±s.e.m.) of risk zone (n=10). DDTC 200 mg kg^{-1} at reperfusion did not significantly modify infarct size (45.9±6.3%, n=6, unpaired t-test).

These studies were amplified in an *in vitro* preparation using SN50, a specific peptide inhibitor of NFκB. Adult male Sprague-Dawley rats (350-450g) were anaesthetised with pentobarbitone (50 mg kg^{-1}), the hearts excised and cardiac myocytes isolated (Jacobson & Piper, 1986). Myocytes were subjected to 45 min simulated ischaemia followed by 180 min re-oxygenation. SN50 (18 μM) was given at re-oxygenation. Cells were labelled with annexin V (AnnV) and propidium iodide (PI) and sorted by flow cytometry. AnnV labelling indicated early apoptosis whereas PI plus AnnV positive cells indicated necrosis. After simulated ischaemia-reoxygenation, 31.4±2.2% of control cells were necrotic and 34.2±3.2% were apoptotic. Treatment with SN50 at re-oxygenation changed neither the percentage of necrotic nor apoptotic cells (26.5±1.7% and 36.1±4.0%, respectively, one-way ANOVA).

We conclude that NFκB activation occurs rapidly at reperfusion. However, this activation is not associated with modulation of cell death during the first 3 hours of reperfusion. These data do not obviate a role of NFκB-mediated effects in later reperfusion which require further studies.

Jacobson, SL & Piper, HM, (1986). *J.Mol.Cell.Cardiol.* **18**(7):661-678.

Xuan, YT et al (1999). *Circ.Res.* **84**(9):1095-109

323P INHIBITING MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING: A NEW PARADIGM FOR MYOCARDIAL PRECONDITIONING?

D J Hausenloy, H L Maddock, G F Baxter & D M Yellon. The Hatter Institute, UCL Hospitals and Medical School, London WC1E 6DB.

The mechanism by which ischaemic preconditioning (IPC) protects the myocardium is unknown, though activating the mitochondrial K_{ATP} channel has been implicated (Garlid et al., 1997). It has been suggested that the mitochondrial permeability transition pore (MPTP) opens in the first few minutes of reperfusion, mediating the cell death of ischaemia-reperfusion injury by apoptosis/necrosis (Griffiths & Halestrap, 1995). Inhibition of MPTP opening at reperfusion with cyclosporin A, has been shown to be cardioprotective (Griffiths & Halestrap, 1993). We hypothesised that IPC or activating the mitochondrial K_{ATP} channel induce cardioprotection by inhibiting MPTP opening at reperfusion.

Male Sprague-Dawley rats (350-450 gms) were anaesthetised with pentobarbitone sodium (50 mg kg^{-1}, i.p.). Hearts were excised and Langendorff perfused at constant pressure with Krebs-Henseleit buffer and subjected to 35 minutes of left coronary artery occlusion and 120 minutes of reperfusion and assigned to the following groups (n=6-12): (1) control; (2) IPC of 2x5 minutes each of global ischaemia prior to lethal ischaemia; (3) 0.2 μM cyclosporin A (CsA, which inhibits MPTP opening) at reperfusion for 15 minutes; (4, 5) pre-treatment with 30 μM diazoxide (Diaz, a mitochondrial K_{ATP} channel opener) or 200 nM 2 chloro-N^6-cyclopentyl-adenosine (CCPA, an adenosine A1 receptor agonist); (6) 20 μM atractyloside (Atr, a MPTP opener) at reperfusion for 15 minutes; (7) IPC+Atr; (8) Diaz+Atr; (9) CCPA+Atr.

Infarct size was determined using triphenyltetrazolium stain and expressed as a percentage of the risk volume. Mitochondria were isolated from adult rat hearts according to a previously described technique (Johnson et al., 1999), and incubated with calcein for 15 minutes. Changes in mitochondrial calcein fluorescence were measured after 10 minutes incubation with calcium 500 μM in the presence or absence of Diaz (30 μM), CCPA (200 nM), CsA (0.2 μM) and with Atr (5 mM) alone. All values are expressed as mean ± SEM. Data were analysed by one-way ANOVA. Differences were considered significant when $P<0.05$.

IPC limited infarct size (19.9±2.6% vs 44.7±2.0% in control, $p<0.01$), as did inhibiting MPTP opening with CsA at reperfusion (24.6±1.9%, $P<0.01$), diazoxide pre-treatment (18.0±1.7%, $P<0.01$) and CCPA pre-treatment (20.4±3.3%, $P<0.01$). Opening the MPTP at reperfusion with atractyloside abolished this protection (47.7±1.8% in IPC+Atr, 42.3±3.2% in Diaz+Atr, 51.2±1.6% in CCPA+Atr). In mitochondria, Diaz (30 μmol/L) decreased calcium-induced MPTP opening from 37.5±4.0% to 18.4±4.1% ($P<0.05$). Atr opened the MPTP as shown by a reduction in calcein fluorescence of 40.1±3.1%. CsA was shown to block both calcium and atractyloside induced MPTP opening. CCPA, as one would expect, had no effect on calcium-induced MPTP opening, as it mediates its action via a cell-surface receptor.

This study suggests IPC or activating the mitochondrial K_{ATP} channel induce protection by preventing MPTP opening at reperfusion.

Garlid KD, Paucek V, Yarovy-Yarovy H, et al. (1997) *Circ Res* ;**81**:1072-1082.
Griffiths EJ & Halestrap AP. (1995) *Biochem J.***307**:93-98.
Griffiths EJ & Halestrap AP. (1993) *J Mol Cell Cardiol.* **23**:1351-1354.
Johnson N, Khan A, Virji S, et al. (1999) *Eur J Biochem.* **263**:353-359.

324P NEUROPEPTIDE Y INCREASES 4-AMINOPYRIDINE-SENSITIVE TRANSIENT OUTWARD POTASSIUM CURRENT IN RAT VENTRICULAR MYOCYTES

M.P. Heredia, M. Fernandez, G. Benito & C. Delgado (introduce by M.I. Colado). Institute of Pharmacology & Toxicology (CSIC-UCM), Univ. Complutense, 28040 Madrid, Spain.

Neuropeptide Y (NPY) is a potent vasoconstrictor. However, its cardiac actions are still under controversy (McDermott *et al.*,1993). Negative inotropic effects of NPY were demonstrated in adult rat cardiomyocytes and the opposite was shown in those of adult guinea-pig (Millar *et al.*, 1991). To explain this discrepancy Millar *et al.* (1991) have postulated that the negative inotropic effect of NPY in rat cardiomyocytes may be coupled to activation of a transient potassium outward current (I_{to}) given that a selective blocker of this current, 4–Aminopyridine (4-AP), abolishes the negative inotropic effect. Therefore the aim of this study is to analyze the modulation of NPY on 4-Aminopyridine-sensitive I_{to} (4-AP I_{to}) in adult ventricular rat myocytes including the NPY receptor subtype involved and the possible participation of an intracellular pathway that comprises a PTX sensitive G protein and PKC mechanisms.

Patch-clamp experiments were performed on enzymatically dissociated ventricular cells obtained from the apical part of the rat heart (Gomez *et. al.*, 1997). I_{to} was obtained by applying depolarizing pulses from a holding potential of –80 mV to voltage tests from –40 to +60 mV in 10 mV increments for 1000 ms at a frequency of 0.2 Hz. I_{to} amplitude was taken as the difference between the peak outward current and the current at the end of the pulse. 4-AP I_{to} was obtained by the subtraction of the currents obtained before and after 3 mM 4-AP application.
The current density-voltage curves showed that at any potential studied 4-AP I_{to} density was significantly higher in cells incubated 3-6 h with 100 nM NPY (n=16) compared with control cells (n=17) (Figure).

In the presence of BIBP 3226 an NPY receptor antagonist that binds selectively to NPY Y1-receptors (Rudolf *et al.*, 1994), the effect of NPY on 4-AP I_{to} density was maintained. However, in the presence of BIIE0246 a highly selective non-peptide NPY Y2-receptor antagonist (Doods *et al.*, 1999), NPY was unable to increase 4-AP I_{to}. In addition, the effect of NPY on 4-AP I_{to} density was prevented by pretreatment with 500 ng/ml pertussis toxin (PTX) and by the specific PKC inhibitor calphostin C (100 nM). Thus, short term exposure to NPY induces an increase of 4-AP I_{to} density in rat ventricular myocytes mediated by Y2 receptors and involving the action of PKC via a PTX-sensitive signalling cascade.

Doods, H.N. *et al.* (1999) *Eur. J. Pharmacol.* **384**, R3-R5.
McDermott, B.J. *et al.* (1993) *Cardiovasc. Res.* **27**, 893-905.
Millar, B.C. *et al.* (1991) *Am. J. Physiol.* **261**, H1727-H1733.
Gomez, A.M. *et. al.* (1997) *Am. J. Physiol.* **272**, H1078-H1086.
Rudolf, K. *et al.* (1994) *Eur. J. Pharmacol.* **271**, R11-R13.

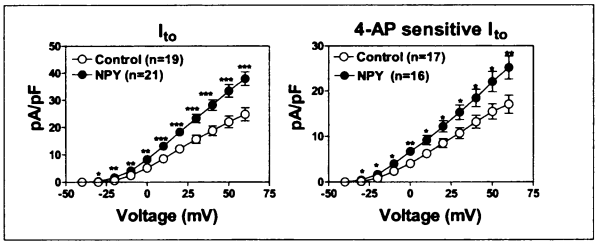


Figure. Graphs showing mean current density of I_{to} (left panel) and 4-AP-sensitive I_{to} (right panel) in control (open circles) and NPY-treated cells (closed circles). Results shown as mean ± s.e.mean. *P<0.05, **P<0.01, ***P<0.001.

CD thanks CICYT (SAF-99-0068) for support. Selective NPY receptor antagonists were gifts from Boehringer Ingelheim.

325P TYPE-B NATRIURETIC PEPTIDE LIMITS INFARCT SIZE IN RAT ISOLATED HEART

S P D'Souza, D M Yellon & G F Baxter. The Hatter Institute, UCL Hospitals and Medical School, London WC1E 6DB

Chronic haemodynamic overload leading to cardiac dilatation triggers the release of atrial natriuretic peptide (ANP) and type-B natriuretic peptide (BNP). ANP and BNP are abundant in cardiomyocytes, in the form of pro-peptides stored in secretory granules. Both peptides bind to natriuretic peptide receptor-A (NPR-A), and subsequent elevation of intracellular cGMP concentration mediates the endocrine actions of ANP and BNP, including vasodilatation and natriuresis. Acute ischaemia or hypoxia can evoke the rapid release of natriuretic peptides from cardiac tissue (Toth et al., 1994; Hama et al., 1995) but the local role of the peptides during ischaemia has not been defined. ANP was shown to be antiarrhythmic in dog heart subjected to acute coronary artery occlusion and reperfusion (Rastegar et al., 2000). We hypothesised that BNP, the predominant natriuretic peptide in ventricular myocardium, exerts an autocrine cytoprotective action during ischaemia, independent of systemic haemodynamic unloading.

Male Sprague Dawley rats (275-400 g) were anaesthetised with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and heparinised (300 i.u.). All hearts were excised and Langendorff perfused with Krebs-Henseleit buffer at 37 °C and subjected to 35 min left coronary artery occlusion and 120 min reperfusion. Control hearts received no further treatment. BNP was perfused at various concentrations commencing 10 min before ischaemia and continued until 30 min reperfusion. Infarct size was determined using triphenyltetrazolium stain and expressed as a percentage of the ischaemic risk zone.

Infarct size data are shown in figure 1.

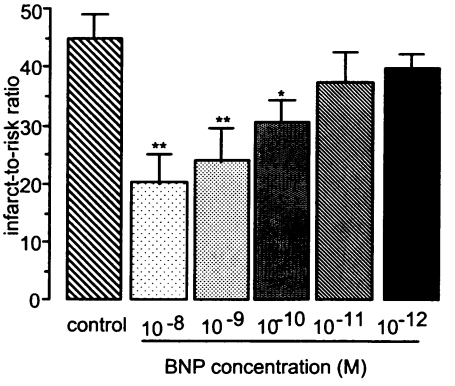


Figure 1. Infarct size. Bars represent mean ± s.e.m. *P < 0.05; ** P < 0.01 v control (1-way ANOVA). n = 8-12 per group

BNP limited infarct size in a concentration-dependent manner, with significant protection being seen at 10^{-8}, 10^{-9} and 10^{-10} M. The risk zone volume averaged 0.35 cm^3 for all the groups. There were no detectable differences in coronary flow rates at these concentrations, suggesting that the cytoprotective action of BNP was not mediated by coronary vasodilatation in this model. We conclude that BNP protects against acute ischaemia-reperfusion injury and may represent an innate cardioprotective response. The molecular mechanism of this previously undefined action of BNP is unknown.

Hama N et al. (1995), Circulation, 92, 1558-1564.
Rastegar A et al. (2000), Cardiovasc Drug Ther, 14, 471-479.

326P THE SIGNALLING MECHANISMS ACTIVATED CONCOMITANTLY WITH THE PROLIFERATIVE OR APOPTOTIC RESPONSES EVOKED BY OXYSTEROLS IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECs)

T. Cindrova[1], J.N. Skepper[2] & L.A. Sellers[3], (introduced by T.-P.D. Fan[3]). Departments of Pathology[1], Anatomy[2] and Pharmacology[3], University of Cambridge.

Oxidation of dietary lipids contributes to the pathogenesis of atherosclerosis. Oxysterols, lysophospholipids and reactive aldehydes are the main toxic components of oxidised low density lipoprotein (oxLDL). OxLDL has a dual effect on cells of the vascular wall, promoting both growth and cell death. Its cytotoxicity has been largely attributed to the oxysterol components (Lizard *et al.*, 1999). Whether oxysterols are pro-atherogenic, anti-atherogenic or benign *in vivo* is unresolved. Here, we exposed HUVECs to oxysterols at a range of concentrations, including 7-ketocholesterol (5 $ngml^{-1}$ - 80 μgml^{-1}), 7-β-OH-cholesterol (1 $ngml^{-1}$ - 80 μgml^{-1}) and 27-OH-cholesterol (1 $ngml^{-1}$ - 80 μgml^{-1}).

High concentrations of oxysterols induced cell death by apoptosis, which was confirmed in live experiments using confocal microscopy. The majority of dying HUVECs stained with Annexin-V-FITC and only subsequently became permeable to propidium iodide (PI). Only a small proportion of dead cells was necrotic (initially staining with PI). Immunofluorescent studies revealed a decrease in cytochrome c in cells treated with oxysterols (compared to non-treated controls). A high proportion of cells stained with anti-active caspase-3 and caspase-9 antibodies, indicating apoptotic death was by the mitochondrial pathway. Nucleosome staining was also increased in oxysterol-treated cells, giving additional confirmation that death was by apoptosis.

The signalling pathways associated with the oxysterol-induced cell death were examined by Western analysis of whole cell extracts (Fig. 1A). Compared to basal levels, all oxysterols induced a marked decrease in phospho-ERK after treatment for 3 hr. A transient increase in phospho-p38 was detected after 1 hr, and surprisingly a sustained phosphorylation of Akt at Ser473 was also detected. Phospho-PKC (pan) levels increased after 3 hr and again were sustained. Total protein levels remained unaffected. Phospho-ERK down-regulation was also con-firmed by immunofluorescent staining.

In contrast, proliferation of serum-starved HUVECs was induced by low concentrations of all three oxysterols as determined by an increase in BrdU incorporation. An increase in phospho-ERK was observed 20 min after treatment (Fig. 1B), whereas Akt and PKC activities remained essentially unchanged.

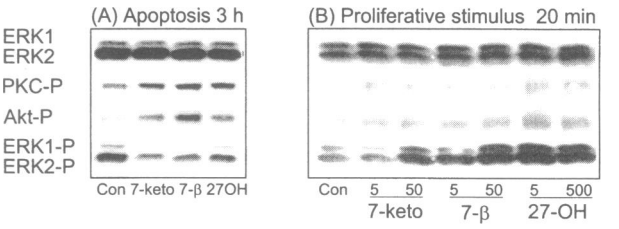


Figure 1. Effect of 7-ketocholesterol (7-keto), 7-βOH-cholesterol (7-β) and 27-OH-cholesterol (27-OH) on the phosphorylation status of PKC, Akt, ERK1 and ERK2 in HUVECs. (A) Treatment with 40 μgml^{-1} or vehicle control (Con) (compounds were dissolved in serum-rich medium). (B) Serum-starved HUVECs treated with oxysterols ($ngml^{-1}$).

In conclusion, oxysterols can mediate opposing effects on HUVEC survival, depending on the administered concentration. Associated with the induction of apoptosis was a marked and sustained decrease in ERK activity, whereas rapid ERK phosphorylation was evoked by concentrations of oxysterols that promoted cell proliferation. Elucidating the mechanisms involved in the biphasic effects of oxysterols will be important for the future therapeutic management of atherosclerosis.

Lizard, G., Monier, S. Cordelet, C., *et al.* (1999). *Arterioscler Thromb Vasc Biol.* 19. 1190-1200

327P INHIBITION OF FURIN ATTENUATES TYPE I COLLAGEN–INDUCED MMP-2 ACTIVATION IN CARDIAC FIBROBLASTS

C. Guo & L. Piacentini. School of Pharmacy, De Montfort University, Leicester LE1 9BH

Activation of cardiac metalloproteinase-2 (MMP-2) has been implicated in ventricular remodelling after myocardial infarction. Furin, a subtilisin/Kex2p-like pro-protein convertase that is up-regulated after myocardial infarction (Sawada *et al,* 1997), is necessary for MMP-2 activation in some cell types (Zigrino *et al,* 2001). We have previously shown that treatment of cardiac fibroblasts with concanavalin A (Con A) activates MMP-2 (Guo and Piacentini, 2001). We have also observed that these cells secrete active MMP-2 when seeded into a type I collagen lattice. In this study, we investigated the effects of a synthetic specific furin inhibitor, Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (FI), on MMP-2 activation in cardiac fibroblasts. Primary cultures of adult rat cardiac fibroblasts were prepared by enzymatic dissociation of left ventricular tissue from male Sprague-Dawley rats (150-350 g). Confluent monolayers of cells were serum-deprived for 48 h and then pretreated with FI (0-100 μM) for 6 hrs before addition of Con A (10 $\mu g\ ml^{-1}$) for a further 18 hrs. Alternatively, suspended cells were preincubated with FI (0-100 μM) for 20 min and then added to a type I collagen lattice solution to give a final collagen concentration and cell density of 1 mg ml^{-1} and 5×10^5 cells ml^{-1}, respectively. The cell-populated polymerised collagen lattices were incubated with FI for a further 48 hrs. MMP activity in media samples and whole cell lysates was assessed using gelatin-zymography followed by densitometric analysis of the lytic bands. Where appropriate, results are expressed as mean ± s.e.m. Each experiment was repeated at least three times using independent cell populations. Statistical analysis between FI- treated and control groups was performed using repeated measure ANOVA followed by Dunnet's post test. FI-treatment of cells seeded into collagen lattices significantly reduced the ratio of active MMP-2 to total MMP-2 gelatinolytic activity (A/T ratio) measured in the cell lysate (figure 1; n= 3, $P<0.05$). A similar concentration-dependent pattern of MMP-2 activity was observed in cell-conditioned media. In contrast, Con A-induced activation of MMP-2 was not modulated by concurrent treatment with FI (figure 1, n=3). These findings suggest that, consistent with findings in human dermal fibroblasts (Zigrino *et al,* 2001), type I collagen-induced activation of MMP-2 in cardiac fibroblasts is partially dependent on furin.

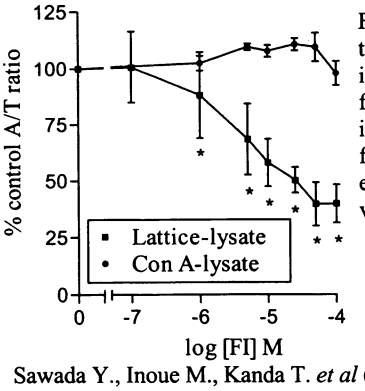


Figure1. Ratio of active to total MMP-2 lytic activity in cell lysates from cardiac fibroblasts treated as indicated. The A/T ratio for each FI concentration is expressed as % control values. * $P < 0.05$.

Sawada Y., Inoue M., Kanda T. *et al* (1997). *FEBS Lett.* 400, 177-182

Zigrino P., Drescher C., Mauch C. (2001). *Eur. J. Cell. Biol.* 80, 68-77

Guo, C., Piacentini, L. (2001). *Br. J. Phamacol.*133, 132P.

328P PROSTAGLANDIN I_2 RECEPTOR-STIMULATION BLOCKS ENDOTHELIN-1-INDUCED ERK PHOSPHORYLATION AND THE SUBSEQUENT PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELLS

R.-C. Li, T. Cindrova, T.-P. D. Fan, J.N. Skepper & L.A. Sellers. Departments of Pharmacology and Anatomy, University of Cambridge, Cambridge CB2 1QJ

Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays a major role in atherogenesis. Prostaglandins are reported to attenuate VSMC proliferation (Umemura *et al.*, 1997; Todaka *et al.*, 1999), but the mechanisms through which this response is mediated are poorly defined. Here we have investigated the effect of the prostacyclin mimetic, iloprost, on the proliferation of rat aortic VSMCs induced by endothelin-1 (ET-1) and fibroblast growth factor-2 (FGF-2). In addition, concomitant changes in the phosphorylation status of key signalling effectors were also identified.

The EP4 receptor-selective agonist, 11-deoxy-PGE1 (1μM) induced a time-dependent phosphorylation of PKC (pan) as well as ERK1 and ERK2 in serum-starved VSMCs, whereas the EP2 receptor agonist, butaprost (1μM), was without effect. Iloprost (1μM) slightly inhibited basal levels of ERK activity in serum-starved VSMCs, but failed to have any effect on that evoked by submaximal concentrations of serum (0.5%). ET-1 (100nM) induced a rapid phosphorylation of PKC, ERK1 and ERK2 in serum-starved VSMCs which remained elevated over basal throughout the duration of the study (4 hr). Iloprost (1μM) had no effect on basal PKC activity or on that evoked by ET-1. The initial activation of ERK induced by ET-1 (over 15 min) was unaffected by co-application with iloprost. However, iloprost inhibited the sustained phase of ET-1-induced ERK phosphorylation and the level obtained after 1 hr fell below basal levels (Fig 1). Similar findings were obtained following co-application of iloprost on the evoked phosphorylation of ERK by FGF-2 (10ng/ml). The ET-1-induced sustained activation of ERK was also abolished by the MEK1 inhibitor, PD98059 (20μM). The expression levels of ERK and PKC were unaffected by all treatments over the time course studied. Confirmation of the inhibitory effect on ET-1 induced ERK phosphorylation by iloprost was obtained by immunofluorescent labelling using confocal microscopy.

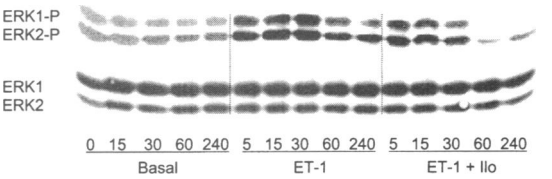


Figure 1. Effect of iloprost (ilo; 1μM) on ET-1-induced (100nM) phosphorylation of ERK1 and ERK2 in serum-starved VSMCs. Changes in the phosphorylation status of ERK were determined using phospho-specific antibodies (top panel). Total protein remained unaffected over the time course studied (shown in min). Data representative of 3 experiments.

An increase in the number (values $x10^3$; n=3) of serum-starved VSMCs was observed 24 hr following application of ET-1 (1μM) (24±2 to 38±1*) which was abolished either by PD98059 (20μM) (25±2#) or iloprost (1μM) (26±1#). Neither drugs had any effect on basal cell numbers (23±2 and 24±3, respectively). (*P<0.01 vs basal; #P<0.01 vs ET-1, Student's t Test).

In conclusion, VSMC proliferation induced by ET-1 is critically dependent on the evoked ERK activity. Stimulation of the IP receptor blocked both responses mediated by ET-1, suggesting the inhibitory mechanism through which prostacyclin can regulate VSMC growth is via the supression of ERK activity. Stimulation of ERK by a EP_4 receptor agonist, suggests that prostaglandins may have a diverse and complex role in VSMC physiology. Elucidating the mechanisms involved in the regulation of VSMC proliferation will be important for future therapeutic management of atherosclerosis. [R-CL and TC thank the Cambridge Overseas Trust for support.]

Todaka, T., Yokoyama, C., Yanamoto, H., *et al.* (1999). Stroke, 30, 419-426.
Umemura, K., Watanabe, S., Kondo, K., *et al.* (1997). Atherosclerosis, 130, 11-16.

329P ROLE OF PHOSPHATIDYLINOSITOL 3-KINASE IN α_2-ADRENOCEPTOR-MEDIATED VASOCONSTRICTION AND ERK ACTIVATION IN ISOLATED PORCINE PALMAR LATERAL VEIN.

R.E Roberts. Institute of Cell Signalling, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH.

Previous studies have shown that the Erk-MAP kinase pathway is involved in α_2-adrenoceptor-mediated vasoconstriction (Roberts, 2001). How α_2-adrenoceptors stimulate the Erk pathway is not clear. One possible mechanism is through activation of phosphatidylinositol 3-kinase (PI 3-K; Hawes *et al.*, 1996). Therefore, the aim of this study was to determine whether PI 3-K is involved in α_2-adrenoceptor-mediated vasoconstriction and Erk activation.

Trotters from pigs of both sexes were obtained from a local abattoir. Palmar lateral veins were dissected into 5 mm segments and mounted in a tissue bath containing Krebs-Henseleit buffer maintained at 37°C, and gassed with 95% O_2/5% CO_2. Contractions were measured using an isometric force transducer linked to a MacLab data acquisition system. After reproducible responses to 60 mM KCl were obtained, cumulative concentration-response curves to the selective α_2-adrenoceptor agonist UK14304 were obtained in the absence or presence of LY294002, an inhibitor of PI 3-K (Vlahos *et al.*, 1994; 1-50 μM in 0.1% DMSO added 1 h beforehand). Alternatively, segments were exposed to a single, maximum concentration of UK14304 (10 μM), in the absence or presence of 50 μM LY294002. After contractions had reached a plateau (3-4 min), tissues were removed from the tissue baths, rapidly frozen, then homogenised. Proteins were separated by SDS-PAGE, and levels of phosphorylated and total Erk, and phosphorylated and total Akt were determined by immunoblotting. Bands were measured by densitometry.

UK14304 produced large contractions of the porcine palmar lateral vein (maximum 81.6 ± 8.9 % of 60 mM KCl response, mean ± s. e. mean, n=12). These were significantly inhibited by 50 μM LY294002 (maximum response in the presence of 50 μM LY294002 25.5 ± 6.8 % of 60 mM KCl response, n=12, p<0.001, ANOVA followed by Bonferroni test). A single concentration of UK14304 (10 μM) caused a significant increase in phosphorylation of Akt, an enzyme downstream of PI 3-K (143.7 ± 17.2 % of control levels, n=12, p<0.01, Student's paired t-test). Taken together, these data suggest that PI 3-K is involved in α_2-adrenoceptor-mediated vasoconstriction. UK14304 (10 μM) also caused a significant increase in phosphorylation of Erk (184.7 ± 27.5 % of control levels, n=6, p<0.025, Student's paired t-test). This phosphorylation was significantly inhibited in the presence of 50 μM LY294002 (97.8 ± 18.3 % of control levels, n=6, p<0.025, Student's paired t-test) suggesting that PI 3-K is upstream of Erk activation. This study demonstrates that α_2-adrenoceptor-mediated vasoconstriction in porcine palmar lateral vein is associated with activation of PI 3-K, and that this activation is upstream of Erk activation.

Hawes, B. E., Luttrell L. M., van Biesen T. *et al.* (1996). *J. Biol. Chem.* **271**, 12133-12136.
Roberts R. E. (2001) *Br. J. Pharmacol.* **133**, 859-866.
Vlahos C. J., Mather W. F., Hui K. Y. *et al.* (1994) *J. Biol. Chem.* **269**, 5241-5248.

Supported by The Wellcome Trust.

330P REGULATION OF EXTRACELLULAR RECEPTOR KINASE 1/2 IN HUMAN VASCULAR SMOOTH MUSCLE CELLS BY G-PROTEIN COUPLED P2Y RECEPTORS AND PDGF RECEPTORS

R. Kumari[1,2] & M.R. Boarder[1]. [1]The Cell Signalling Laboratory, Department of Biological Sciences, De Montfort University, Leicester and [2]Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, U.K.

Regulation of intimal smooth muscle proliferation is a key event in the development of vascular diseases such as atherosclerosis and restenosis following angioplasty. We have previously (White *et al* 2000) shown PDGF to act as a mitogen, increasing DNA synthesis in human saphenous vein vascular smooth muscle cells (VSMC) and also increasing extracellular receptor kinase 1&2 (ERK1/2), which is a member of mitogen activated protein kinase family (MAPK). ATP and UTP have been shown to be mitogenic in rat vascular smooth muscle cells (VSMCs) (Harper *et al*, 1998), but in human VSMC we have reported that in cultured human VSMCs UTP displays anti-proliferative properties and ATP displays pro-proliferative effects (White *et al* 2000). This study further investigates the role of ERK 1/2 in the nucleotide signalling pathways leading to control of proliferation in human VSMCs.

Primary cultures (p3-p7) of human SV VSMCs were grown in 6 or 96 well plates and serum starved at 80% confluence for 48 hours. For western blot cells were stimulated for 5-60 min at 37°C with agonists. Samples were prepared in ice-cold lysis buffer, separated by electrophoresis and blots probed with anti-phospho specific ERK antibodies. [^{3}H] Thymidine incorporation into DNA was used as an index of proliferation following a 1-hour stimulation with agonists. Cells were pre-incubated with inhibitors for 30 minutes where indicated. Data from blots was collected by directly imaging chemi-illuminesence using ECL reagent. One way ANOVA was used to statistically analyse the data.

PDGF increased ERK phosphorylation in a dose dependant manner (range 0.03-3nM PDGF). 1nM PDGF significantly increased levels of ERK phosphorylation (397.4±42.5% of basal $P<0.05$ n=3). UTP and ATP (each at 300μM) alone had no influence on both basal and PDGF induced ERK phosphorylation after 5 minutes of stimulation. However ATP potentiated the PDGF response when cells were stimulated for 40-60 minutes with both ATP and PDGF present (485.1±129.9% of PDGF response, $P<0.05$). This is consistent with previously reported increase in DNA synthesis when cells were stimulated for 60 minutes with ATP and PDGF. Increasing ATP concentrations (range 3μM-1mM) potentiated the ERK response generated by 1nM PDGF in a dose dependent manner (EC_{50}=3.77±13.7μM, n=3, $p<0.05$). UTP did not influence ERK activity stimulated by PDGF in this way.

PD98059 is an inhibitor ERK1/2 pathway. Cells were pre-incubated with increasing concentrations of PD98059 (range 3μM-1mM) prior to stimulation with 1nM PDGF. The increase in ERK phosphorylation produced by PDGF was inhibited in a dose dependent manner in the presence of this inhibitor (IC_{50}=58.9±7.5μM, n=3, $p<0.001$). PD98059 also inhibited [^{3}H] thymidine incorporation into DNA stimulated by PDGF in a dose dependent manner (IC_{50}=39.09±19.25μM, n=3, $P<0.001$). Increases in ERK activation and DNA synthesis generated by co-stimulation with ATP and PDGF were also inhibited.

These results suggest that the ERK1/2 pathway is necessary for the coupling of P2Y receptors stimulated by ATP to the control of proliferation in human VSMCs. UTP influences are independent of the ERK pathway.

Harper, S., Webb, T.E., Charlton, S.J., Ng, L.L. *et al.* 1998. *Br. J. Pharmacology, 124(4), 703-710*
White, P.J., Kumari, R., Porter, K.E., London, N.J.M *et al.* 2000. *Amer. J. Physiology, 279, H2735-H2742.*

331P P2Y RECEPTOR REGULATION OF HEPATOCYTES: DISTINCT ACTION OF ADP AND 2-METHYLTHIO ADP

C.J. Dixon, J.F. Hall & M.R. Boarder. Cell Signalling Laboratory, Department of Biological Sciences, De Montfort University, Leicester, UK.

Hepatocyte function is known to be regulated by multiple P2Y receptors. Previous results suggest that ADP and 2MeSADP act via $P2Y_1$ receptors, giving a different cytosolic Ca^{2+} response to that elicited by ATP (Dixon et al, 1995; Dixon 2000). Here, we have studied the (poly)inositol phosphate response to ADP, and the role played in this response by agonist interconversion in the extracellular compartment.

Hepatocytes were prepared by collagenase digestion of rat livers (Dixon et al, 1995) and cultured in 24-well culture dishes, with William's medium E. Total [^{3}H]inositol (poly)phosphates ($InsP_x$) were measured in [^{3}H]inositol labelled cells in the presence of lithium. Agonists were added to cells without change of medium and were present for 20 min. Data are dpm of [^{3}H]$InsP_x$ stimulated by agonists minus unstimulated controls (mean ± s.e.mean, n = 3). Hexokinase was included in the incubation at 3 units/well where indicated. In parallel experiments agonist interconversion was monitored by HPLC analysis of medium 20 min after adding agonists.

Stimulation of [^{3}H]inositol labelled cells with ADP led to a concentration-dependent increase in $InsP_x$ which was partial compared to the response to ATP at the maximal concentration (300 μM) used: ADP, 3675 ± 48; ATP 4062 ± 27 ($P<0.01$). UTP and ITP also gave substantial concentration-dependent increases in $InsP_x$ formation. However, 2-methylthio ADP (2MeSADP) failed to stimulate, or gave a much smaller maximal stimulation of $InsP_x$ accumulation (e.g. control, 474 ± 45; 300 μM 2MeSADP, 992 ± 115). To investigate a role for $P2Y_1$ receptors in the ADP response we used the selective antagonist A3P5P at 100 μM: this had no significant effect on the concentration response curve for ADP stimulation of $InsP_x$ accumulation. To ask whether ADP conversion to ATP is involved in the response to ADP, hexokinase treatment was used to remove ATP. Hexokinase altered the ADP concentration-response curve to ADP ($P<0.01$, 2-way ANOVA), reducing the response to 100 μM (ADP 3368 ± 7, ADP + hexokinase 1457 ± 238; $P<0.01$). HPLC analysis showed the nucleotide agonists used (e.g. ATP, 2MeSADP, UTP, UDP) were all stable during incubation with the cells for 20 min under the conditions of our experiments with the exception of ADP, which was very actively converted to AMP and ATP in the extracellular compartment. Hexokinase reduced or eliminated the ATP concentration, forming more AMP: e.g. 20 min incubation of 300 μM ADP: no hexokinase - AMP 125 μM, ADP 125 μM, ATP 94 μM; with hexokinase – AMP 160 μM, ADP 68 μM, ATP, 14 μM.

These results suggest that the response of cultured hepatocytes to ADP is not via the $P2Y_1$ receptor. Hepatocytes show a remarkable and selective capacity to modify ADP in the extracellular space, forming ATP and AMP (presumably by extracellular adenylate kinase), and the response of the cells is likely to be a consequence of the resulting mixture of extracellular nucleotides.

Dixon, CJ, Cobbold, PH & Green AK (1995) *Br J Pharmacol* 116, 1979-1984
Dixon, CJ (2000) *Br. J. Pharmacol.* 130, 664-668.

332P A BIOINFORMATICS ANALYSIS OF THE STRUCTURE AND EXPRESSION OF THE A-TYPE ENDOTHELIN RECEPTOR (EDNRA) GENE

Nigel Brand[1], Robina Azam[2], Alistair Pate[2], Magdi H. Yacoub[1,2] & Adrian H. Chester[2]. Cardiothoracic Surgery, N.H.L.I., Faculty of Medicine, Imperial College of Science, Technology & Medicine, [1]Dovehouse Street, London SW3 6LY and [2]Heart Science Centre, Harefield Hospital, Harefield, Middlesex UB9 6JH Email: n.brand@ic.ac.uk;

The endothelin receptor A gene (EDNRA) is a single copy gene subject to alternative mRNA splicing which resides on chromosome 4 in man and has been previously mapped as an eight-exon gene spanning more than 40-kb of DNA (Hosada *et al*, 1992). Alternative splicing of the EDNRA transcript theoretically allows the expression of several novel protein isoforms. We have begun to quantify the expression of alternative splice isoforms in human vascular tissue by using RealTime PCR and have taken a bioinformatics approach to determine more information about the EDNRA gene.

EDNRA alternative transcripts were analysed by reverse transcription-polymerase chain reaction (RT-PCR) using gene-specific primers and total mRNA from human saphenous vein as template. GenBank nucleotide databases were screened at the NCBI website (http://www.ncbi.nlm.nih.gov/) using the BLAST search tool. Protein sequences were predicted from DNA sequences using the BCM search launcher site (http://searchlauncher.bcm.tmc.edu/).

RT-PCR reveals that saphenous vein expresses several smaller EDNRA transcripts in addition to the wild type (i.e. full-length) mRNA. By scanning expression sequence tag (EST) cDNA databases we were able to identify 10 ESTs comprising partial cDNA sequences derived from the EDNRA wild-type mRNA. Suprisingly, a number of these contain genomic DNA from intron 2, suggesting mis-splicing at the junction (i.e. exon2/intron 2 junction) occurs frequently. None of the ESTs represent alternatively spliced isoforms, including other possible combinations that could be generated by splicing of exons downstream of exon 4, suggesting that alternative splicing is a rare occurence. We have localized the EDNRA gene on two large (>150 kb) genomic DNA clones deposited in the *high throughput genome sequencing* (*htgs*) database. Our analysis confirms that the EDNRA gene is dispersed over 50 kb of DNA. Over 20 SNPs (single nucleotide polymorphism) have been recorded for the EDNRA, most of which fall outside the protein coding region.

We have accurately determined the size of the EDNRA gene by aligning two large genomic DNA clones deposited in the human databases with gene and cDNA structure data originally reported by Hosada *et al* (1992). Comparison of our sequence with an entry for a 1.1 Mb chromosome 4 working draft sequence containing the EDNRA locus, deposited in the database by the NCBI Annotation Project, suggests that the prediction of gene structure by computer-based assembly of large overlapping genomic DNA sequences should be qualified by information derived by other, more "bench-based" means.

This study is funded by the British Heart Foundation (PG 2000020) and Harefield Research Foundation

Hosada K. *et al* (1992) *J. Biol. Chem.* 267: 18797-804.

333P PHARMACOLOGICAL CHARACTERISATION OF RECEPTOR-MEDIATED AND STORE-DEPENDENT CALCIUM INFLUX IN THE HUMAN PROMYELOCYTIC LEUKAEMIC CELL LINE (HL60)

P. Bahra, C.T. Poll, J. Westwick & S.W. Li. Novartis Respiratory Research Centre, Horsham, West Sussex, RH12 5AB. UK.

Receptor agonists such as purinergic nucleotides and formyl-methionyl leucyl phenylalanine (fMLP) stimulate intracellular calcium (Ca^{2+}) release and extracellular Ca^{2+} influx through plasma membrane Ca^{2+} channels in HL60 cells (Cowen *et al.*, 1989 & Demaurex *et al.*, 1992). These stimuli activate a non-selective cation current (Krautwurst *et al.*, 1993) in HL60 cells suggesting that this current may play a role in Ca^{2+} entry in these cells. This current was potently inhibited by the non-selective cation channel (NSCC) blocker LOE 908 (IC_{50} = 40nM, Krautwurst *et al.*, 1993). Lanthanum (La^{3+}) has also been reported to be a cation channel blocker in HL60 cells (Demaurex *et al.*, 1992). The aim of this study was to compare the effect of the putative Ca^{2+} and cation channel blockers SK&F 96365 (Merritt *et al.*, 1990), LOE 908 and the lanthanide cations, La^{3+} and gadolinium (Gd^{3+}), on receptor-stimulated (UTP) and store-dependent (thapsigargin) Ca^{2+} influx in HL60 cells using the Ca^{2+} re-addition protocol.

Changes in intracellular free Ca^{2+} concentration were measured at room temperature (RT) in Fluo-4 (Molecular Probes)-loaded HL60 cells (ATCC, $1.5x10^5$ per well) in 96 well plates using FLIPR (FLuorescence Imaging Plate Reader, Molecular Devices). Cells were stimulated with maximally effective concentrations of UTP or thapsigargin (inhibitor of endoplasmic reticulum Ca^{2+}-ATPase) in Hanks Buffered Salt Solution containing 0.07mM $CaCl_2$ and 1mM $MgCl_2$ (Bahra *et al.*, 2001). Ca^{2+} influx (measured as area under the curve) was quantified following addition of 1mM extracellular $CaCl_2$ ($[CaCl_2]_{ex}$) 130 seconds after the cells were stimulated with UTP (10μM) or thapsigargin (1μM). Cells were pre-treated with LOE 908 (QuChem, Northern Ireland), SK&F 96365, $LaCl_3$ or $GdCl_3$ (Sigma Aldrich) for 2 min (RT) prior to stimulation with UTP or thapsigargin. Results are expressed as mean IC_{50} values or percentage inhibition ± s.e.mean and n represents the number of individual experiments performed. Statistical significance was assumed when $P<0.05$ using an unpaired Student's t-test.

SK&F 96365, Gd^{3+} and La^{3+} inhibited UTP (10μM)-stimulated Ca^{2+} influx with IC_{50} values of 2.2±0.2μM (n=3), 316±10μM (n=3) and 808±62μM (n=3) respectively. LOE 908 only partially inhibited the response reaching a maximum inhibition of 47.3±2.1% (100μM, $P<0.05$ compared with the control UTP response, n=3). A similar profile of inhibition for these channel blockers was observed for thapsigargin (1μM)-stimulated Ca^{2+} influx with IC_{50} values for SK&F 96365, Gd^{3+} and La^{3+} of 1.8±0.3μM (n=3), 537±80μM (n=3) and 817±76μM (n=3) respectively. The maximum inhibition achieved by LOE 908 against thapsigargin-stimulated Ca^{2+} influx was 21.5±5.5% (100μM, $P>0.05$ compared with the control thapsigargin response, n=3).

The results of this study suggest that similar Ca^{2+} entry pathways are activated by UTP and thapsigargin in HL60 cells. These data also indicate that other Ca^{2+} permeable channels may be activated in HL60 cells in addition to LOE 908-sensitive NSCCs (Krautwurst *et al.*, 1993). Further pharmacological characterisation of these channels could be addressed by the combined use of channel blockers such as SK&F 96365 and LOE 908, as previously described in rat aortic smooth muscle cells (A7r5, Iwamuro *et al.*, 1999).

Bahra, P., Poll, C.T., Westwick, J. *et al.*, (2001) *Br. J. Pharmacol.* In press.
Cowen, D.S., Lazarus, H.M., Shurin, S.B. *et al.*, (1989) *J. Clin. Invest.* **83(5)**, 1651-1660
Demaurex, N., Lew, D.P., Krause K.H. (1992) *J. Biol. Chem.* **267**, 2318-2324
Iwamuro, Y., Miwa, S., Zhang, X.F. *et al.*, (1999) *Br. J. Pharmacol.* **126**, 1107-1114
Krautwurst, D., Hescheler, J., Arndts, D. *et al.*, (1993) *Mol. Pharmacol.* **43**, 655-659
Merritt, J.E., Armstrong, P.W., Benham, C.D. *et al.*, (1990) *Biochem. J.* **271**, 515-522

334P DEVELOPMENT OF A LUCIFERASE REPORTER GENE HIGH THROUGHPUT SCREEN FOR 7-TRANSMEMBRANE RECEPTOR ANTAGONIST IDENTIFICATION

L. Jenkinson[1], J. Stables[2], S. Rees[2], R. Vivier[2], J. Coote[3], S. Brown[4], A. Begum[4], A. Wise[1] & S. Wilson[1]. [1]Systems Research, [2]Molecular Screening and [3]GEPB, GSK Medicines R&D Centre, Stevenage, Herts, SG1 2NY; [4]Neurology CEDD, GSK, Harlow North, Third avenue, Essex CM19 5AW.

The aim of this work was to establish high throughput 384-well luciferase reporter gene assays suitable for antagonist identification at the C5a and EP1 7-transmembrane receptors (7TMR). The nuclear factor activation of T-cells (NFAT) luciferase gene reporter assay has been developed to detect agonist-mediated activation of 7TMR (Alam and Cook). In this assay the NFAT transcription factor binding site is positioned upstream of the luciferase gene. Upon Gq-coupled 7TMR activation and subsequent calcium mobilisation, NFAT is dephosphorylated, binds to the NFAT transcription factor binding site leading to luciferase expression. Luciferase catalyses the oxygenation of luciferin to luminescent oxyluciferin, enabling its detection. Co-expression of the promiscuous G-protein $G\alpha_{16}$ in this assay system has enabled non-Gq coupled receptors to be examined. Agonist assays in this format have proved to be robust and reliable and have been used as a basis to develop similar systems to detect antagonist compounds.

Chinese hamster ovary cells stably expressing the NFAT-luciferase reporter gene were further transfected with cDNA encoding C5a or EP1 receptor genes. For the C5a receptor cell line the host cells also stably expressed $G\alpha_{16}$. Cells were grown to 80% confluency and quiesced for 24 hours in serum free media, harvested, washed and plated into 384-well plates preloaded with agonist and antagonist. Following a 4 hour incubation at 37°C luciferase accumulation was measured.

During assay optimisation, cells were shown to tolerate 0.6% DMSO and performed better when grown in flasks compared to roller bottles. Optimal cell density for C5a cells was $1x10^5$ cells/ml and for EP1 $3x10^5$ cells/ml. Luminometer read times were optimised to 15 and 30 seconds for the EP1 and C5a assays respectively. C5a and EP1 receptor agonists (C5a and PGE2) both caused concentration-dependent increases in luciferase accumulation with pEC50s (n= minimum of 3) of 8 and 8.5 for PGE2 and C5a respectively. In addition receptor selective antagonists at each receptor both caused concentration-dependent inhibition of C5a and PGE2 stimulated luciferase accumulation with pIC50s (n=minimum of 3) of 6.5 and 8.5 respectively.

To examine assay robustness and potential performance in HTS mode a random set of 1280 compounds was tested in duplicate and the data generated used to predict the hit rate. Hit rates from these compound sets were 12% for EP1 and 29% for C5a. Correlation of inhibition rate between the cell lines was low suggesting that a parallel screening strategy could not be used to identify and exclude non-specific hits.

Although the NFAT luciferase reporter gene assay provides a sensitive, homogeneous assay ideal for high throughput screening the predicted high hit rates and difficulties in validating any hit compounds by parallel screening preclude the use of such an assay for screening purposes.

Alam, J. & Cook, J. (1990). Anal. Biochem., 188, 245-254

335P 5-$HT_{2C\ (VSV)}$ RECEPTOR-MEDIATED ACTIVATION OF INOSITOL PHOSPHATE ACCUMULATION AND ARACHIDONIC ACID RELEASE IN CHO CELLS: NO EVIDENCE OF AGONIST-DIRECTED TRAFFICKING

C.J. Dimech, [1]N.H. Allen, [1]R.H.P. Porter, [1]M.J. Sheardown & P.G. Strange. School of Animal and Microbial Sciences, University of Reading, P. O. Box 228, Whiteknights, Reading, RG6 6AJ and [1]Vernalis Research Ltd, Oakdene Court, 613 Reading Road, Winnersh, Wokingham, RG41 5UA.

The 5-HT_{2C} receptor has been reported to couple independently to the stimulation of inositol phosphate (IP) accumulation and the activation of arachidonic acid (AA) production (Berg *et al.*, 1994). Recently, it was demonstrated that the rank order of agonist efficacy at the human 5-HT_{2C} receptor was dependent on which of the two responses was measured (Berg *et al.*, 1998). A subset of agonists preferentially activated IP accumulation, and another preferentially activated AA release, providing experimental support for the hypothesis of agonist-directed trafficking of receptor stimulus (Kenakin, 1995). The purpose of this study was to investigate this observation.

Human 5-HT_{2C} receptors (VSV isoform) were stably expressed in Chinese hamster ovary (CHO) cells. Cells were seeded in 12 well plates and labelled with 1 μCi ml^{-1} myo-[^{3}H]inositol for 24 hours and with 0.1 μCi ml^{-1} [^{14}C]arachidonic acid for 4 hours in the same well, at 37 °C. Cells were washed twice with Hank's buffered salt solution supplemented with 20 mM HEPES, 20 mM LiCl and 0.1% fatty acid free BSA (experimental medium). Cells were then treated with either vehicle or drug for 25 minutes at 37 °C. Extracellular medium was removed for measurement of [^{14}C] content with liquid scintillation counting. 10 mM formic acid (4 °C) was added for 30 minutes to extract the accumulated [^{3}H]inositol phosphates. The extract was applied to ion-exchange columns (containing AG 1-X8 formate dowex). [^{3}H]mono-inositol phosphates ([^{3}H]IP_1) were eluted from the column with 0.2 mM ammonium formate / 0.1 M formic acid. The [^{3}H] content of the [^{3}H]IP_1eluate was quantified with liquid scintillation counting. GraphPad Prism was used to analyse data.

The potencies (pEC_{50}) and efficacies (% of 5-HT) of a series of agonists to elicit [^{3}H]IP_1 accumulation and [^{14}C]AA release coupled to the activation of 5-$HT_{2C\ (VSV)}$ receptors expressed in CHO cells were measured, and are summarised in Table 1.

The rank order of pEC_{50} values for [^{3}H]IP_1 accumulation was 5-HT > mCPP > DOI > TFMPP > LSD > quipazine > MK212, and for [^{14}C]AA release was 5-HT > mCPP > LSD DOI TFMPP > MK212 quipazine. The rank order of efficacy values for [^{3}H]IP_1 accumulation was 5-HT MK212 > mCPP TFMPP > DOI quipazine > LSD, and for [^{14}C]AA release was 5-HT > MK212 > mCPP > TFMPP > quipazine > DOI > LSD.

Table 1. Agonist stimulation of [^{3}H]IP_1 accumulation and [^{14}C]AA release in CHO cells expressing the 5-$HT_{2C\ (VSV)}$ receptor. Data are expressed as mean ± s.e.m, n 3. * $p<0.05$ versus [^{3}H]IP_1 accumulation, paired student's t-test.

	pEC_{50}		Efficacy (% of 5-HT)		Hill slope	
Drug	IP	AA	IP	AA	IP	AA
5-HT	7.90 ± 0.03	7.83 ± 0.04	100	100	1.1 ± 0.1	1.2 ± 0.1
MK212	6.08 ± 0.05	6.07 ± 0.06	98.5 ± 8.8	83.8 ± 2.9*	1.1 ± 0.1	1.4 ± 0.3
mCPP	6.83 ± 0.10	6.82 ± 0.02	64.3 ± 3.5	61.7 ± 2.3	1.7 ± 0.4	1.7 ± 0.4
TFMPP	6.61 ± 0.06	6.58 ± 0.09	61.5 ± 4.0	48.8 ± 2.3*	1.0 ± 0.0	1.0 ± 0.0
quipazine	6.25 ± 0.23	6.06 ± 0.20	39.3 ± 3.7	35.6 ± 9.7	1.8 ± 0.4	1.3 ± 0.4
(±)-DOI	6.69 ± 0.06	6.63 ± 0.10	42.3 ± 3.8	27.3 ± 5.0*	1.0 ± 0.0	1.0 ± 0.0
LSD	6.47 ± 0.28	6.66 ± 0.22	19.6 ± 2.6	14.1 ± 1.0	1.0 ± 0.0	1.0 ± 0.0

All agonists tested displayed similar potencies for the stimulation of IP_1 accumulation and AA release, and the rank orders of potencies for the two responses were very similar. All the agonists were more efficacious at stimulating IP_1 accumulation than AA release. However, no agonist was capable of preferentially activating AA release compared to IP_1 accumulation. As such, these results do not support the idea that agonists are capable of selectively trafficking the cellular responses coupled to the 5-$HT_{2C\ (VSV)}$ receptor expressed in CHO cells.

Berg K.A. *et al.* (1994). *Mol. Pharmacol.* **46**, 477-485.
Berg K.A. *et al.* (1998). *Mol. Pharmacol.* **54**, 94-104.
Kenakin T. (1995). *Trends Pharmacol Sci.* **16**, 232-238.

This work was supported by a BBSRC CASE studentship.

336P THE EFFECTS OF (-)-ISOPRENALINE BUT NOT OF (-)-CGP12177 ARE MARKEDLY REDUCED AT GLY-389 β_1-ADRENOCEPTORS COMPARED TO ARG-389 β_1-ADRENOCEPTORS

S.S. Joseph[1], J.A. Lynham[1], A.A. Grace[2], W.H. Colledge[1] & A.J. Kaumann[1]. Departments of [1]Physiology and [2]Biochemistry, University of Cambridge, U.K.

Several clinically used β-adrenoceptor (βAR) blocking agents, designated non-conventional partial agonists (NCPA), cause cardiostimulant effects at concentrations considerably greater than those that antagonise the effects of catecholamines (Kaumann, 1989). The agonist effects of NCPA are resistant to blockade by (-)-propranolol. (-)-CGP12177 ((-)-4-(3-tertiary butylamino-2-hydroxypropoxy) benzimidazol-2-one), an experimental NCPA, increases cyclic AMP through both recombinant β_1AR and β_2AR (Pak & Fishman, 1996). The cardiostimulant effects of (-)-CGP12177 persist in β_2AR knockout mice but are absent in β_1AR/β_2AR double knockout mice, indicating an obligatory role of β_1AR (Kaumann et al., 2001). β_1AR of normal individuals present a polymorphism at amino acid position 389, Arg or Gly, with allele frequency 0.74 and 0.26 respectively. (-)-Isoprenaline stimulates the adenylyl cyclase more markedly through recombinant Arg-389 β_1AR than through recombinant Gly-389 β_1AR (Mason et al., 1999). To investigate whether polymorphism affects the (-)-propranolol-resistant state, we compared the effects of (-)-isoprenaline and (-)-CGP12177 on recombinant Arg-389 β_1AR (327±8 fmol.mg^{-1}) and Gly-389 β_1AR (340±6 fmol.mg^{-1}), transfected into CHO cells. Cyclic AMP was determined in CHO cells exposed to 1 mM 3-isobutyl-1-methylxanthine. Basal cyclic AMP levels were not different in CHO cells expressing the Arg-389 β_1AR (18.3±2.4 pmol.mg^{-1}) or Gly-389 β_1AR(18.4±2.2 pmol.mg^{-1}). Concentration-effect curves were determined. Data of table 1 were from 5 independent assays, carried out in duplicate for each group. Maximum effects, E_{max}, were expressed as multiples of basal cyclic AMP. E_{max} for (-)-isoprenaline was reduced 12 times more than the E_{max} of (-)-CGP12177 at the Gly-389 β_1AR, compared to the Arg-389 β_1AR.

Table 1 Agonist responses and potencies (mean ± s.e.m.)

β_1AR	(-)-Isoprenaline		(-)-CGP12177	
	-logEC_{50}	E_{max}	-logEC_{50}	E_{max}
Arg-389	7.93±0.15	97.5±8.9	7.74±0.16	6.3±0.9
Gly-389	9.58±0.18	3.4±0.4	8.11±0.15	2.7±0.3

Compared to Arg-389 homozygous individuals, homozygous Gly-389 individuals may exhibit more reduced β_1AR responses to catecholamines than to NCPAs.

Supported by the British Heart Foundation.

Kaumann, A.J. (1989) *Trends Pharmacol. Sci.* **10**, 316-320.

Kaumann, A.J. et al. (2001) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **363**, 87-93.

Mason, D. A. et al. (1999) *J. Biol. Chem.* **274**, 12670-12674.

337P SINGLE NUCLEOTIDE POLYMORPHISMS IN THE HUMAN ADENOSINE A_{2B} RECEPTOR GENE

Gino van Heeke, Rachael Seamons, Jean-Yves Metais, John R. Fozard, Stephen Goff[+], Amanda Wheatley* & Ian P. Hall*. Novartis Horsham Research Centre, Horsham, UK. [+]Torrey Mesa Research Institute, La Jolla, CA, USA, *Division of Therapeutics, University Hospital of Nottingham, NG7 2UH, UK.

A role for adenosine in asthma has been postulated for many years. Of particular significance is the marked hyperreactivity of the airways of asthmatics to adenosine which shows a close relationship to the underlying disease process. Adenosine manifests its wide spectrum of pharmacological activities by activation of cell surface receptors of which four subtypes (A_1, A_{2A}, A_{2B}, A_3) are recognised. Of these, it is the A_{2B} receptor which is present in human lung mast cells and is believed to be responsible for the release of mediators and the synergistic interaction between adenosine and allergen (Peachell *et al.*, 1991). On this basis the A_{2B} receptor has been implicated in the pathophysiology of asthma (Feoktistov *et al.*, 1998). Single nucleotide polymorphisms (SNPs) are essential genetic markers for disease association studies and pharmacogenetic analyses. While the literature indicates SNPs in the A_{2A} receptor (Deckert *et al.*, 1996), there is no such information on genetic variation in the A_{2B} receptor gene. The aim of this study was to analyse and identify SNPs in the human A_{2B} receptor and define their prevalence in a Caucasian population.

Genomic DNA sequencing and PCR were used as the basis for SNP identification. Genomic DNA was isolated from 10 mL of peripheral blood from 96 volunteers recruited on-site at the Novartis Horsham Research Centre. Eleven pairs of oligonucleotide primers were designed to amplify overlapping fragments covering the entire A_{2B} receptor coding sequence, the intron-exon boundaries, the 5' and 3' UTR, and an additional 960 bp of the region immediately upstream of the first exon. Ten ng of amplified and purified DNA was used in a double-strand cycle-sequencing protocol using ABI BigDye™ reagents and the reaction products separated on an automated ABI 377 DNA sequencer. Raw DNA sequence data was analysed using the PHRED/PHRAP software package for automated base calling and sequence assembly, and viewed using CONSED. DNA sequence variants were identified by visual inspection of each individual sequence trace and comparison to its complementary strand read and to the consensus sequence determined by CONSED.

The data revealed that in this population of 96 subjects (192 chromosomes) the A_{2B} receptor does not contain any SNPs in the cDNA sequence nor in the intron-exon boundaries. In contrast, the promoter region of the gene contains three SNPs. One was found at position –466 at a frequency of 4.2% and introduced a *BamHI* restriction enzyme site (GGCTCC to GGATCC). A second one, which eliminated a *BbrPI* restriction enzyme site, occurred at position –696 (CCACGTG to CCATGTG). The third was detected at position –679 (GAACCT to GAGCCT). SNPs 2 and 3 each occurred at a frequency of 2.1%.

The coding region of the A_{2B} receptor does not encode any SNPs, however, two of the 3 promoter SNPs create a restriction fragment length polymorphism (RFLP) which will be a useful diagnostic tool for future association studies.

Deckert, J.*et al.* (1996) *J. Neural Transm.*, **103**, 1447-1455.

Feoktistov, I. *et al.* (1998) *Trends Pharmacol. Sci.*, **19**, 148-153.

Peachell, P.T. *et al.* (1991) J. *Pharmacol. exp. Ther.*, **256**, 717-726.

338P IDENTIFICATION OF REGULATORY SHORT OPEN READING FRAMES (SORFS) IN THE HUMAN HISTAMINE H1 RECEPTOR GENE

Caroline Swan & Ian P. Hall, Division of Therapeutics, University Hospital of Nottingham, Nottingham NG7 2UH.

Regulation of expression of the human histamine H1 receptor gene in airway epithelium and airway smooth muscle is potentially important in the pathogenesis of allergic responses in the airways. The aims of the studies described here were (i) to determine the arrangement of the human H1 receptor gene 5'untranslated region (5'UTR) and (ii) to define potential regulatory elements within the gene.

A previous study (De Backer & Loonen, 1998) described a 5.8 kb intron in the H1 receptor gene. We used genomic DNA and cDNA from human airway smooth muscle cells (HASM) (Daykin & Widdop, 1993) to determine the genomic arrangement of this region. In addition to confirming the presence of the 5.8kb intron at –36 bp from the start codon, we found an additional 364 bp intron, the splice site being present at –122 bp in the cDNA sequence. This sequence was also found on Genbank clone GI14278703. Four further differences to Genbank entry AJ000742 (De Backer & Loonen) were observed in cDNA from HASM, a human airway epithelial cell line (BEAS2B) and human placenta. These were G at –6 bp; A at –81bp; A deletion at –92bp; G deletion at –101. In addition, 13 bp of non-genomic polylinker sequence were found at the 5' end of the published sequence. Updated sequence has been submitted to Genbank (AF420434). The 5' UTR of this gene also contains two potential short open reading frames (SORFs) commencing at positions –144 and –85bp.

To test the potential regulatory effects of the SORFs four constructs containing 198 bp of the H1 gene promoter sequence, plus the 5'UTR, cloned upstream of a luciferase reporter gene were made. One construct contained 'wild type' cDNA and three others cDNA in which either one, or both, of the SORF start codons had been altered to CTG to prevent expression. Transfections were performed in BEAS2B cells and primary cultures of HASM using a *Renilla* luciferase reporter gene vector to control for variations in transfection efficiency. Mutation of each of the SORF ATGs resulted in an increase in reporter gene expression in BEAS 2B cells. The more 5' SORF knockout caused a 1.28 $\pm$0.04 fold increase in expression (all figures mean $\pm$ SEM), whereas the more 3' SORF knockout produced a 1.53 $\pm$0.03 fold increase. Mutation of both SORF ATGs in the same construct resulted in a 2.26 $\pm$0.07 fold increase cf the wild type construct (n=8, $p<0.01$ for all changes by one way Anova with Bonferroni's correction). A similar trend was seen in parallel experiments performed using the same constructs in primary cultures of HASM, (1.47 fold $\pm$ 0.26; 1.42 fold $\pm$ 0.43; 2.18 fold $\pm$ 0.33 respectively), although changes did not reach statistical significance, probably due to the inherent variability in reporter gene expression seen in transfections in these cells.

These data suggest that 5' UTR SORFs may play a negative regulatory role in expression of the human H1 receptor gene in airway cells. The potential importance of these mechanisms to levels of H1 receptor expression in the airways remains to be determined.

Daykin, K., Widdop, S., et al. Eur. J. Pharmacol. (Mol. Pharm.) 264 135-140 (1993).
De Backer, M.D., Loonen, I., et al. Biochem. J. 335 663-670 (1998).

339P CHARACTERISATION OF THE HUMAN HISTAMINE H_1 RECEPTOR IN BEAS2B CELLS

Julie Hawley, Randolph Corteling & Ian P Hall. Division of Therapeutics, Queens Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK.

Histamine is an important inflammatory mediator in human airways. It is able to contract human airway smooth muscle via stimulation of H_1 receptors and increase epithelial permeability (Hill *et al.*, 1997). The BEAS2B cell line is derived from human bronchial epithelium and has many characteristics of human airway epithelial cells ex vivo (Penn *et al.*, 1994). The aim of the current study was to define the effects of stimulation of BEAS2B cells with histamine and to characterise the histamine receptor present on these cells.

BEAS2B cells were cultured in DMEM + 10% foetal calf serum. Responses to agonists were measured in 24 well culture plates as previously described in cells prelabelled with [^{3}H]-myo-inositol (Hawley *et al.*, 1995). The effects of histamine on intracellular Ca^{2+} were studied in single BEAS2B cells loaded for 30min with Fura 2 AM (6µM): data were analysed using the Improvision software package.

Initial studies showed that BEAS2B cells demonstrated a 56.5±7.4 fold increase in total [^{3}H]-inositol phosphate production in response to histamine (100µM, 30min) (EC50 = 7.8µM, n=4). In contrast, no increase in [^{3}H]-inositol phosphate formation was observed following stimulation with bradykinin (1µM), UTP (1mM), 5-HT (1mM) or carbachol (1mM). NaF (20mM) produced a 34.8±5.0 fold increase cf basal (n=4). The time course of total [^{3}H]-inositol phosphate formation was essentially linear for periods of up to 30 min, and all further experiments were therefore performed at this time point.

In order to define the histamine receptor subtype underlying this response, BEAS2B cells were preincubated with a range of antagonists for 30 min prior to stimulation with histamine (100µM). The table shows the Kd values obtained for the antagonists studied. Tiotidine (1µM) and thioperamide (100nM) were without effect (n=4). Stimulation of Fura 2 AM loaded BEAS2B cells with histamine resulted in an increase in intracellular free Ca^{2+} (EC50 = 30µM, n = 17). The response to 100µM histamine was inhibited by preincubation with mepyramine (10µM).

Antagonist Kd(nM)	*Mean*	*SEM*	n
(+)Chlorpheniramine	12.46	5.03	4
Promethazine	2.82	0.19	4
Triprolidine	1.11	0.13	4
Chlorpromazine	3.42	2.36	4
Chlorcyclizine	2.25	0.35	4
Mepyramine	2.97	0.32	4
Ketotifen	0.006	0.002	4

These data demonstrate the presence of an H_1 receptor coupled to phospholipase C in BEAS2B cells and suggest this cell line may be a useful model system to study regulation of histamine H_1 receptors in an airway epithelial cell system.

J.Hawley *et al.*, (1995) Biochem. Pharmacol, 49(7);1005-1011.
S.J.Hill *et al.*, (1997) Pharmacol Rev, 49(3);253-278.
R.B Penn *et al.*, (1994) Am J Respir Cell Mol Biol, 11(4);496-505.

340P INVESTIGATION INTO THE CORRELATION BETWEEN EFFICACY AND K_L:K_H AFFINITY RATIO FOR THE DOPAMINE D_{2S} RECEPTOR

E.J. Spragg, S.J. Charlton & C.M. Scaramellini Carter. Novartis Horsham Research Centre, West Sussex, RH12 5AB.

It has been hypothesised (Kent *et al.*, 1980) that the ratio of the low affinity binding constant, K_L, to the high affinity binding constant, K_H, gives a measure of efficacy for agonists at G protein coupled receptors (GPCRs). We tested eight D_2 agonists at the D_{2S} receptor (CHO-D_{2S} membranes) in both a functional assay (GTPγS binding), and a radioligand binding assay. In several studies (e.g. Egan *et al.*, 2000) K_L has been determined by displacement of antagonist radioligand, and K_H by displacement of agonist radioligand. In this study displacement of an antagonist radioligand by agonist resulted in biphasic displacement curves, from which both K_L and K_H could be determined.

GTPγS binding was determined using a scintillation proximity assay (SPA). Membranes (10 μg) were incubated (30-90 min, room temperature) with agonist, in a total volume of 200 μl, in the presence of 300 pM [^{35}S]GTPγS and 5 μM GDP, together with wheatgerm agglutinin coated poly(vinyltoluene) SPA beads (1.5 mg). After centrifugation (377 g, 10 min) the plate was read on TopCount. The binding of dopamine at the D_{2S} receptor was studied by competition against 2 nM [^{3}H]methyl-nspiperone (10 μg membrane, in a total volume of 200 μl, 2 hr incubation, room temperature). The incubation was terminated by filtration of membranes onto GF/C filter plates, using a Brandel harvester. Scintillant was added, and the plate read on TopCount.

Of the eight D_2 agonists tested, only five gave clearly biphasic curves (F test, $p<0.05$ vs. one site binding curve, GraphPad Prism 3.0), from which K_L and K_H could be determined. Attempts to relate efficacy in the functional assay, with K_L:K_H in the radioligand binding assay did not result in any correlation, suggesting that in the D_{2S} receptor system K_L:K_H does not give a measure of efficacy. Attempts to obtain a correlation between K_L:K_H and efficacy at the dopamine D_{2L} receptor have also failed (Gardner & Strange, 1998).

Egan C. *et al.* (2000) *Synapse*, **35**: 144-150
Gardner B. & Strange P.G. (1998) *Brit. J. Pharmacol.*, **124**: 978-984
Kent R.S. *et al.* (1980) *Mol. Pharmacol.*, **17**: 14-23

Table 1. Agonist properties in functional assay ([^{35}S]GTPγS binding) and in radioligand binding assay. Values are mean ± sem, n = 3.
Abbreviations used:
NPA, R-(-)-propylnorapomorphine
PPHT, [±]-2-[N-phenylethyl-N-propyl]-amino-5-hydroxytetraline

ligand	p[A]50	% efficacy	pK_i	pK_L	pK_H	K_L:K_H
NPA	8.6 ± 0.2	115.8 ± 3.3		6.8 ± 0.2	8.4 ± 0.3	39.8
pergolide	8.2 ± 0.1	57.5 ± 6.0	6.6 ± 0.2	-	-	-
PPHT	8.0 ± 0.1	50.4 ± 5.5		6.1 ± 0.2	7.7 ± 0.2	39.8
lisuride	7.6 ± 0.2	25.0 ± 1.5	6.4 ± 0.5	-	-	-
apomorphine	7.5 ± 0.0	61.1 ± 5.6		5.7 ± 0.0	7.6 ± 0.3	79.4
bromocriptine	7.3 ± 0.2	40.8 ± 3.6	6.8 ± 0.2	-	-	-
dopamine	6.5 ± 0.0	93.0 ± 3.1		4.4 ± 0.1	6.2 ± 0.4	63.1
ropinerole	6.0 ± 0.1	61.0 ± 7.2		4.2 ± 0.3	6.6 ± 0.3	251.2

341P AFFINITY ESTIMATES FOR DARIFENACIN, TOLTERODINE AND OXYBUTYNIN AT M_2 AND M_3 MUSCARINIC RECEPTORS IN MEMBRANE AND INTACT CHINESE HAMSTER OVARY CELL PREPARATIONS

Carl P. Nelson[1], Paul Gupta[2], Carolyn M. Napier[2], R.A. John Challiss[1], [1]Dept. of Cell Physiology & Pharmacology, University of Leicester, Leicester LE1 9HN, and [2]Pfizer Central Research, Sandwich, Kent, CT13 9NJ.

The M_2 muscarinic acetylcholine (mACh) receptor is the predominant subtype in the bladder, but it is the minority M_3 subtype that functionally mediates bladder contraction (Eglen *et al.*, 1996) and is a therapeutic target in the management of overactive bladder (OAB). The aim of this study was to characterize the activities of selective and non-selective mACh receptor antagonists at cloned human M_2 and M_3 receptors.

All studies were carried out in Chinese hamster ovary (CHO) cells that recombinantly express human M_2 or M_3 mACh receptors. [^{3}H]-*N*-methylscopolamine was used in competition assays, involving either intact cell monolayers or membrane preparations, to generate displacement curves for each mACh receptor antagonist (Table 1). Functional studies in intact cell monolayers were performed to assess (*i*) inhibition of [^{3}H]-inositol phosphate accumulation stimulated by acetyl-β-methylcholine (MCh; 3 μM) in CHO-m3 cells; or (*ii*) reversal of the inhibition of forskolin-stimulated adenylyl cyclase activity caused by MCh (1 μM) in CHO-m2 cells. IC_{50} values were used to calculate K_i values from the binding data, and binding constant (K_b) estimates from the functional data (Craig, 1993).

In radioligand binding experiments in cell membranes, atropine and tolterodine were non-selective, while darifenacin (39.4-fold) and oxybutynin (8.2-fold) displayed selectivity for the M_3 versus M_2 receptor subtypes (Table 1). Only darifenacin was M_3 selective in intact cells, and in general, antagonist affinities at both receptor subtypes were diminished. In functional assays, darifenacin was again found to be the most M_3 selective antagonist (23.9-fold) followed by the weakly M_3 selective oxybutynin (2.4-fold) (Table1).

Table 1 mACh receptor antagonist K_i and K_b values: mean (s.e.m.), in nM, from ≥3 experiments.

		Membranes		Intact cells	
		M_2	M_3	M_2	M_3
Atropine	K_i	1.02 (0.23)	0.92 (0.32)	0.74 (0.14)	3.95 (0.94)
	K_b	-	-	0.48(0.14)	0.94 (0.31)
Darifenacin	K_i	24.9 (7.0)	0.63 (0.06)	103 (12)	7.95 (1.81)
	K_b	-	-	54.4 (17.6)	2.27 (0.11)
Oxybutynin	K_i	10.3 (2.5)	1.26 (0.20)	66.0 (15.8)	94.3 (34.7)
	K_b	-	-	58.9 (9.8)	24.2 (1.3)
Tolterodine	K_i	1.97 (0.29)	1.77 (0.55)	3.42 (0.78)	22.4 (4.7)
	K_b	-	-	3.05 (0.70)	7.8 (1.76)

In conclusion, all of the mACh receptor antagonists exhibited higher affinities for M_2 and M_3 mACh receptors in isolated membrane preparations than in intact cells. Oxybutynin displayed M_3 selectivity in membranes, but appeared non-selective in intact cells. Tolterodine was non-selective in membrane assays, and M_2 selective in intact cells. Darifenacin was M_3-selective in both binding and functional assays. The selectivity of darifenacin for M_3 over M_2 mACh receptors may reduce the incidence of tachycardia when treating OAB, and overall, produce a superior clinical profile.

We thank Rajendra Mistry for assistance with the cyclic AMP and [^{3}H]-inositol phosphate assays.

Craig, D.A. (1993) *Trends Pharmacol. Sci.*, **14**, 89-91.
Eglen, R.M., Hegde, S.S., Watson, N. (1996) *Pharmacol. Rev.*, **48**, 531-565.

342P LIDOCAINE BLOCK OF Nav 1.3 IS MODULATED BY CO-EXPRESSION OF β1 AND β3 SUBUNITS

B.Shah, A.E. Dinn*, J.E.John*, R.D. Pinnock, K. Lee & M.K. Patel*. Pfizer Global Research & Development, Cambridge, CB2 2QB, U.K. *Dept. Internal Medicine, (Cardiovascular) University of Virginia HSC, Charlottesville, VA, USA.

Lidocaine, a local anaesthetic causes blockade of voltage gated sodium channels via a complex mechanism including a tonic (resting) and a phasic (use-dependant) block (Hille 1977). These processes may be modulated by sodium channel auxiliary subunits. In view of this, we have examined the effect of lidocaine on the embryonic Nav 1.3 expressed alone or in combination with β1 and β3 subunits in *Xenopus* oocytes.

Oocytes were microinjected with Nav1.3 with and without β1 and β3 cRNA. Two-electrode voltage clamp recordings were performed within 3-6 days. Oocytes were continually perfused with ND96 solution. Lidocaine, dissolved in ND96, was applied by continuous perfusion. Averaged data are presented as means ±S.E.M. Statistical analyses were performed using a Student's unpaired t test; $P<0.05$ considered significant.

Lidocaine dose response curves were generated on Nav 1.3 currents elicited by depolarising steps from –100mV to –10mV (n=3-6). Lidocaine inhibited Nav1.3 alone with an EC50 of 1.2 ± 0.2mM. β3 co-expression caused a shift in the EC50 to 4.9 ± 2.3mM whilst β1 co-expression caused a greater shift of EC50 to 10.7 ± 2.9mM ($P<0.05$ compared with Nav 1.3 alone). Hill slopes for Nav 1.3 and Nav 1.3 + β1 were –1.03 ± 0.28 and –0.99 ± 0.02 respectively indicating a single binding site. In contrast, the Hill slope for Nav 1.3 + β3 was found to be –0.75 ± 0.08 suggesting more than one independent binding site. Lidocaine (100μM) shifted $V_{1/2}$ for Nav 1.3 by -9.2 ± 2.7mV. A Similar shift in $V_{1/2}$ was seen with β3. Interestingly, a greater shift in $V_{1/2}$ (-22.0 ± 2.1mV) was seen with β1 co-expression ($P<0.01$ compared with Nav 1.3 alone and Nav 1.3 + β3). Lidocaine (1mM) reduced the percentage of fast mode recovery of Nav 1.3 by 39.5±1% which was greater than that for β1 and β3 co-expression (63.5±3%, 62.2±1% respectively; $P<0.005$ compared with Nav 1.3 alone). The fast time constant (τ_1) was significantly reduced ($P<0.05$) with β1 co-expression (52.3+/-12ms) compared to Nav 1.3 alone (93.4±24) and β3 co-expression (95.1±2ms). Lidocaines use-dependant actions were to significantly ($P<0.05$) reduce the proportion of channels gating with a slower time constant. Proportion of slow gating in Nav 1.3 was reduced by lidocaine (1mM) from 93 ± 10% to 63 ± 12%. Quantitatively similar gating changes were observed with Nav1.3 + β3 (from 94 ± 11% to 66 ± 8%) and Nav1.3 + β1 (from 36 ± 17% to 15 ± 17%).

In conclusion, we have shown that β1 and β3 co-expression differentially modulates the effects of lidocaine on Nav 1.3 channels. Lidocaines effects were more pronounced with β3 compared to β1. Since, β1 and β3 mRNA have different patterns of distribution in rat brain, DRG and spinal cord lidocaine may be used specifically to target certain types of sodium channel complex in these tissues.

Hille B (1977). J Gen Physiol 69; 497-515

343P ACTIVATION KINETICS OF RAT AND HUMAN KV2.1 POTASSIUM CHANNEL: AN N TERMINAL DETERMINANT

Min Ju, Louisa R Rashleigh, Sue J Ormond & Dennis Wray, School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK

The human and rat forms of the Kv2.1 potassium channel have identical amino acids over the membrane-spanning regions, but differ only in the N and C terminal sequences (Frech et al., 1989; Albrecht *et al.*, 1993). However, the activation of rat Kv2.1 is much faster than for the human channel. In the N-terminal region there are only two amino acids that are different between the two channels (at positions 67 and 75). Here, we have investigated a possible role of these two N-terminal residues (Q and E respectively for rat, E and D respectively for human) (Leadbitter & Wray, 2000).

For this, cDNA mutants were made using PCR-based site-directed mutagenesis corresponding to Q67E and E75D in the rat channel, and E67Q and D75E in the human channel. cRNAs for these mutants and for the wild type channels were injected into *Xenopus* oocytes, and two-electrode voltage-clamp recordings were made 1-2 days later at room temperature. Depolarising pulses were applied at 0.1Hz from a holding potential of –80mV.

The current/voltage curves for wild type and mutant channels were not significantly different from each other whether for rat or human.

Mutation E67Q in the human channel did not significantly affect the 10-90% activation time as compared with human wild type at test potentials of 0mV to 70mV. On the other hand, mutation D75E in the human channel showed faster activation time than for human wild type (eg at 0mV test potential, 72±6ms, n= 9 human wild type; 38±6ms, n=12 human D75E, means ± SEM, $P<0.05$, t-test). This suggests that, for the human channel (ie with human C-terminus), residue 75 is important in determining activation time.

On the other hand, for the rat channel, mutation Q67E in the rat channel showed a slowing of activation as compared with rat wild type (eg at 0mV, 43 ± 17ms, n=9 rat wild type; 153 ± 6ms, n=9 rat Q67E, $P<0.05$), while there was no change in activation time for rat mutant E75D as compared with rat wild type. Thus, for the rat channel (ie with rat C-terminus), it is residue 67 that is important for activation kinetics.

These results suggest that both of the residues located in the N-terminal region are of importance in determining the activation kinetics of the Kv2.1 channel, but that the nature of the C terminus (ie whether rat or human) is also important.

We thank the Wellcome Trust for support.

References

Albrecht, B., Lorra, C., Stocker, M. & Pongs, O. (1993) *Receptors and and Channels*, **1**, 99-110.

Frech, G.C., VanDongen M.J., Schuster, G., Brown, A.M. & Joho, R.H. (1989) *Nature*, **340**, 642-645.

Leadbitter, E. & Wray, D. (2000) *J. Physiol.*, **527**, 122P.

344P CHARACTERISING ATP RELEASE FROM EACH VARICOSITY ON A NERVE TERMINAL BRANCH ON AN IMPULSE-TO-IMPULSE BASIS IN THE MOUSE ISOLATED VAS DEFERENS

K.L. Brain, S.J. Trout & T.C. Cunnane, University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK

A technique to detect simultaneously the release of the neurotransmitter ATP from individual varicosities on the same nerve terminal branch has recently been developed (Brain *et al.*, 2001). Here we present further studies on the characteristic features of release on an impulse-to-impulse basis.

Mice (Balb/C) were humanely killed. The Ca^{2+} indicator Oregon Green 488 BAPTA-1 10 kD dextran was applied to the cut end of the isolated vas deferens for 8 - 10 hours to load nerve terminals orthogradely (Brain & Bennett, 1997) and to load smooth muscle cells. The preparations were examined using a Leica inverted confocal microscope, with a temporal resolution of either 200 ms or 400 ms per frame. The Ca^{2+} concentration in the smooth muscle cells ($[Ca^{2+}]_{sm}$) and nerve terminal varicosities ($[Ca^{2+}]_v$) was monitored simultaneously. Each action potential always elicited a $[Ca^{2+}]_v$ transient. However, the release mechanism in each varicosity was only activated intermittently and produced a focal $[Ca^{2+}]_{sm}$ transient in the adjacent smooth muscle cell. These transients result from the release of a packet of ATP and have been termed Neuroeffector Ca^{2+} Transients (NCTs; Brain *et al.*, 2001).

The probability of detecting a NCT following each field stimulus (P_{NCT}) applied at a frequency of 1 Hz was 60 ± 20% greater than at 0.1 Hz stimulation (number of smooth muscle cells, $n_{sm} = 8$; number of preparations, $n_p = 5$; $P < 0.05$). This frequency-dependent facilitation of P_{NCT} was greater in those varicosities with a low P_{NCT} at 0.1 Hz stimulation (130 ± 30% facilitation from those sites with P_{NCT} 0.02; 38 ± 16% facilitation from those sites with $P_{NCT} > 0.02$; unpaired two-tailed t-test $P < 0.05$). This difference was significant even after correction for regression to the mean.

When stimulating at 1 Hz, the competitive α_2-adrenoceptor antagonist yohimbine (10 μM) increased P_{NCT} by 55 ± 21% ($n_{sm} = 4$; $n_p = 3$; $P < 0.05$). This finding implies that NA was released from or close to the varicosities responsible for the purinergic NCTs and that secretory varicosities are equipped with functional α_2-adrenoceptor-mediated inhibitory mechanisms. In some smooth muscle cells, the occurrence of a NCT was strongly correlated with the simultaneous occurrence of a NCT in an adjacent smooth muscle cell (Fisher's Exact $P < 0.001$; number of pairs = 6; $n_p = 4$). The existence of such synchronous NCTs suggests that locally released ATP acts on more than one muscle cell, implying that autonomic 'junctions' in some instances may involve more than one smooth muscle cell. Spontaneous $[Ca^{2+}]_v$ transients were occasionally observed which were synchronous with spontaneous NCTs in the underlying smooth muscle cell ($n_{sm} = 4$; $n_p = 3$). These observations suggest that spontaneous elevations in $[Ca^{2+}]_v$ sometimes precede and therefore presumably initiate neurotransmitter release.

This technique now allows the study of autonomic neurotransmission with an unprecedented spatial resolution.

Brain, K.L. & Bennett, M.R. (1997). *J. Physiol.* **502**:521-536.
Brain, K.L. *et al.* (2001), J.Physiol. in the press.

345P EFFECTS OF MDMA AND CATHINONE ON NORADRENALINE EVOKED CONTRACTIONS OF RAT RIGHT VENTRICLE

Robert Buber, Linda Cleary & James R. Docherty. Department of Physiology, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland

We have recently shown that methylenedioxymethamphetamine (MDMA) and cocaine significantly increase the potency of noradrenaline, but not isoprenaline, at augmenting the force of contraction of 1 Hz paced rat ventricular strips (Al-Sahli et al., 2001). This suggests that the actions of MDMA on the heart cannot be explained simply in terms of displacing noradrenaline from nerve terminals. In this study, we wish to establish whether cathinone, an amphetamine-like agent from the Khat plant, has similar actions.

Following overdose of CO_2, strips of right ventricle from male Wistar rats (250-350g) were placed in Krebs-Henseleit solution at 37°C and set up between platinum electrodes under 2 g tension and paced at a frequency of 1 Hz (supramaximal voltage, 0.5 msec pulses). Cumulative concentration-response curves to noradrenaline or isoprenaline was carried out in 1 log units increments, beginning with 10 nM, before and after 30 min exposure to test drug or vehicle.

Noradrenaline significantly increased stimulation-evoked contractions with a pD_2 (-log EC_{50}) of 5.64±0.07 (mean ±s.e.m, n=18). Cocaine (10 μM), MDMA (10 μM) and cathinone (10 μM) significantly increased the potency of noradrenaline to 6.31±0.11, 6.42±0.13 and 6.47±0.23 (n=6, each), respectively as compared with vehicle (Analysis of Variance with Dunnett's or Tukey's post-test). Isoprenaline significantly increased stimulation-evoked contractions with a pD_2 of 7.12±0.12 (mean ±s.e.m, n=18). Cocaine, MDMA or cathinone did not significantly affect the response to isoprenaline as compared with the effects of vehicle. Test drugs did not affect the maximum increase in the stimulation-evoked contraction produced by isoprenaline (vehicle 244.0±27.6% of control; cathinone 10 μM 239.6±17.9% of control; n=6 each). or noradrenaline (vehicle 205.3±29.0% of control; cathinone 10 μM 204.9±35.7% of control; n=6 each).

Although cocaine, MDMA or cathinone increased the potency of noradrenaline, the potency of the agonist isoprenaline, which is not a substrate for the noradrenaline transporter, was not increased by these agents. Hence, MDMA and cathinone share with cocaine an action which may involve competitive blockade of the noradrenaline transporter, rather than simply displacement of noradrenaline from nerve terminals. Since cocaine use is linked to an increased incidence of myocardial infarction (Mittleman et al., 1999), these results may have implications in terms of cardiac morbidity of MDMA and cathinone in man.

Supported by the Irish Heart Foundation

Al-Sahli, W. et al., 2001. Eur. J. Pharmacol. 422, 169-174.
Mittleman, M.A. et al., 1999. Circulation 99, 2737-2741.

346P MDMA INDUCES LOCALISED INCREASES IN ARC mRNA EXPRESSION IN RAT BRAIN IN A DOSE-DEPENDENT FASHION.

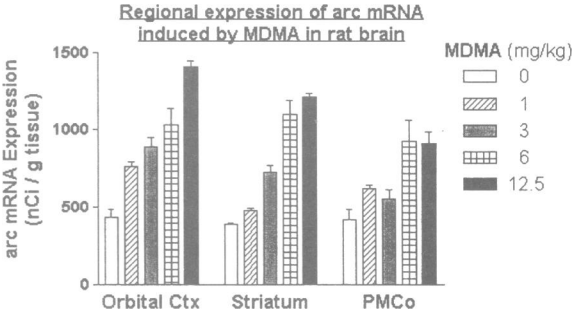
J.C.Aston, T.J.R. Beveridge & J.M. Elliott. School of Pharmacy, De Montfort University, Leicester LE1 9BH

Arc (activity-regulated cytoskeleton-associated gene) is an effector immediate early gene whose mRNA is selectively localised in neuronal dendrites. Its expression is induced by neuronal excitation and by stimulation of 5-HT_{2A} (Pei et al., 2000) and D_1 receptors (Kodama et al., 1998). MDMA (3,4-methylenedioxymeth-amphetamine) is an amphetamine analogue which induces the release of monoamines, principally 5-HT and dopamine (Shulgin, 1986). In this study we have investigated the regional effects of MDMA at varying doses on arc mRNA expression in rat brain.

Male Dark Agouti rats (190-210g) were administered (i.p.) either saline (1ml/kg) or MDMA (1, 3, 6 or 12.5 mg/kg). Rats were killed 2 hours later and the brains isolated and immediately frozen in cooled isopentane. Arc mRNA expression was analysed by in situ hybridisation histochemistry using [^{35}S]-dATP labelled oligonucleotide probe as described previously (Pei et al., 2000). Relative abundance of arc mRNA in selected areas was determined by densitometric quantification of autoradiograms using NIH-Image software. Statistical analysis of the results was made using 1-way or 2-way ANOVA as appropriate and Newman-Keuls post-hoc test.

MDMA induced a regional and dose-dependent increase in arc mRNA expression in rat brain. In the frontal cortex, arc expression was evident in both orbital and cingulate areas but was significantly greater in orbital cortex (222% v 115% above basal; $p<0.05$) at the highest MDMA dose tested. Within parietal cortex arc mRNA expression was localised to areas corresponding approximately to layer II and a more diffuse pattern around layers V/VI. The maximal increase in expression above basal was significantly greater in layer II than layers V/VI (221% and 171% respectively, $p<0.01$).

The striatum displayed a diffuse pattern of expression in the caudate-putamen, which at the highest dose showed a 215% increase above basal. In hippocampus, MDMA induced a dose-dependent expression of arc in the CA1 region, rising to 131% above basal, whereas CA2, CA3 and dentate gyrus showed no significant increase. Within the amygdala nuclei, expression was localised to the posteromedial cortical amygdala (PMCo), and increased to 120% above basal at the highest dose tested.



We conclude that acute administration of MDMA induces arc mRNA expression in localised areas of the rat brain in a dose-dependent manner. Previous reports of arc mRNA induction by DOI (Pei et al., 2000) and methamphetamine (Kodama et al., 1998) suggest that the effects of MDMA may be mediated by release of serotonin and dopamine. This will be clarified by future studies.

Kodama, M., Akiyama, K., Ujike, H. Shimizu, Y., *et al.* (1998) *Brain Res.*, **64**, 796, 273-283
Pei, Q., Lewis, L., Sprakes, M.E., Jones, E.J., *et al.* (2000) *Neuropharmacol.*, **39**, 463-470
Shulgin, A.T. (1986) *J. Psychoactive Drugs*, **18**, 291-304

347P *IN VIVO*, DUAL-PROBE, MICRODIALYSIS STUDY OF THE EFFECTS OF LOCAL INFUSION OF *d*-AMPHETAMINE ON NORADRENALINE EFFLUX IN RAT HYPOTHALAMUS AND FRONTAL CORTEX

S.M. Géranton, D. Heal[1] and S.C. Stanford, Department of Pharmacology, University College London, London UK and [1] RenaSci, Nottingham, UK

In our recent studies, the magnitude and time course of the increase in extracellular noradrenaline content ('NA efflux') caused by sibutramine, a NA and 5-HT reuptake inhibitor, differed in the rat hypothalamus (HYP) and frontal cortex (FCx) (Wortley *et al.*, 1999). Evidence that there is greater suppression of NA release by presynaptic α_2-adrenoceptors in the HYP than in the FCx partly explained these observations. Here, *in vivo* dual-probe microdialysis was used to investigate whether a similar regional difference in NA efflux is found after administration of *d*-amphetamine (*d*-AMP), an anorectic agent, which increases monoamine release.

Probes were implanted in both the FCx (mm: AP + 3.5, L + 1.5, DV – 4.0) and the HYP (mm: AP – 1.8, L – 0.4, DV – 9.2) of male SD rats (240-320g) under halothane anaesthesia. Experiments started the next day, after rats had recovered from the surgery. Probes were perfused with modified Ringer's solution and, after collecting 4 basal samples, rats were separated into two groups. The first (n=10) received *d*-AMP by reverse dialysis, at a concentration of 10μM and then 100μM. The second group (n=10/11) received the α_2 receptor antagonist atipamezole (1mg.kg^{-1}, i.p.), followed by the same regimen as the first group. Samples were analysed by HPLC-ECD and the data were analysed by split-plot ANOVA.

In the first group, NA efflux increased after local infusion of *d*-AMP at 10μM and again at 100μM, in both the HYP and FCx. The increase observed during the 10μM infusion was greater in the HYP than in the FCx ($F_{1,6} = 7.53$, $P = 0.034$ (0-80 min); maximum net increase: 24± 8 and 15 ± 5 fmol/20 min, respectively). There was no difference between the increase observed at 10 and 100μM in the HYP (data not shown) but, in the FCx, the increase at 100μM was greater than that at 10μM ($F_{1,18} = 6.828$, $P = 0.018$, 0-120min; maximum net increase: 44 ± 8 and 15 ± 5 fmol/20 min, respectively). Systemic injection of atipamezole alone did not affect noradrenaline efflux but, when followed by local infusion of 10μM *d*-AMP, there was a greater increase in the FCx, when compared with the effect of *d*-AMP alone ($F_{1,17} = 4.687$; $P = 0.045$ (0-120 min)), and there was no longer any difference in the increase in noradrenaline efflux in these two brain regions (data not shown). There was no regional difference in NA efflux after infusion of 100μM *d*-AMP for either group of rats. Also, in both brain areas, there was no difference in the increase observed after infusion of 100μM whether or not the rats had received atipamezole first.

These results suggest that the increase of NA efflux in the FCx after administration of 10μM *d*-AMP is blunted by activation of presynaptic α_2-adrenoceptors. This could enable the FCx to maintain its releasable pool of NA when exposed to the lower concentration of *d*-AMP. At the higher concentration, there is no apparent regulation *via* α_2-adrenoceptors suggesting that this efflux is attributed to impulse independent release. Finally, α_2-adrenoceptors had no influence on NA efflux in the HYP, at either concentration of *d*-AMP.

Wortley *et al.* (1999) *Br. J. Pharmacol.*, 127: 1860-6

348P METHAMPHETAMINE INDUCES A LONG TERM LOSS OF NEUROFILAMENT PROTEIN NF68 IN MOUSE STRIATUM

[1]V. Sanchez, [2]M. Zeine, [1]J, Camarero, [1]E. O'Shea, [2]L. Bosca, [3]A.R. Green & [1]M.I. Colado. [1]Dept. Farmacol., F. Medicina, [2]Instituto de Bioquimica (CSIC-UCM), F. Farmacia, Univ. Complutense, 28040 Madrid, Spain & [3]School of Pharmacy, De Montfort Univ., Leicester, LE1 9BH

Administration of methamphetamine (METH) to mice induces neurodegeneration of dopamine (DA) nerve terminals in the striatum (Ricaurte *et al.*, 1984). Damage is reflected by a long lasting depletion of DA content and a reduction in the density of DA uptake sites and tyrosine hydroxylase activity (Wagner *et al.*, 1980). Neurofilament proteins are the main components of the axonal cytoskeleton and play a crucial role in the determination of neuronal morphology and maintenance of axonal transport (Hoffman & Lasek, 1975). Recently, it has been suggested that these proteins are involved in neurodegenerative processes such as Parkinson Disease (Ray *et al.*, 2001).

We have now examined whether METH administration produces selective and long term changes in the levels of the neurofilament protein NF68 in the mouse striatum.

Adult male C57BL/6J mice (25-30 g) were injected with METH (3 mg kg^{-1}, i.p.) or saline at 3 h interval for a total of 3 injections. Measurements of quantitative immunoreactivity of NF68 in striatal and cortical tissue were conducted at several points after the last METH injection by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis. The PVDF membranes were incubated with monoclonal anti-NF68 and anti-mouse IgG as primary and secondary antibodies, respectively. Concentrations of striatal DA and cortical 5-HT were determined 7 d after treatment by h.p.l.c. coupled with electrochemical detection.

Seven days after METH treatment, mice showed a reduction of about 40% in the immunoreactivity for NF68 in the striatum, compared to saline-treated group (Figure). No reduction was seen in the cortex. No difference was observed when immunoreactivity measurements were made 1 h and 24 h after treatment. METH administration induced a substantial loss (65%) in the striatal concentration of DA and no change in the cortical concentration of 5-HT 7 d later. The present results indicate that repeated administration of METH induces a long lasting and regionally selective loss of NF68 in the striatum of mice which correlates with the loss of DA and could contribute to degeneration of both nerve terminals and axons.

Hoffman, P.N. & Lasek, R.J. (1975) *J. Cell. Biol.* **66**, 351-366.

Ray, S.W. *et al.* (2001) *Ann. N.Y. Acad. Sci.* **914**, 275-283.

Ricaurte, G.A. *et al.* (1984) *Brain Res.* **303**, 359-364.

Wagner, G.C. *et al.* (1980) *Brain Res.* **181**, 151-160.

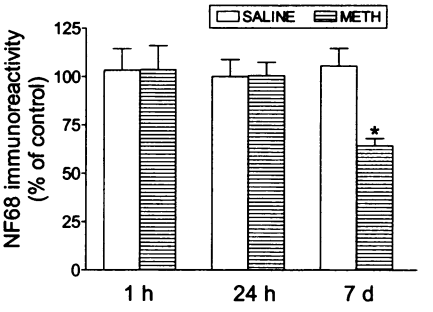


Figure. NF68 immunoreactivity in striatum of mice receiving 3 injections of METH (3 mg kg^{-1}, i.p.) 3 h apart. Samples were examined for NF68 protein content at 1 h, 24 h and 7 d after treatment. Results shown as % change vs saline in each immunoblot (mean ± s.e.mean, n=4-6). Different from saline: *P<0.01 (Newman Keuls test).

MIC thanks Plan Nacional sobre Drogas for support.

349P A SELENIUM DEFICIENT DIET POTENTIATES THE LONG TERM LOSS OF STRIATAL DOPAMINE INDUCED BY MDMA IN MICE AND INDUCES DEPLETION OF CORTICAL 5-HT

[1]J. Camarero, [1]V. Sanchez, [1]E, O'Shea, [1]E. Velasquez, [2]A.R. Green & [1]M.I. Colado. [1]Dept. Farmacol., F. Medicina, Univ. Complutense, 28040 Madrid, Spain & [2]School of Pharmacy, De Monfort Univ., Leicester, LE1 9BH

Administration of 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') to mice produces a long term depletion of the striatal dopamine (DA) content, indicative of neurotoxic degeneration, but has little effect on 5-HT containing neurones (O'Shea *et al.*, 2001). Experimental evidence indicates that neurotoxicity is due to oxidative stress: a) neuronal nitric oxide inhibitors prevent neuronal damage and the rise in striatal hydroxyl radical formation induced by MDMA (Colado *et al.*, 2001), b) CuZn superoxide dismutase (SOD) transgenic mice are resistant to the neurotoxic action of MDMA (Cadet *et al.*, 1995) and c) MDMA causes a decrease in SOD and glutathione peroxidase (GPx) activities and an increase in lipid peroxidation in regions of the brain (Jayanthi *et al.*, 1999). We have now examined the influence of a selenium (Se) diet on the long term effect of MDMA on DA and 5-HT concentrations in the brain of mice, Se being a cofactor to facilitate GPx activity.

Two groups of adult male C57BL/6J mice (20-25 g) were fed either a Se-deficient diet (<0.02 ppm) or a Se-replete diet (0.2 ppm) for 8 weeks. After 7 weeks, both groups received MDMA (15 mg kg^{-1}, i.p.) 3 times at 3 h intervals. Control animals were given saline. Rectal temperature was measured during MDMA administration. Seven days later concentrations of striatal DA and cortical 5-HT were measured as well as GPx activity in striatum and cortex as previously described (Colado *et al.*, 1999; Flohe & Gunzler, 1984).

MDMA administration increased rectal temperature by approx. 3°C and 2°C above saline-injected in both Se-deficient or Se-replete treated mice after the second and third injection, respectively. Seven days later MDMA had produced a substantial depletion in the striatal DA concentration in both groups, although the depletion was considerably larger in the Se-deficient mice (Figure). In contrast, a long term loss on cortical 5-HT concentration was only evident in Se-deficient mice (Figure). GPx activity was reduced by 30% in striatum and cortex of mice maintained with Se-deficient diet. These results indicate that Se-deficiency impairs the cellular antioxidant status of the mouse brain and that this effect not only potentiates the dopaminergic toxicity induced by MDMA but also facilitates the appearance of damage to 5-HT containing neurones.

Cadet, J.L. *et al.*, (1995) *Synapse* **21**, 169-176.
Colado, M.I. *et al.*, (1999) *Br. J. Pharmacol.* **126**, 911-924.
Colado, M.I. *et al.*, (2001) *Br. J. Pharmacol.* **134**, 1711-1723.
Flohe, L. & Günzler, W.A. (1984) *Meth. Enzymol.* **105**, 114-121.
Jayanthi, S. *et al.* (1999) *Neuroscience* **91**, 1379-1387.
O'Shea, E. *et al.* (2001) *Neuropharmacology* **40**, 65-74.

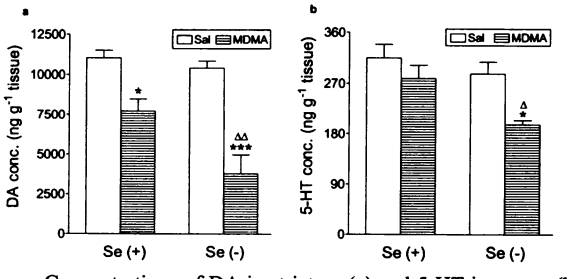


Figure. Concentrations of DA in striatum (a) and 5-HT in cortex (b) of mice fed a Se-deficient or Se-replete diet for 8 weeks and given 3 injections of MDMA. Results shown as mean ± s.e.mean, n=5-9. Different from the corresponding saline group: *P<0.05, ***P<0.001. Different from Se-replete + MDMA group: $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01.

MIC thanks Plan Nacional sobre Drogas for support.

350P CITRUS FLAVONOID TANGERETIN ACCUMULATES IN THE BRAIN AND PRE-TREATMENT PROTECTS AGAINST DOPAMINERGIC NEURONAL LOSS IN A RAT MODEL OF PARKINSON'S DISEASE

K.P.Datla[1], M.A. Christidou[2], W.W. Widmer[3], H.K. Rooparai[2] & D.T. Dexter[1]. [1]Dept Neuroinflammation, Imperial College School of Science, Technology and Medicine, London W6 8RF, [2]Dept Neuropathology, Inst of Psychiatry, London SE5 8AF. [3]Department of Citrus, Lake Alfred, Florida, U.S.A.

Although the precise mechanisms contributing to the loss of nigrostriatal dopaminergic neurons in Parkinson's disease (PD) are unclear, increased oxidative stress is thought to be involved (Olanov & Tatton, 1999). One way of reducing the oxidative stress is to scavenge the reactive oxygen species. Many plant polyphenolics, such as flavonoids, were shown to have free radical scavenging activity *in vitro* (Pietta 2000). However, no information is available on 1) whether they cross the blood-brain barrier and 2) reduce oxidative stress, *in vivo*. Here, we report the bio-distribution and *in vivo* neuroprotective effects of a citrus flavonoid, tangeretin, using 6-OHDA lesion rat model of PD.

In male Sprague-Dawley rats (225 ± 25 g)treated with tangeretin (10 mg/kg/dayx28; p.o.; n=6), 1 h after the last dose highest concentrations were detected in the brain compared to peripheral organs. Within the brain, concentrations of tangeretin showed considerable variation. Brain stem and cerebellum showed the lowest concentrations (0.17±0.05 and 0.27±0.05 ng/mg tissue respectively) whilst hippocampus, striatum and hypothalamus showed highest concentrations (2.0±0.23, 2.36±0.25 and 3.88±0.48 ng/mg tissue respec-tively). In the periphery, liver had highest concentration (0.59±0.09 ng/mg), while the kidney lowest concentrations (0.05±0.01 ng/mg). Levels in heart (0.12±0.02 ng/mg), lung (0.14±0.01 ng/mg) and plasma (0.11±0.02 ng/ml) showed similar concentrations.

6-OHDA (8 μg) was injected unilaterally onto the median forebrain bundle under anaesthesia in tangeretin (20 mg/kg/day x 4 days, p.o; last dose 1 hr before lesioning) or vehicle treated rats (n=6). 7 days later brains were collected and assayed for tyrosine hydroxylase positive cells (TH+cells) in the substantia nigra (SNpc) and dopamine levels in the striata. The number of TH+cells in SNpc and dopamine levels in striata were significantly decreased with 6-OHDA lesioning. However, the loss of TH+cells and dopamine in the vehicle-treated group was significantly higher than the tangeretin-treated group, suggesting neuroprotection by tangeretin (Table 1).

Table 1. Effects of tangeretin pre-treatment on TH+cells and dopamine levels.

	control side	lesion side
	TH+cells in the substantia nigra	
vehicle	136 ± 9	59 ± 8*
tangeretin	163 ± 8	136 ± 9*,a
	Striatal dopamine levels (μg/mg)	
vehicle	1.05 ± 0.03	0.39 ± 0.1[b]
tangeretin	1.10 ± 0.04	0.77 ± 0.09[b,c]

* $p<0.005$; [b] $p<0.02$ vs. control side, paired Student's t-test; [a] $p<0.0005$ and [c] $p<0.05$ Vs lesioned-side vehicle-treated group, unpaired Student's t-test; after significant ANOVA.

These studies, for the first time, give evidence for tangeretin's capacity to cross the blood-brain barrier and pretreatment-induced protection of dopaminergic neuronal loss in the 6-OHDA-lesion rat model of PD.

Olanow CW & Tatton WG (1999) Ann Rev Neurosci 22:123-144

Pietta P-G (2000) J. Nat Prod 63: 1035-1042; 3. Datla KP, et al., (2001) Movement Disord 16:424-434.

351P CITICOLINE IS NEUROPROTECTIVE BY REDUCING GLUTAMATE LEVELS AFTER PERMANENT FOCAL CEREBRAL ISCHAEMIA IN RATS.

A. Cárdenas, J. De Cristóbal, J.Castillo[1], A.Dávalos[2], J.C. Leza, M.A. Moro, P. Lorenzo & I. Lizasoain. Pharmacology Department, School of Medicine, Universidad Complutense, Madrid. [1]Neurology Service, Hospital Clínico Universitario, Santiago de Compostela. [2]Hospital Universitari, Doctor Josep Trueta, Girona.

Citicoline is a neuroprotector (Kakihana et al.,1988; Schäbitz et al., 1996) that activates the biosynthesis of structural phospholipids of the neural membranes and of the glia, raises the cerebral metabolism and accelerates the re-absorption of edema (Weiss, 1995); however, the action mechanism in cerebral ischemia is not fully understood. Thus, we have studied the effect of citicoline on serum levels of an excitatory amino acid (glutamate) and of an inhibitory amino acid (GABA), in a model of permanent middle cerebral artery occlusion (MCAO, De Cristóbal et al., 2001) in rats subject to various treatment protocols.

Male Fisher rats (≈ 250g) were used (5 sham, 10 MCAO, 10 with citicoline administered during the 4 days prior to the MCAO, 10 with citicoline administered immediately prior to the MCAO and 10 with citicoline administered immediately before and at 24 hours following MCAO). In all cases, 4g/kg of citicoline was administered in each dose by intra-peritoneal injection. Blood samples were obtained 1 hour prior to the MCAO and at 30 minutes, and 2, 4, 6, 8, 24, 36 and 48 hours afterwards. The size of the infarct was determined in 9 sections of 2 mm of the brain of the animal, extracted at 48 hours.

Basal serum glutamate levels (142 ± 10 $ng.ml^{-1}$) increased up to maximal levels (657 ± 39) at 24 hours following the MCAO. The levels of glutamate was significantly lower in the group of animals treated with citicoline immediately before and at 24 hours following the MCAO (209 ± 17 vs. 657 ± 39; n=10 $p<0.001$) and the infarct size was significantly related to the concentration of glutamate at 24 h (n=10, $p<0.001$). There were no significant variations in the levels of GABA in the groups of animals treated with citicoline.

In a model of MCAO, citicoline originates a reduction in the serum levels of glutamate related with a reduction in the infarct size.

De Cristóbal J, Moro MA, Davalos A, *et al* (2001). *J. Neurochem.* 79, 456-459

Kakihama M, Fukuda N, Suno M, *et al* (1998) *Stroke* 19:217-222.

Schäbitz WR, Weber J, Takano K, *et al* (1996) *J Neurol Sci* 138:21-25.

Weiss GB. (1995) *Life Sci.* 56:637-660.

352P INTRA-VITREAL INJECTION OF TETANUS NEUROTOXIN (TeNT) PREVENTS MONOCULAR DEPRIVATION (MD) -INDUCED APOPTOSIS IN THE LATERAL GENICULATE NUCLEUS (LGN) OF NEW-BORN RATS

C. Nucci, S. Piccirilli[2], R. Nisticò[2], L. Cerulli[1] & G. Bagetta[2,3]. Dept. of Biopathol. Univ. of Rome "Tor Vergata", Via Raimondo 00173 Rome, [2]Mondino-Tor Vergata Center for Exp Neurobiol Univ of Rome «Tor Vergata», Rome, [3]Dept of Pharmaco-Biology, Calabria Univ, 87030 Cosenza Italy.

In new-born rats MD increases citrulline, the co-product of nitric oxide (NO) synthesis, and causes apoptosis in the contralateral LGN; antagonists of glutamate receptor abolish citrulline accumulation and prevent neuronal death (Nucci *et al.*, 2000). In the deprived eye, retrobulbar surgical lesion of the optic nerve prevents apoptosis in the LGN, supporting the hypothesis that MD induces optic nerve terminals to release excess glutamate in the LGN where it causes neuronal death. If this is correct then intra-vitreal injection of TeNT, a neurotoxin which blocks neurotransmitter release (see Schiavo *et al.*, 2000), should prevent MD-induced apoptosis in the LGN.

For the experiments we used Long Evans, new-born rats (20 ± 0.5g); the surgical procedures were carried out under ether anaesthesia. At post-natal day 14, the right eyelids were sutured for 48 h; age-matched, non deprived, rats were used as control (n=6 per group). For test studies, TeNT (0.1 mg/ml) was injected into the vitreus of the right eye of rats at post-natal day 12, using a 5µl Hamilton syringe (2 µl volume) (n=12). After 48 h, (an approximate time required for the toxin to reach the LGN via the optic nerve) the right eyelids of six animals were sutured for 48 hours. Serial brain coronal sections (15µm) were processed for *in situ* detection of DNA fragmentation according to the TUNEL technique (Gavrieli *et al.*, 1992). Morphological characteristics of adjacent brain sections were assessed under light microscopy using haematoxylin and eosin (H&E) staining. DNA fragmentation (TUNEL positive cells) was observed in areas of the sections (n=6 per brain) corresponding to (Pellegrino *et al.*, 1981) the LGN connected with the deprived (for 48h) eye (Tab. 1). Nuclear chromatin marginalization and condensation, typical features of apoptosis, were observed in adjacent H&E stained sections. Intravitreal injection of TeNT, 48 h before MD, significantly reduced the number of TUNEL positive cells (Tab. 1). Injection of TeNT alone did not evoke apoptosis in the LGN.

Table. 1 intravitreal TeNT prevents MD-evoked apoptosis in the LGN connected with the deprived eye

Experimental condition	TUNEL positive cells
Control	0.44±0.24
MD for 48h	3.77±0.11*
TeNT+MD for 48h	0.62±0.10
TeNT	0.44±0.11

$p<0.05$ vs age-matched control (Student's «t» test)

In conclusion, our present data support the original hypothesis (Nucci *et al.*, 2000) that during development MD leads optic nerve terminals to release excessive glutamate in the deprived LGN where it elevates NO and causes neuronal apoptosis.

Gavrieli, Y. *et al.* (1992) *J Cell Biol* 119,493-501.
Nucci, C. *et al.* (2000) *Biochem. Bhiophys Res. Comm.* 278, 360-367.
Pellegrino, L.J. *et al.* (1981) *A Stereotaxic Atlas of the Rat Brain.* Plenum Press, London.
Schiavo, G. *et al.* (2000) *Physiol Rev* 80, 717-766.

Financial support from the CNR to G.B. and MURST to C.N.

353P DOPAMINE AUTOXIDATION INDUCES NEURONAL DEATH THROUGH GENERATION OF H_2O_2.

R. Pedrosa & P. Soares-da-Silva. Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

Recently, it was been postulated that during the preclinical phase of Parkinson's disease the dopamine (DA) turnover may increase to compensate the loss of dopaminergic neurons (Foley et al, 2000). This increase in the rate of DA utilization is likely to accelerate the neurodegenerative process through the generation of quinones, semiquinones and hydrogen peroxide (H_2O_2), as a result of oxidative DA metabolism (Graham, 1978). The present study examined the effects of various non-enzymatic antioxidants, antioxidant enzymes, free radical scavengers and a strong iron chelator on the autoxidation and neurotoxicity of DA.

Neuro 2A cells (ATCC CCL-131) were grown at 37° C in a humidified atmosphere (5% CO_2) in Minimum Essential Medium supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B and 100 µg ml^{-1} streptomycin. DA was quantified by means of high pressure liquid chromatography with electrochemical detection, as previously reported (Soares-da-Silva et al, 1994). Viability was measured using the cell viability probe calcein AM (Molecular Probes). H_2O_2 quantification was measured fluorometrically using the Amplex Red assay kit (Molecular Probes). Caspase 3 activity was measured using the Caspase 3 assay kit #2 (Molecular Probes). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

DA levels in DA working solutions stored for 48 h in cell culture conditions (5% CO_2 atmosphere at 37°C) were markedly lower than in fresh solution (69 ± 5% reduction), this being prevented by ascorbic acid (AA), gluthatione (GSH), sodium metabisulfite and N-acetyl-cysteine (NAC). Neuro 2A cell death during the exposure to DA was concentration and time dependent. Incubation of cells with DA 1mM for 48h was accompained by a decrease in viable cells (85 ± 4% reduction), this being prevented by AA, GSH, sodium metabisulfite and NAC (approximately 50% cell viability). However, the free radical scavengers N-tert-butyl-α-phenylnitrone and a strong iron chelator, deferoxamine, did not protect cells against DA-induced toxicity. This suggests an important role of H_2O_2 and DA quinones on DA-induced cytotoxicity, and exclude the involvement of Fenton reaction and oxygen radicals such as $^{\bullet}OH$ and $O_2^{-\bullet}$. The generation of H_2O_2 by autoxidation of DA was time dependent. The production of H_2O_2 by autoxidation of 1mM DA at 48h was 151 ± 14 µM, this value being reduced to 17 ± 3 µM, in the presence of AA. Toxicity induced by 1mM DA, at 48h was prevented by catalase (48 ± 9% viable cells). DA-induced cell death was not accompanied by increases in caspase 3 activity. These findings suggest that DA autoxidation may induce neuronal death through the generation of H_2O_2.

Foley, P., et al (2000). *J. Neurol.*, **247**, II82-II94

Graham D.G. (1978). *Mol. Pharmacol.*, **14**, 633-643;

Soares-da-Silva, P., et al (1994). *Br. J. Pharmacol.*, **112**, 611-615.

354P GENERATION OF L-DOPA QUINONES AND H_2O_2 AS MAJOR MECHANISMS IN L-DOPA INDUCED NEURONAL DEATH: IMPLICATIONS IN PARKINSON'S DISEASE

R. Pedrosa & P. Soares-da-Silva. Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

One potential problem with the use of L-DOPA in treatment of Parkinson's disease arises from the fact that L-DOPA metabolism or autoxidation can give rise to radical species, hydrogen peroxide (H_2O_2), semiquinones, and quinones (Graham, 1978). The present study evaluated the neurotoxic profile of L-DOPA in Neuro 2A cells. The effects of various non-enzymatic antioxidants, antioxidant enzymes, free radical scavengers and a strong iron chelator were assessed to define the different mechanisms through the which L-DOPA induced cell death.

Neuro 2A cells (ATCC CCL-131), with derived from a mouse neuroblastoma, were grown at 37° C in a humidified atmosphere (5% CO_2) in Minimum Essential Medium supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B and 100 µg ml^{-1} streptomycin. L-DOPA was quantified by means of high pressure liquid chromatography with electrochemical detection, as previously reported (Soares-da-Silva et al., 1994). Viability was measured using the cell viability probe calcein AM (Molecular Probes). H_2O_2 quantification was measured fluorometrically using the Amplex Red assay kit (Molecular Probes). Caspase 3 activity was measured using the Caspase 3 assay kit #2 (Molecular Probes). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

L-DOPA levels in L-DOPA working solutions stored for 48 h in cell culture conditions (5% CO_2 atmosphere at 37°C) were markedly lower than in fresh solution (68±1% reduction), this being prevented by ascorbic acid (AA), gluthatione (GSH), sodium metabisulfite and N-acetyl-cysteine (NAC). Neuro 2A cell death during the exposure to L-DOPA was concentration and time dependent. Incubation of cells with L-DOPA 1mM for 48h was accompanied by a decrease in viable cells (90±4% reduction), this being prevented by AA (200 µM), GSH (1 mM), sodium metabisulfite (200 µM) and NAC (1 mM) (approximately 70% cell viability). However, the free radical scavengers N-tert-butyl-α-phenylnitrone (1 mM) and a strong iron chelator, deferoxamine (1, 10, 100 µM), did not protect cells against L-DOPA-induced toxicity. This suggests an important role of H_2O_2 and L-DOPA quinones on L-DOPA-induced cytotoxicity, and excludes the involvement of Fenton reaction and oxygen radicals such as $^{\bullet}OH$ and $O_2^{-\bullet}$. The generation of H_2O_2 by autoxidation of L-DOPA was time dependent. The production of H_2O_2 by autoxidation of 1mM L-DOPA at 48h was 119±17 µM, this value being reduced to 14±1 µM in the presence of AA (200 µM). Toxicity induced by 1mM L-DOPA, at 48h was prevented by catalase (40±1% viable cells). L-DOPA-induced cell death was also accompanied by increases in caspase 3 activity, but AA did not prevent this. These findings suggest that L-DOPA quinones and H_2O_2 generated by autoxidation play a major role in neuronal death.

Graham, D. G. (1978). *Mol. Pharmacol.*, **14**, 633-643.

Soares-da-Silva, P., et al (1994). *Br. J. Pharmacol.*, **112**, 611-615.

355P DEVELOPMENTAL CHANGE IN INHIBITORY PRESYNAPTIC MUSCARINIC RECEPTOR SUBTYPE IN RAT OLFACTORY CORTEX

B. Whalley & A. Constanti Dept Pharmacology, The School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX.

The mammalian olfactory cortex is responsible for the assimilation of responses to odours and the integration of these responses into learning and memory (Hasselmo & Bower, 1990). We have previously shown that excitatory synaptic transmission in this brain area is strongly suppressed by activation of presynaptic muscarinic M_1 receptors. Moreover, synaptic responses elicited by lateral olfactory tract (LOT) stimulation are less susceptible to muscarinic inhibition than those evoked by stimulation of intrinsic fibres (Hasselmo & Bower, 1990). We have examined whether the muscarinic receptor subtype mediating this response was subject to developmental change. Olfactory cortex brain slices were prepared from either adult (P>40) or immature (P+13 to P+24) Wistar rats as previously described (Postlethwaite *et al.*, 1998). Stable intracellular recordings were made from presumed 'deep' pyramidal cells using 2 M K acetate-filled microelectrodes (resistance 50-80 MΩ). The muscarinic agonist, oxotremorine-M (OXO-M; 10 µM) was bath-applied and synaptic responses elicited using two bipolar stimulating electrodes (submaximal stimulus range 3-30 V), placed in layer I to stimulate afferent LOT fibres, and in layer II-III to stimulate intrinsic fibres.

Fourteen adult and 22 immature neurones were recorded (average resting potentials = -83.8 mV (adult) and -82.7 mV (immature) respectively). In all recordings from adult or immature slices, LOT-evoked synaptic responses were unaffected by OXO-M. However, those elicited by intrinsic fibre stimulation were consistently depressed. Adult slices displayed a significantly greater degree of synaptic depression in the presence of OXO-M (77.1 ± 6.2%) than immature animals (24.8 ± 5.4%), (mean percentage depressions ± s.e. mean of peak EPSP amplitude evoked at -90 mV ($p < 0.01$ by Wilcoxon signed rank test)). Application of the M_1-selective muscarinic antagonists, pirenzepine (100 nM: 7/7 adult and 12/12 immature cells) or telenzepine (20 nM: 7/7 adult and 10/10 immature cells) reversed the synaptic inhibition of intrinsic fibres induced by OXO-M in adults but not immature cells. In contrast, application of the M_2-selective muscarinic antagonists, methoctramine (300 nM: 7/7 adult and 12/12 immature cells) or AFDX-116 (1 µM: 7/7 adult and 10/10 immature cells) reversed the synaptic inhibition of intrinsic fibres induced by OXO-M in immature but not in adult cells.

Our investigation has confirmed that LOT terminals appear to lack the inhibitory muscarinic receptors present on intrinsic fibres. In addition, we have shown that the presynaptic muscarinic receptor population on the intrinsic fibres changes developmentally, being primarily of the M_2 subtype in the immature animals, but of the M_1 subtype in adult animals. The presynaptic M_2 receptors of the immature animals also mediate a smaller inhibitory effect upon synaptic transmission than the M_1 receptors found in adults. This developmental difference may be important in governing the appearance of epileptiform behaviour seen in some immature cells in OXO-M (Postlethwaite *et al.*, 1998), since the reduced inhibitory effect of the activated M_2 receptors in the immature slices may allow a threshold to be breached, making the immature cell network more prone to bursting.

Hasselmo, M.E. & Bower, J.M. (1990) *J. Neurophysiol.* **64**:179-190

Postlethwaite, M., Constanti, A. & Libri, V. (1998) *J. Neurophysiol.* **79**:2003-2012

356P AN IMMUNOHISTOCHEMICAL STUDY OF THE DISTRIBUTION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR (nAChR) β4 SUBUNIT IN ADULT RAT BRAIN.

Laura R. Johnson, Non M. Evans, Suchira Bose, Peter J. Craig, Michael J. O'Neill, Emanuele Sher, *Cecilia Gotti, Stephen G. Volsen, (introduced by John R. Boot). Eli Lilly & Co. Ltd., Lilly Research Centre, Erl Wood Manor, Windlesham, Surrey, GU20 6PH. * Department of Medical Pharmacology, University of Milan, Italy.

Nicotinic receptors have been implicated in a number of central nervous system disorders. A greater understanding of the distribution of the subtypes is important in evaluating their roles in such disorders.

The nAChRβ4 subunit was first described by Duvoisin *et al.* (1989), who reported expression of the β4 gene in the medial habenula of adult rat brains. Further in-situ hybridisation histochemistry studies by Dineley-Miller and Patrick (1992) revealed abundant β4 mRNA throughout the rat brain. In this study, a rabbit polyclonal antibody raised against a peptide mapping the intracellular loop of the rat β4 receptor was fully characterised and used for immunolocalisation studies in paraffin sections of adult rat brains.

The specificity of the antibody was characterised by western blot analysis of rat brain extracts and HEK293 cells transfected with the human β4 protein in combination with an α subunit. A 50kDa band appeared only in lanes containing the rat extracts, suggesting that the antibody was species specific. The β4 protein is known to be highly homologous in sequence to the β2 protein. Immunofluoresence studies in COS-7 cells transiently transfected with either rat α4β4 or rat α4β2 revealed that the antibody did not recognise the β2 protein. Taken together, these data confirm that the antibody is specific for the rat β4 protein.

Imunohistochemical localisation studies were performed on 8μm sections from rat brains (male, Sprague-Dawley 150-200 g) taken at 9 coronal levels that included all main cell bodies, nuclei and terminal structures. A commercially available avidin-biotin immunoperoxidase kit (ABC elite, Vector Laboratories) was used and the sections were incubated overnight at 4°C with 0.5μg/ml of the anti β4 antibody. The specificity of the immunostaining was confirmed by using the antibody pre-adsorbed with a 5-fold excess of its immunising peptide.

Strong β4 immunoreactivity was observed in optic and olfactory nuclei, the interpeduncular nucleus, the pontine nucleus, the pineal gland and in the Purkinje cells. Moderate staining was observed in the frontal, cingulate and retrosplenial cortex, throughout layer IV of the isocortex, the mammillary nuclei, the medial habenula nucleus and in many hindbrain nuclei such as the mesencephalic trigeminal nucleus, the pedunculopontine tegmental nucleus and the dorsal raphe nucleus. Weaker staining was observed in the piriform cortex, amygdaloid nuclei and the arcuate nucleus. Very weak reactivity was noted in the CA1 region of the hippocampal formation. Some staining of cells was also observed in white matter tracts such as the lateral olfactory tract, the optic tract and the cerebral peduncle.

In conclusion, the β4 protein appears to be localised in discrete areas throughout the rat brain. Evaluation of this distribution may provide clues to the functional role of this receptor subtype.

Dineley-Miller K. & Patrick J. (1992) *Mol. Brain Res.* 16, 339-344.

Duvoisin R.M., *et al.* (1989) *Neuron*, 3, 487-496

357P ALPHA-2 ADRENOCEPTOR AGONIST DEXMEDETOMIDINE-INDUCED HYPNOSIS ACTIVATES OREXIN-CONTAINING NEURONES IN THE PERIFORNICAL AREA OF FISCHER RATS

Nelson L, Lu J*, Guo T, Noormohamed F, Maze M, Saper CS*
Department of Anaesthesia, Imperial College, London; *Department of Neurology, Harvard Medical School, Boston

The α_2 adrenoceptor agonist dexmedetomidine (DEX) induces sedation with unique rousability properties (Hall *et al.*, 2000). Its mechanism of hypnotic action is hypothesized to converge on the neural substrates modulating NREM sleep. Orexin (hypocretin) is an arousal-promoting (Piper *et al.*, 2000) neuropeptide synthesised in neurones in the perifornical region of the lateral hypothalamus, innervating the cortex, basal forebrain and LDT/ PPT cholinergic cells and ascending monoaminergic arousal systems (Date *et al.*, 1999). c-Fos, an immediate early gene product commonly used as a marker of activation , expression is high at night (waking cycle) or with central stimulants modafinil or amphetamine (Scammell *et al.*, 2000), and low during day (Estabrooke *et al.*, 2001). We aimed to determine whether the arousal-permitting features of DEX are mediated by orexinergic cells.

Adult male Fischer rats implanted with EEG and EMG electrodes were anaesthetised for two hours (7:00-9:00pm, rodent waking cycle) with DEX (50 μg/kg, i.p.), muscimol (MUS; $GABA_A$ receptor agonist; 5 mg/kg, i.p.) or saline control. Animals were administered chloral hydrate (500 mg/kg, i.p.) and transcardially perfused with 100 ml 0.9% saline and 500 ml 10% formalin. Brains were removed, post fixed in formalin for 4 hours, equilibrated in 20% sucrose and cryosectioned (1:5 series, 30 μm). Sections were double-labelled for c-Fos (1:100,000, rabbit; Oncogene) and orexin-A (1:5,000, rabbit; Chemicon) and visualized with 3,3'-diaminobenzidine (DAB; Sigma) under light microscopy.

56.5% of orexin containing cells in animals anaesthetised with DEX (n=2) and 24% with MUS (n=2) were c-Fos positive. During wakefulness (n=3), about 47% of the cells were c-Fos positive. (*Figure 1*)

It is surprising to observe elevated c-Fos expression in wake-active orexinergic neurones induced by a hypnotic anaesthetic agent. Only 20-25% of orexinergic neurons are c-Fos positive in naturally sleeping animals (Estabrooke *et al.*, 2001). DEX-sedated subjects are uniquely more "arousable" than subjects equally sedated with GABA-mediated agents (Hall *et al.*, 2000). This orexinergic activation during the sedated state opposes the hypnotic action of the drug itself. A balance between these counteractive effects may explain the uniquely quick switch from sedation to arousal that is often seen during DEX hypnosis.

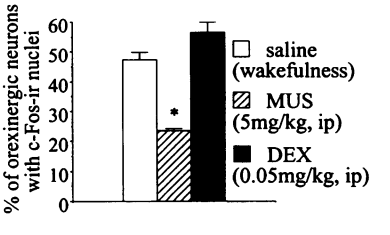


Figure 1: c-Fos expression in perifornical orexin neurones during DEX and MUS induced hypnosis. *p<0.001 (ANOVA, Neuman-Keuls)

Date Y *et al.*, PNAS USA 96(2):748-53, 1999
Estabrooke IV *et al.*, J Neurosci 21(5):1656-1662, 2001
Hall JE *et al.*, Anesth Analg 90:699-705, 2000
Piper DC *et al.*, Eur J Neurosci 12(2):726-730, 2000
Scammell TE *et al.*, J Neurosci 20(22):8620-8628, 2000

358P EFFECT OF A CYCLODEXTRIN-LOFEPRAMINE COMPLEX ON THE MOTILITY OF *TETRAHYMENA PYRIFORMIS*

Darcy P.[1,2], Kelly J.P.,[2] Leonard B.E.[2] & Henry J.A.[1]
[1]Academic Department of Accident and Emergency Medicine, Imperial College School of Medicine, St. Mary's Hospital, London, England. [2]Department of Pharmacology, National University of Ireland, Galway, Ireland.

Previous attempts to measure the *in-vitro* effects of lofepramine have been frustrated by its poor aqueous solubility. We here examined two approaches: to create a cyclodextrin-lofepramine complex and to dissolve the drug in ethanol.

Four agents were studied: lofepramine hydrochloride, desipramine hydrochloride, amitriptyline base and lignocaine hydrochloride. Each compound was enclosed in cyclodextrin to facilitate solubility in the aqueous medium. Cyclodextrin complexes were prepared according to Loukas (1997). The effect on the swimming speed of the protozoan *Tetrahymena pyriformis* was measured using a video image analysis system (Cassidy and Henry 1986, 1989). This organism is sensitive to lipid soluble anaesthetic agents, as demonstrated by Nunn *et al.* (1974) and Al-Saadi et al. (1981). The concentration at which the swimming speed of *Tetrahymena* was reduced by 50% (IC_{50}) for each drug was then correlated with its respective octanol-water partition coefficient value (Pow).

The IC_{50} of lofepramine was 357.40 $\pm$ 25.00 mM, while the values for the other drugs studied were: amitriptyline 1.26 $\pm$ 0.29 mM, desipramine 75.99 $\pm$ 14.40 mM, 85.73 $\pm$ 18.30 mM. There was a significant correlation between the IC_{50} values and the Pow values. Lofepramine was soluble in a 20% ethyl alcohol solution, and although the presence of alcohol caused a small reduction in the motility of *Tetrahymena*, it was possible to produce an IC_{50} curve for the drug. The value found was 436.2 $\pm$ 61.0mM. Although the use of a concentrated ethanol solution for dissolving a drug might be acceptable for studies involving a protozoan cellular model, it would be of little use in a mammalian model.

We conclude that cyclodextrin solubilisation of lofepramine is suitable as a delivery method for the drug in *in-vitro* experimental situations. This concept can be applied to other lipophilic drugs with low aqueous solubility.

Al-Saadi, D.M.A. *et al.* (1981) J. Pharm. Pharmacol. 33, (Supplement): 93P.

Cassidy, S.L. & Henry, J.A. (1986) Fd. Chem. Toxicol. 24, 807-809.

Cassidy, S. L. & Henry, J.A. (1989) Toxicol. In Vitro 3, 181-187.

Loukas, Y. L. (1997) J. Pharm. Biomed. Anal. 16, 275-280.

Nunn, J.F. *et al.* (1974) Cell Science 15, 537-554.

359P INFLUENCE OF FLUOXETINE UPON EXTRACELLULAR LEVELS OF SEROTONIN IN RAT BRAIN REGIONS: AN *IN VIVO* CONCOMITANT ELECTROCHEMICAL AND ELECTROPHYSIOLOGICAL STUDY

C. Lazzarini, E. Vecchiato, M. Corsi & F. Crespi. Biology Dept., Psychiatry-CEDD, GlaxoSmithKline S.p.A., Medicines Research Centre, via Fleming 4, Verona, Italy.

There is evidence for reduced activity of the serotonergic system in the CNS in patients with unipolar depression. Serotonin (5-HT) is the major target of the new antidepressant drugs such as Selective Serotonin Reuptake Inhibitors (SSRI) (Blier & de Montigny, 1994). It has been proposed that i) fluoxetine (SSRI) selectively inhibits the 5-HT transporter increasing the synaptic concentration of 5-HT, and ii) a negative feedback that involves the activation of somato-dendritic $5\text{-}HT_{1A}$ autoreceptors is responsible for the sub-sequent inhibition of 5-HT release by forebrain serotonergic nerve terminals (Briley and Moret, 1993).

In order to analyse this hypothesis *in vivo* Differential Pulse Voltammetry (DPV) has been performed in anaesthetised (urethane 1.2g/kg ip) adult CD male rats (230-250g). In order to selectively measure extracellular 5-HT levels Nafion coated carbon fibre electrodes (Nafion-CFE) (Crespi *et al.*, 1988) were placed in frontal cortex, hippocampus, amygdala or raphe dorsalis nucleus. In addition, extracellular electrophysiological recordings were made from raphe dorsalis nucleus in parallel with the voltammetric recordings by means of the same Nafion-CFE as described previously (Crespi *et al.*, 1995). Rats were treated with a single dose of fluoxetine (Sigma) either locally (1µl, 100µM) or systemically (20mg/kg i.p.).

Local fluoxetine injection into frontal cortex or hippocampus (n=4 each) was followed by significant ($p<0.05$) increase up to 134±4% and 198±18% of extracellular 5-HT levels, respectively. In contrast, systemic fluoxetine was followed by significant ($p<0.05$) decrease to 30±8% or 43±3% of extracellular 5-HT levels monitored in amygdala or in raphe dorsalis nucleus, respectively (n=4 each). Furthermore, concomitant electrophysiological recordings in raphe dorsalis nucleus indicated a rapid (5 – 10 minutes), significant ($p<0.05$) decrease of cell firing to 28±11% of control values. Very similar findings have been obtained in raphe dorsalis nucleus in parallel experiments in which the anaesthetised rats were treated with the $5\text{-}HT_{1A}$ receptor agonist 8-OHDPAT (30µg/kg s.c., n=4). All the data presented are means ± sem. They are compared with corresponding vehicle control, and submitted to Dunnett's test.

These *in vivo* results support the feasibility of using DPV to analyse the influence of SSRI upon central 5-HT system. In addition, they confirm the feasibility of combining voltammetry and electrophysiology at a single electrode. Finally, local fluoxetine injection in frontal cortex or in hippocampus resulted in increased local extracellular 5-HT levels, supporting the reported influence of this SSRI upon the 5-HT transporter. On the other hand it appeared that fluoxetine has a predominant influence upon $5\text{-}HT_{1A}$ receptors when injected systemically, resulting in decreased extracellular 5-HT levels as already reported by Briley and Moret (1993).

Blier, P., de Montigny, C. (1994) TiPS 15, 220-226.

Briley, M. and Moret, C. (1993) Clin. Neuropharmacol. 16,387-400.

Crespi, F., England, T., Ratti, E. et al., (1995) Neurosci. Lett. 188, 33-36.

Crespi, F., Martin, K., Marsden, C.A. (1988) Neurosci. 27, 885-896.

360P DEVELOPMENT OF TOLERANCE TO THE SEDATIVE & ANTINOCICEPTIVE PROPERTIES OF α_2 ADRENERGIC AGONISTS IS DEPENDENT ON NEURONAL NITRIC OXIDE SYNTHASE (nNOS)

Y.K. Amin[1], M.F. Davies[2], F.H. Noormohamed[1] & M. Maze[1]
Dept. of Anaesthestics, [1]Imperial College, London; [2]Stanford University, USA

Studies reveal that the sedative and analgesic effects of α_2 adrenergic agonists diminish over time, a form of synaptic plasticity known as tolerance. Nitric oxide synthase (NOS) is pivotal in some forms of synaptic plasticity, which suggests possible involvement in tolerance to the hypnotic and analgesic effects of α_2 adrenoreceptor agonists. This study aimed to investigate whether nNOS is involved in the development of tolerance to the sedative and antinociceptive actions of a highly selective α_2 adrenoreceptor agonist dexmedetomidine (dex).

Spontaneous locomotor activity (beam crossings; BC) was assessed as a measure of the hypnotic response. Antinociceptive response was measured by the Hargreaves hind paw test (maximum possible effect; %MPE). Adult male 129/BL6 mice wild type (WT) and 129/BL6 nNOS knockout (KO) mice weighing 20-40g were chronically infused for 6 days with either dex (10 μg/kg/hr), or dex plus NOS inhibitor Nω-nitro-L-arginine (NO_2-arg) (4μg/kg/hr). Effects on antinociception and sedation to the subsequent administration of acute dex were compared. Thereafter, the effects of chronically infused dex (10μg/kg/hr), in KO and matching generations of WT mice were compared.

Data were analysed using ANOVA followed by post hoc Bonferroni comparisons. N= 8-10 for each cohort. Results are expressed as mean ±SD. A p value of <0.05, is regarded as statistically significant.

Inhibition of NOS by a) administering NO_2-arg b) or the lack of the nNOS isoenzyme prevents the development of tolerance to the sedative effects of dex. Tolerance to the chronic administration of dex (94±33; BC) is shown by no change in sedative response to acute admin of dex, when compared to controls chronic infusion of saline(22±16; BC) or NO_2-arg(38±36; BC). The cohort chronically administered dex + NO_2-arg was responsive to acute dex compared to control. Acute dex admin significantly decreased activity in the KO cohort chronically treated with dex (27±31; BC), as compared to control (105±107; BC). Antinociceptive response (%MPE) to dex which had dex and co-administration of dex+NO_2-arg (57±9) was statistically different to antinociceptive response to the control (81±14), which would indicate that tolerance to the antinociceptive effects of dex had developed. However, the difference in response between this group and the chronic dex (3±10) infusion group, implies that partial prevention of tolerance to the antinociceptive actions of dex has occurred. This is further evident in the KO cohort, where a difference between responses to the pre- and post-acute dex in both WT (45±4, 4±5) & KO (59±13, 35±7) cohorts was observed. In addition, a different response was seen between the two post-acute dex groups, WT & KO, implying that partial prevention occurred.

The absence of nNOS iso-enzyme or co-admin of a NOS inhibitor prevents the development of tolerance to the sedative properties of dex and partially prevents the development of tolerance to the antinociceptive actions of dex. Development of tolerance to the sedative and antinociceptive actions of systemically administered α_2 adrenoreceptor agonists involves nNOS.

361P EFFECT OF TRAMADOL AND ITS DERIVATIVES ON ANALGESIA AND EMESIS

R.M. Huckle, R.J. Naylor[1], B.R. Tuladhar[1] & S.S. Rowton[2]. Celltech R&D Ltd, Granta Park, Gt Abington, Cambridge CB1 6GS. [1]The School of Pharmacy, University of Bradford, Bradford, W Yorks, BD7 1DP. [2]Covance Laboratories Ltd, Otley Rd, Harrogate, N Yorks, HG3 1PY

The enantiomers of the analgesic tramadol (T) each display differing affinities for various receptors. (+) T is an agonist of opiate receptors and preferentially inhibits 5-HT reuptake, whereas (-) T mainly inhibits noradrenaline reuptake. The actions of these 2 enantiomers have been said to be synergistic and result in the analgesic effect of the racemate (+/-) T (Raffa *et al*, 1992). The most common side effects seen with administration of (+/-) T are nausea and dizziness (Desmeules, 2000). (+/-), (+), (-) T and O-desmethyl tramadol (ODT) were examined in models of analgesia (tail-flick and Randall-Selitto in the rat) and a model of emesis (retching and vomiting in the ferret).

Assessment of analgesia: Male Wistar rats (120-250 g) were used in these studies. (+/-), (+), (-) T (+) ODT and (-) ODT were formulated in 0.9% w/v saline and dosed orally at a constant dose volume of 10 ml/kg.

Tail-Flick - For each animal the reaction time in response to an infrared beam with an intensity setting of 20 (Tail-Flick Unit, Ugo Basile) was measured at the -0, +0.5, +1, +2 hours.

Randall-Selitto - Immediately following oral administration, 0.1ml of a 20% w/v suspension of Brewer's yeast in saline was injected subcutaneously into the plantar surface of the left hind paw to induce hyperalgesia. The right paw was injected similarly with saline (0.1ml) in order to serve as a control for each animal. For each animal the pain threshold of the left and right hind paw was assessed using an analgesiometer (Ugo Basile) and measured at -0, +0.5, +1, +2 hours. In both models of analgesia T and its derivatives displayed a similar rank order of potency (see Table 1).

Emesis: T and its derivatives were examined for potential to induce emesis (retching and vomiting) in the ferret when observed over a 6 hour period following an oral administration (in 2mls/kg).

Table 1 Effect of tramadol (T) and its derivatives on analgesia in the rat and emesis in the ferret

Treatment (po)	ED50 (mg/kg) (mean ± SEM n=3-6)			
	Tail Flick (0.5h post-dose)	Randall-Selitto		
		Inflamed	Non-Inflamed	Ferret Emesis
(+/-) T	20 ± 1.6	20 ± 2.7	40 ± 3.5	120 ± 8.5
(+) T	7.5 ± 1.5	10 ± 0.8	15 ± 1.6	25 ± 2.2
(-) T	>100	75 ± 4.5	75 ± 5.2	>200
(+) ODT	5.0 ± 0.8	5.0 ± 0.5	10 ± 1.5	2.5 ± 0.5
(-) ODT	>200	100 ± 8.5	100 ± 7.8	>100

These results are generally in agreement with previous studies (Raffa *et al*, 1992 and 1993) that show (+) T is more potent an analgesic than (-) T. In the analgesia models the enantiomers appear to interact in a complimentary manner. Whilst (+) T and (+) ODT are most potent in the analgesia models, they also cause emesis. In fact (+/-) T is less emetic than would be predicted from the potency of (+) T and (+) ODT. Further investigations into non-racemic ratios of T may reveal improved tolerability (as regards nausea) whilst maintaining analgesic efficacy.

Desmeules, J.A., (2000) *Eur J Pain* 4 Suppl A 15-21

Raffa, R.B. *et al.*, (1992) *J Pharmacol Exp Ther* 260 275-285

Raffa, R.B. *et al.*, (1993) *J Pharmacol Exp Ther* 267 331-340

362P ANTI-EMETIC EFFECT OF (-) TRAMADOL

R.M. Huckle, B.R. Tuladhar[1] & R.J. Naylor[1] Celltech R&D Ltd, Granta Park, Gt Abington, Cambridge CB1 6GS. [1]The School of Pharmacy, University of Bradford, Bradford, W Yorks, BD7 1DP.

Nausea is a known side effect of tramadol (Desmeules, 2000) and other analgesic opioid drugs. (+/-) Tramadol (T) has been used clinically as the racemate and in the ferret model of emesis, (+) but not (-) T was found to induce retching and vomiting (see Huckle *et al.*, this meeting). Of particular interest, (+/-) T was a less potent emetogen than expected, indicating that the (-) isomer may actually attenuate emesis induced by the (+) isomer. This possibility was investigated in the present study.

Male ferrets were routinely fed a pellet SDS Diet 'C' (E) with water available *ad libitum*. *Suncus murinus* were routinely allowed free access to water and Aquatic 3 trout pellets. *Suncus* and ferrets were also fed cat food (Kitekat Supreme, Pedigree Foods) three times a week. Animal rooms were maintained at 22-24 °C with artificial lighting 0700 to 1900h. For the induction and measurement of emesis, animals were transferred to individual holding cages and the ferrets each allowed 100g of cat food. Animal behaviour was recorded via CCTV and analysed at the completion of the study. Emesis was characterised by rhythmic abdominal contractions that were either associated with the oral expulsion of solid or liquid material from the gastrointestinal tract (ie vomiting) or not associated with the passage of material (ie retching movements). Effects on motion sickness were examined in *Suncus*, which was induced by a 10 minute period of horizontal movement (2Hz, 40 mm displacement). Preliminary experiments established an optimal anti-emetic pre-treatment time for (-) T of 180 minutes against (+) T. This was used for each emetogen. Prior to the emetogenic challenge, animals received vehicle or (-) T. Cisplatin was administered as a single injection on day 1 and animals observed over a 3 day period.

Table 1 The anti-emetic effect of (-) T (180 min pre-treatment, administered orally in the ferret and i.p. in *Suncus*) on drug induced emesis in the ferret and motion induced emesis in *Suncus*.

(-) Tramadol pretreatment (mg/kg) po	Emetogen (mg/kg)	% change from vehicle group mean (R+V)	Incidence of emesis n
10	(+) Tramadol	-93*	1/4
30	(50 po)	-97*	1/4
50		-100*	0/4
3	Cisplatin [0-23h]	-23*	5/5
10	(5.0 ip)	-83*	5/5
50		-82*	5/5
3	Cisplatin [24-47h]	-15*	5/5
10		-84*	5/5
50		-76*	5/5
3	Cisplatin [48-72h]	-22	5/5
10		-84*	5/5
50		-75*	5/5
10 (ip)	Motion	-95*	2/6

*P<0.0001 vs vehicle control by ANOVA with post-analysis by Bonferroni/Dunnett's t-test.

The present study has shown that in contrast to the emetic effect of (+) T in the ferret, the (-) isomer will not induce emesis but will actually antagonise the emetic effects of (+) T. This has important implications for an understanding of the nausea induced by T racemate in the clinic where it is available as the racemate. In addition, the anti-emetic effect of the (-) enantiomer was extended to an antagonism of cisplatin emesis in the ferret and also to motion induced sickness in *Suncus murinus*. This provides the rationale to support the testing of (-) T in clinical conditions such as post-operative nausea and vomiting and other indications.

Desmeules, J.A., (2000) *Eur J Pain* **4 Suppl A** 15-21
Huckle, R.M. *et al.* (2001) *Br J Pharmacol* (in press)

363P KINDLING INDUCED IN MICE BY REPEATED ADMINISTRATION OF SUB-CONVULSANT DOSES OF NICOTINE

J.F. Bastlund, W.P. Watson & C. Sánchez Neuropharmacology Department, H. Lundbeck A/S, Ottiliavej 9, 2500 Valby, Copenhagen, Denmark.

Repeated administration of sub-convulsive doses of pro-convulsive compounds can, over time, induce a convulsive state in an animal, i.e. cause kindling. Compounds commonly used to induce kindling are leptazol, PTZ or FG 7142 (Little *et al.*, 1987), all of which act at the $GABA_A$/benzodiazepine receptor complex. In the following experiments we investigated whether nicotine, a drug not acting directly at GABA receptors, could induce kindling.

Separate groups of 8 male NMRI mice (22g at start of experiment) were used. The animals were injected either every weekday or every other day (Mon, Wed, Fri) for 3 weeks, with nicotine bitartrate (6.0, 7.0 or 7.5 mg/kg, IP) or saline vehicle. Nicotine doses were chosen to be around the minimal convulsant dose in drug naïve animals (data not shown).

Each mouse in the study was individually identified, so that its behaviour could be followed for the whole experiment. Following injection with the convulsant drug, the mice were placed singly in plastic cages, and their behaviour was noted for the next 15 min. The incidence of clonic convulsions was recorded and the results were presented as the percent of the group exhibiting clonic convulsions.

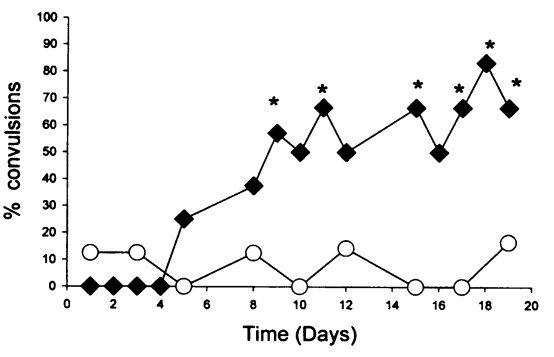
Our study demonstrated that injecting mice with nicotine, 7mg/kg every weekday, but not every other day, caused an increase in the number of mice convulsing over time (fig 1). In the group receiving daily injections, significantly more animals convulsed on days 9,11,15,17,18 and 19 than did in the saline treated group – which showed no convulsions ($P < 0.05$; Fisher's exact probability test).

There was no increase in the number of mice convulsing when nicotine was given at 6 mg/kg, with either time schedule, whilst those given 7.5 mg/kg showed a marked increase in mortality over time (data not shown).

These experiments demonstrate a schedule for induction of kindling by nicotine, and will permit further investigation of antiepileptic compounds on the mechanisms of kindling itself, rather than interactions at the GABA receptor.

Little, H.J., Nutt, D.J. & Taylor, S.C. (1987) *Neuropharmacology*, 26, 25-31.

Figure 1: Effect of repeated dosing with nicotine (7mg/kg IP) on incidence of clonic convulsions. Drug was given Mon to Fri (closed symbols) or Mon, Wed, Fri (open symbols). (* $P < 0.05$ c.f. saline)



364P COMPARISON OF THE ACTIONS OF LEVETIRACETAM AND TIAGABINE ON KINDLING INDUCED BY NICOTINE OR PTZ

W.P. Watson, J.F. Bastlund & C. Sánchez. Neuropharmacology Department, H. Lundbeck A/S, Ottiliavej 9, 2500 Valby, Copenhagen, Denmark.

We have previously demonstrated that daily administration of 7mg/kg nicotine bitartrate induced kindling in male NMRI mice (Bastlund *et al.*, this meeting). In the following experiments we investigated the effect of the antiepileptic drugs tiagabine and levetiracetam on the development of kindling induced by nicotine, and compared this with their action on kindling induced by pentylenetetrazole (PTZ).

Groups of 10-12 male NMRI mice (22 g at the start of the experiment) were used. Each mouse in the study was individually identified, so that its behaviour could be followed for the whole experiment. Animals were kindled by repeated injection IP of either 7 mg/kg nicotine bitartrate every weekday for two weeks or by repeated injection IP. of 37 mg/kg PTZ Mondays, Wednesdays and Fridays for 19 days. Mice in both the nicotine and PTZ kindling experiments were pre-treated SC with either tiagabine (0.5, 1.0 or 2.0 mg/kg), levetiracetam (5, 10 or 20 mg/kg) or saline vehicle, 30 minutes prior to injection of the convulsant drug.

Following injection of the convulsant drug, the mice were placed in individual cages, and the incidence of clonic convulsions was noted for the next 15 minutes. A plot was made of convulsions *versus* time for each mouse, and the results are shown in table 1 as the mean (± s.e.) of the area under this time-effect curve for each treatment group. In addition, the number of animals convulsing in each group on the last experimental day was compared to the number convulsing in the vehicle group with Fisher's exact probability test.

The results in table 1 show that levetiracetam significantly attenuated both PTZ- and nicotine- induced kindling, at all doses tested. However, when tiagabine was used, a difference was seen between the two models. The lowest dose of tiagabine (0.5 mg/kg) significantly decreased the area under the PTZ kindling curve ($P < 0.05$), whereas only the 2 mg/kg dose decreased the area under the nicotine kindling curve ($P < 0.05$).

These experiments demonstrated that both levetiracetam and tiagabine had a beneficial effect on the development of kindling induced by convulsant drugs. The results may imply that both levetiracetam and tiagabine are acting on the process of kindling development *per se*, and may aid our understanding of the progression of epileptic phenomena.

Bastlund, J.F., Watson, W.P and Sánchez C (2001) Current meeting of British Pharmacological Society.

Table 1: Mean ± s.e. area under time-effect curve for PTZ and nicotine kindled mice, pretreated with levetiracetam (LEV) or tiagabine (TIA) (* = Number of convulsions on last day P< 0.05 c.f. Vehicle group – Fishers exact test)

LEV	PTZ	Nicotine	TIA	PTZ	Nicotine
Vehicle	3.4±0.6	2.4±0.7	*Vehicle*	3.4±0.6	2.4±0.7
5 mg/kg	0.3±0.3*	0.9±0.3*	*0.5 mg/kg*	0.5±0.5*	2.4±0.6
10 mg/kg	0.5±0.4*	0.0±0.0*	*1.0 mg/kg*	0.3±0.3*	1.3±0.4
20 mg/kg	0.2±0.2*	0.4±0.2*	*2.0 mg/kg*	0.0±0.0*	1.1±0.2*

365P PROFILE OF CLINICALLY EMPLOYED ANTIEPILEPTICS AGAINST SOMAN-INDUCED EPILEPTIFORM ACTIVITY IN THE GUINEA-PIG HIPPOCAMPAL SLICE

P.K. Harrison, R.D. Sheridan, A.C. Green, I.R. Scott, & J.E.H. Tattersall, Dstl Chemical & Biological Sciences, Porton Down, Salisbury, SP4 0JQ

An important feature of organophosphorus (OP) intoxication is seizure activity (which is initiated by inhibition of brain acetylcholinesterase). Although OP-induced seizures are generally treated with a benzodiazepine, it is possible that other classes of anticonvulsant may give better seizure control. We have previously described a guinea-pig hippocampal slice model in which the irreversible anticholinesterase OP, soman, reliably induces epileptiform bursting (Harrison *et al.*, 2000). The aim of the present study was to evaluate several established and new antiepileptics for their activity in this model.

Neuronal activity in *str. pyramidale* of area CA1 was recorded extracellularly in 500 µm thick hippocampal slices isolated from male Dunkin Hartley guinea-pigs and maintained in a submersion type recording chamber at 31°C. Data are expressed as mean ± s.e. mean.

Application of 0.1 µM soman led to the appearance of synchronised bursting activity in the majority of slices tested (latency to onset: 21.3 ± 1.6 min; discharge frequency 5-15 min after initial appearance of epileptiform activity: 2.5 ± 0.2 bursts/min; $n = 90$ slices).

Test compounds were applied to the slices 15 min after the appearance of soman-induced bursting, and their anti-epileptiform effects were assessed by measuring the burst frequency during a 10 min epoch (30-40 min after drug application) and comparing it to the burst frequency over the 10 min epoch immediately before drug addition.

The effects of antiepileptic compounds are shown in figure 1. Retigabine, diazepam, topiramate and carbamazepine all suppressed the soman-induced epileptiform activity. Whilst neither felbamate nor clomethiazole reduced the burst rate, both showed a marked ability to reduce the duration of the 'burst' event, an effect also seen with carbamazepine and occasionally with diazepam. The antiepileptic drug levetiracetam had little effect.

These findings demonstrate that epileptiform bursting induced by soman can be inhibited by a variety of antiepileptic drugs, with the novel antiepileptic drug retigabine clearly possessing the most potent activity. These results provide some justification for future *in vivo* evaluation of novel antiepileptic compounds in the management of OP-related seizures.

Supported by the UK Ministry of Defence.

Harrison, P.K. *et al.*, (2000) *Eur. J. Neurosci.*, 12, 209 Suppl.

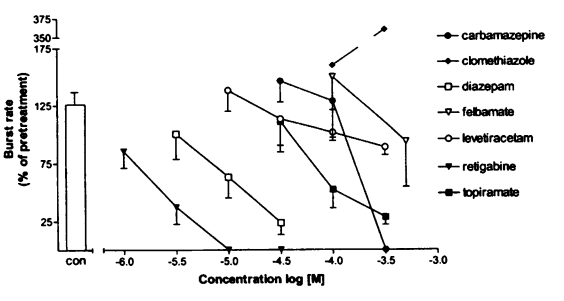


Figure 1 The effect of antiepileptic drugs on the soman-induced epileptiform activity. All points are the mean of 3-6 separate experiments ± SEM. The bar represents the change in rate observed without any intervention. For clarity the error bars for 100 and 300 µM clomethiazole (± 31.5 & ± 175.6, respectively) have been omitted

366P EFFECTS OF CHRONIC ADMINISTRATION OF THE $GABA_B$ AGONIST BACLOFEN ON FOOD INTAKE AND BODY WEIGHT IN RATS

S.M. Patel & I.S. Ebenezer. School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, Hants, PO1 2DT.

It has been previously demonstrated that acute central or systemic administration of the $GABA_B$ agonist baclofen increases food intake in rats (Ebenezer, 1990, Ebenezer and Pringle, 1992). The present study was undertaken to investigate the effects of chronic administration of baclofen on food intake and body weight in rats.

Adult male Wistar rats (n=12; b. wt. at the start of the study = 400 – 420g) were divided into two equal groups. The rats were weighed each morning. At 0900h the animals were injected i.p. with either saline solution (Control Group) or baclofen (1 mg kg^{-1}; Treatment Group). The rats were put into separate experimental boxes and presented with food and water, and food intake measured, as described previously (Ebenezer, 1990). At 17.00h the rats were injected i.p. with either vehicle or baclofen (1 mg kg^{-1}), according to their treatment group, and placed back in their home cages with free access to food and water. The rats received two daily injections of either vehicle or baclofen, with the exception of weekends when they received only one injection in the morning. Food intake and body weight were not measured during the weekend periods. The experiment was carried out over a period of 25 days. The dose of baclofen used in this study has previously been shown to increase food intake in rats (Ebenezer and Pringle, 1992).

Fig. 1A shows the effects of baclofen on cumulative food intake, measured 60 min after administration, during the 25 day dosing period of the study. Baclofen (1 mg kg^{-1}) significantly increased daily food intake. Tolerance did not develop to the hyperphagic effect of baclofen with chronic administration. The body weight of control rats increased by approximately 2g a day during the course of the experiment. The effects of baclofen on changes in body weight are shown in Fig. 1B. There were no significant effects of baclofen on body weight in the treated rats compared with control animals over the experimental period.

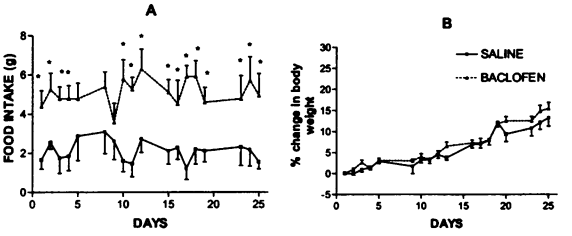


Fig.1. A. Effects of baclofen on food intake measured 60 min after administration. B. Effects of baclofen on percentage changes in body weight from pretreatment values. Vertical lines represent ± s.e.mean. *P<0.05.

The results of this study show that (i) tolerance does not develop to the hyperphagic effects of baclofen with chronic administration, and (ii) there is no effect on body weight following chronic administration of the drug. These results suggest that although baclofen causes an acute increase in feeding following administration, this is not reflected in weight gain. One possibility is that the animals accurately regulate their food intake over a 24h period as shown in the pig (Ebenezer and Baldwin, 1990) to prevent increases in body weight. Another possibility is that baclofen may increase metabolic rate.

Ebenezer, I.S. (1990) NeuroReport 1, 73 - 76
Ebenezer, I.S. and Pringle, A.K. (1992) Neuropharmacol. 31, 39 - 42.
Ebenezer, I.S. & Baldwin, B.A. (1990) Br. J. Pharmacol. 101, 559 - 562

367P SYNERGY BETWEEN CHOLECYSTOKININ (CCK) AND CALCITONIN GENE-RELATED PEPTIDE (CGRP) ON SUPPRESSION OF FOOD INTAKE IN RATS

M Price, S.D. Murray & I.S. Ebenezer. School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, PO1 2DT.

It has been previously been demonstrated that i.p. administration of CCK or CGRP reduce food intake in rats (Gibbs et al., 1973; Price et al., 2000). As both CCK and CGRP are released from the small intestine during the ingestion of a meal, these peptides have been separately mooted to act as peripheral satiety factors (Gibbs et al., 1973; Price et al., 1998). The present study was undertaken to investigate the effects of administering both peptides together on the suppression of food intake in rats.

Experiment 1. Male Wistar rats (n=8; b. wt. 260 - 320g) that were fasted for 22h a day, were injected i.p. with either physiological saline solution or rat(r) CGRP (10 or 20 μg kg^{-1}) and presented with food, as described previously (Ebenezer, 1990). Food intake was measured at intervals over 120 min. A repeated measures design was used with each rat receiving all doses of saline and CGRP; at least 3 days separated successive trials. Experiment 2. Male Wistar rats (n=8; b. wt. 280 – 320g) were fasted for 22h a day and received the following treatments: saline, CCK-8S (2 μg kg^{-1}), rCGRP(20 μg kg^{-1}), or CCK (5 μg kg^{-1})+ CGRP (20 μg kg^{-1}). A repeated measures design was used with each rat receiving all treatments. Food intake was measured, as described for Experiment 1. The dose of CCK used in this experiment has previously been shown in our laboratory and elsewhere, to produce reliable suppression of feeding (Gibbs et al., 1973). The data from both experiments were analysed by ANOVA for repeated measures followed by *post-hoc* test.

The results obtained in Experiment 1 show that rCGRP produces a dose-related decrease in food intake (Fig. 1A). The 20 μg kg^{-1} dose significantly decreased cumulative feeding for up to 60 min. The results from Experiment 2 are shown in Fig. 2. Both CCK-8S (2 μg kg^{-1}) and CGRP (20 μg kg^{-1}) significantly reduced cumulative food intake for up to 60 min. However, the combined doses of CCK-8S and rCGRP significantly reduced cumulative food intake throughout the 120 min measurement period. Moreover, the suppression in food intake at, for example, 60 min, was significantly greater than that for either CCK-8S or rCGRP alone, both in terms of the amount of food consumed by the rats and the period over which this hypophagia is apparent (see Fig. 1B).

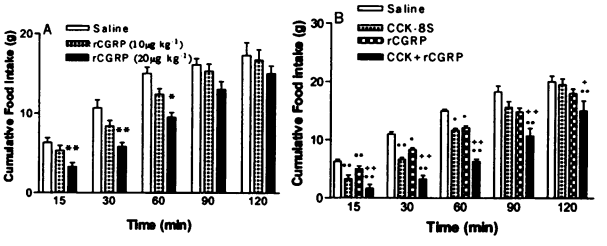


Fig.1. A. Dose-related effects of rCGRP on food intake in rats. B. Effects of CCK-8S (2 μg kg^{-1}), rCGRP (2 μg kg^{-1}), and CCK-8S+rCGRP on food intake in rats. Vertical lines rep. + s.e.mean. **P<0.01 *P<0.05 vs Saline, $^{++}$P<0.01 $^{+}$P<0.05 combination dose vs CCK and rCGRP.

We have recently demonstrated that selective CCK antagonists do not affect CGRP inhibition of food intake (Price, Dacke and Ebenezer, unpublished results), indicating that CGRP and CCK inhibit food intake via distinct pathways. The present results suggest that under physiological conditions CCK and CGRP may act synergistically to produce satiety.

Gibbs, J. et al. (1973) J. Comp. Physiol. Psychol., 84, 448 - 495.
Price, M. et al. (2000) Br. J. Pharmacol., 129, 148P.
Price, M. et al. (1998) J. Physiol., 513, 8P

368P EFFECTS OF 8-HYDROXY-2-(DI-N-PROPYLAMINO)-TETRALIN (8-OH-DPAT) ON WATER INTAKE IN RATS

I.S. Ebenezer & R. Tite. Neuropharmacology Research Group, School of Pharmacy, University of Portsmouth, Portsmouth, U.K.

It have been previously found that while $5HT_{1A}$ receptor agonists, such as 8-OH-DPAT, gepirone and isapirone increase food intake in non-deprived rats (Dourish et al., 1985), they decrease feeding in food deprived rats by a $5\text{-}HT_{1A}$ receptor mediated mechanism (Arkle and Ebenezer, 2000). However, there is a marked paucity of information on the effects of these drugs on water intake. Thus, the present study, was undertaken to investigate the effects of water intake in rats.

Experiment 1. Male Wistar rats (n=6, b. wt. 300 - 380g) were deprived of water for 16h each day and injected s.c. with saline or 8-OH-DPAT (12.5, 25, 50, 100 or 200 μg kg^{-1}). 15 min later they were placed individually in experimental cages where they had free access to water, as described previously (Ebenezer et al., 1992), and water intake measured at intervals over 60 min. A repeated measures design was used with each animal receiving all doses of drug. 4 - 5 days separated successive trials. Experiment 2. Non-deprived male Wistar rats (n=8, b. wt. 300 – 340g) were treated with hypertonic saline (16% w/v) to induced cellular drinking (see Ebenezer et al., 1992, for details) 30 min before exposure to water. 15 min prior to exposure, they were injected s.c. with either saline or 8-OH-DPAT (100 μg kg^{-1}). Water intake was measured, as for Experiment 1. Experiment 3. Male Wistar rats (n=8; b. wt. 320 – 350g) were deprived of water for 16h a day. They received the following treatments: saline followed by saline; the $5HT_{1A}$ receptor antagonist WAY 100635 (300 μg kg^{-1}; WAY; see Arkle and Ebenezer, 2000) followed by saline, saline followed by 8-OH-DPAT (100 μg kg^{-1}), or WAY followed by 8-OH-DPAT. Both injections were give s.c. The first injection was given 30 min and the second injection 15 min prior to exposure to water. A similar procedure, as described for Experiment 1, was used.

The data for each experiment was analysed by ANOVA.

Both water-deprived rats (Expts. 1 & 3) and rats treated with hypertonic saline (Expt. 2), consumed between 85 and 90% of their water in the first 15 min after exposure. 8-OH-DPAT produced a dose-related decrease in water consumption in water-deprived rats, with significant inhibition at doses of 100 and 200 μg kg^{-1} (water intake (ml) ± s.e. mean at 15 min: saline = 13.4 ± 1.3; 8-OH-DPAT 100 μg kg^{-1} = 5.8 ±1.3; 200 μg kg^{-1} = 1.7 ±1.2, P<0.01, in each case). Similarly, 8-OH-DPAT (100 μg kg^{-1}) significantly reduced water intake at 15 min from a control mean ± s.e. mean of 8.4 ± 1.3 ml to 2.9 ± 1.0 ml (P<0.01) in rats given hypertonic saline (Expt. 2).

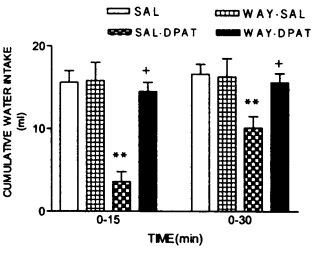


Figure 1. Effect of WAY 100635 on the inhibition of water intake by 8-OH-DPAT (100 μg kg^{-1}). Vertical lines rep. + s.e. mean. **P<0.01 vs saline +P<0.05 WAY+DPAT vs SAL-DPAT. Abbrevations: SAL=saline; DPAT=8-OH-DPAT; WAY = WAY 100635.

The results obtained in Expt. 3 (see Fig. 1) show that the inhibitory effect of 8-OH-DPAT on water consumption is reversed by WAY, thus indicating that the drug-induced hypodipsia is mediated by $5\text{-}HT_{1A}$ receptors. The relationship between these findings and the suppressant effects of 8-OH-DPAT on food intake is not known. One possibility is that $5\text{-}HT_{1A}$ receptor agonists affect motivated behaviours, in general, rather than having selective effects on aspects of ingestive behaviour (see Ebenezer, 1994).

Arkle, M & Ebenezer, I.S. (2000) Eur. J. Pharmacol., 408, 273 – 276
Dourish, C.T. et al. (1985) Brain Res. Bull.,15, 377 – 384.
Ebenezer, I.S. et al. (1992) Gen. Pharmacol., 23, 375 - 379
Ebenezer, I.S. (1994) Br. J. Pharmacol. 112, 648P.

369P EFFECT OF TACE/ADAM 17 INHIBITION IN RAT MIXED CORTICAL CULTURES AFTER GLUTAMATE EXPOSURE

Olivia Hurtado, José L.M. Madrigal, Ignacio Lizasoain, Paz Fernández-Tomé[1], Juan C. Leza, Pedro Lorenzo & María A. Moro. Departamento de Farmacología, and [1]Instituto Mixto de Farmacología-Toxicología (CSIC), Facultad de Medicina, Universidad Complutense de Madrid (UCM), 28040 Madrid, Spain.

The role of the TNF-α convertase (TACE/ADAM17) in the adult nervous system remains poorly understood. Both physiological and pathological roles for the protease activity of TACE/ADAM17 have been indicated (for rev., see Killar *et al.*, 1999) and its CNS expression has been shown (Kärkkäinen *et al.*, 2000). We demonstrated that TACE/ADAM17 is up-regulated in rat forebrain slices exposed to oxygen-glucose deprivation, a paradigm of cerebral ischaemia (Hurtado *et al.*, 2001). In order to further explore TACE/TNF-α pathway in CNS pathology, we have decided to use rat mixed cortical cultures exposed to glutamate, to investigate the expression of TACE, and the effects of TACE inhibition on apoptosis after glutamate exposure.

Primary cultures of mixed cortical cells were performed as described (Moro *et al.*, 1998). Cells were exposed to 10 μM glutamate for 5 min. Then, cells were incubated either in the absence or the presence of the TACE inhibitor BB3103 (0.1-1 μM; Middelhoven *et al.*, 1997) for 2 h. After this time, TACE activity was monitored as the ability to cleave a pro-peptide containing the cleavage site in pro-TNF-α (Hurtado *et al.*, 2001) or by measuring TNF-α levels in the incubation medium by ELISA. TACE protein was measured by immunoblotting, and caspase-3 activity was measured fluorometrically with a commercial kit.

TACE activity in glutamate-exposed cells was higher than in control cortical cultures (1.3±0.1 vs. 2.0±0.1RFU in control and glutamate-exposed cells, respectively, n=4, P<0.05). In addition, TNF-α levels in glutamate-exposed cells were higher (3.4±0.3pg/ml, n=6) than in control cells (1.7±0.3, n=6, P<0.05). Immunoblotting showed the presence of TACE in control cultures and its up-regulation (1.5-fold) after exposure to glutamate. The TACE inhibitor BB3103 (0.1 μM) inhibited both glutamate-induced increase in peptide cleavage (0.7 ± 0.1 RFU, n=4, P<0.05) and in TNF-α levels (1.3±0.1pg/ml, n=6, P<0.05). Exposure to glutamate did not increase caspase-3 activity when compared with control cultures (4.7± 1.1 vs. 6.2±1.0 pmol/min mg prot in control and glutamate-exposed cells, respectively, n= 4, P>0.05). However, BB3103 (0.1 μM) caused an increase in caspase-3 activity after exposure to glutamate (9.8± 1.0 pmol/min mg prot).

Taken together, our data indicate that TACE/ADAM17 is up-regulated in rat cortical cultures after exposure to glutamate, and that the subsequent increase in TNF-α may play an anti-apoptotic role in this setting.

Killar L, White J, Black R *et al* (1999) Ann NY Acad Sci 878, 442-452.
Kärkkäinen L, Rybnikova E, Pelto-Huikko M *et al* (2000) Cell. Neurosci. 15, 547-560.
Hurtado O, Cárdenas A, Lizasoain I, *et al* (2001). Neuropharmacology 40, 1093-1101.
Middelhoven PJ, Ager A, Roos D *et al* (1997). FEBS Lett. 414, 14-18.
Moro MA, Fernández-Tomé P, Leza JC *et al* (1998). Neuropharmacology 37:1071-1079.

370P DISTRIBUTION OF NMDA AND AMPA GLUTAMATE RECEPTOR SUBUNITS IN RAT PREFRONTAL CORTEX AND THE EFFECT OF CHRONIC IMIPRAMINE TREATMENT ON THE GluR2 SUBUNIT

S. Nazir, G.J. Michael & A. T. Michael-Titus, Department of Neuroscience, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary College, University of London, Mile End Road, London E1 4NS

Significant neuronal and glial loss has been reported in the prefrontal cortex in major depression (Rajkowska *et al.*, 1999). Antidepressants decrease the level of mRNA for N-methyl – D-aspartate (NMDA) glutamate receptor subunits in mouse frontal cortex (Skolnick, 1999) and we have shown that imipramine reduces glutamate overflow in rat prefrontal cortex (Michael-Titus *et al.*, 2000). Here we examine the distribution of NMDA and amino-3-hydroxy-5-methylisoxazole- 4-propionate (AMPA) glutamate receptor subunits in the prefrontal cortex of control rats and after treatment with imipramine and we show the effect of the antidepressant on the AMPA GluR2 subunit. Particular attention was paid to the medial prefrontal cortex and the prelimbic area, because of its suggested analogy with the primate prefrontal region.

Male Wistar rats (150-200 g) were treated for 21 days with saline (controls) or imipramine (15 mg/kg, i.p.). 24 hours after the last administration animals were perfused under anaesthesia with 4% paraformaldehyde. The tissue was post-fixed, cryoprotected and blocked and 40 μm sections were cut. Tissue was pre-incubated with donkey serum, and then with polyclonal primary antibody (NR1 1:500; NR2A 1:2000; NR2B 1:1000; GluR1 1:2000; GluR2 1:1000; GluR2/3 1:4000). Sections were washed and incubated with biotinylated donkey anti-rabbit IgG (1:400). After incubation with Vectastain Elite ABC immunoperoxidase reagent, sections were stained with 3,3- diaminobenzidine.

For semi-quantitative analysis, cells were counted (x40) blind in three non-overlapping frames. Data were compared using Student's *t* test.

All subunits were widely distributed in both the motor and prelimbic areas of prefrontal cortex; however, distinctive patterns of labelling were recognised. Dense NR1 immunoreactivity was seen in all areas of motor and prelimbic cortices, with no major differences in the laminar distribution patterns across areas. Immunoreactive neurones were mostly pyramidal cells. NR2B exhibited strong neuropil staining. GluR1 staining was associated with numerous non-pyramidal neurones. GluR2 staining was present in pyramidal cells and was intense in apical dendrites. In imipramine-treated animals the number of GluR2 immunoreactive cells in layer II and layer V of the prelimbic cortex was increased by 21% and 25% respectively, *vs.* controls ($P<0.05$). No such differences were seen in the motor cortex (layers II and V) of the treated animals.

Our study shows that antidepressant treatment may lead to changes in AMPA receptors, notably an increase in the GluR2 subunit, which controls calcium permeability. This increase in AMPA receptors that are calcium impermeable may alter AMPA-mediated transmission and may confer increased resistance to the excitotoxicity associated with increased cytosolic calcium. Interestingly, this effect appears to affect significantly a cortical area associated with depression.

Rajkowska, G., Miguel-Hidalgo, J.J., Wei, J. *et al.* (1999) *Biol. Psychiatry*, 45, 1085-1098.
Skolnick, P. (1999) *Eur. J. Pharmacol.*, 375, 31-40.
Michael-Titus *et al.* (2000) *Neuroscience*, 100, 681-684.

371P MODULATION OF THE RELEASE OF ENDOGENOUS DOPAMINE FROM RAT STRIATAL SLICES BY AGONISTS OF THE STRYCHNINE-INSENSITIVE GLYCINE RECEPTOR SITE OF THE NMDA RECEPTOR.

S Bennett & B Gronier, De Montfort University, Leicester

In the central nervous system, dopamine activity is heavily modulated by glutamate/NMDA neurotransmission and evidence suggests that this interaction plays a role in the aetiology and treatment of schizophrenic disorders. Furthermore, some agonists of the strychnine-insensitive glycine receptor site of the NMDA receptor, such as glycine, D-serine and D-cycloserine have recently been studied in clinical trials as an adjunct to conventional neuroleptics and were found to reduce negative symptoms (Heresco-Levi 2000).

We have examined whether these compounds can modulate the endogenous release of dopamine in both basal conditions and during NMDA receptor stimulation, in rat striatal slices. Freshly prepared slices were incubated in oxygenated Mg^{++}-free Krebs medium at 37 °C. Samples of medium were collected at 5 min intervals and endogenous dopamine levels were measured using HPLC. After equilibration, NMDA (10^{-6}M) was subsequently applied for 5 min at 30 min interval. Glycine (10^{-6}M), D-serine (10^{-6}M) and D-cycloserine (10^{-6}M) were introduced 15 min after the first NMDA stimulation. The effects of the co-agonists were assessed by comparing both basal and stimulated dopamine release, before and during their application in the medium using paired-Student's t test.

In control conditions (no agonists added), NMDA promoted the release of endogenous dopamine to levels 794+/-24 % over baseline (n=26), and the two subsequent NMDA stimulations were of similar amplitude (102±12% variation, n=4, $p<0.9$). D-serine, produced a significant increase (193±44%, n=12, $p<0.04$) of basal dopamine outflow, while glycine and D-cycloserine were devoid of effects. The application of D-serine (n=8) and D-cycloserine (n=5) to the medium significantly reduced the NMDA response by 30±5% and 38±6% respectively ($p<0.04$). Conversely, NMDA-evoked release was not significantly modified in the presence of glycine.

These results indicate that striatal dopamine release can be modulated by D-serine. The fact that D-serine increases basal dopamine release may suggest the presence in striatum of a functional native NMDA receptor subtype modulating terminal dopamine release that can be activated by D-serine alone in the absence of glutamatergic coagonist. Evidence exists for the presence of such a receptor subtype in the rat brain (Paudice et al 1998). The inhibitory, rather than activatory, effects of D-serine and D-cycloserine are probably related to an interaction with GABAergic neurones, since their effects are blocked by bicuculline (data not shown). Finally, the lack of effect of glycine could be due to its being rapidly taken up by a selective transport system, for which D-serine has no affinity (Berger et al 1998).

Overall, these data indicate that these agonists could modulate dopaminergic transmission, either by increasing tonic dopamine release or by reducing the phasic release dependent on NMDA receptors.

Berger A, Dieudonne S & Ascher P (1998) *J. Neurophysiol* 80: 3336-3340

Paudice P, Gemignani A & Raiteri M (1998) *Eur J Neurosci*, 10, 2934-2944

Heresco-Levi U (2000) *Int J Neuropsychopharm* 3, 243-258

372P EFFECT OF NORADRENALINE AND 5-HT ON EXCITATORY AMINO ACID RELEASE IN RAT PREFRONTAL CORTEX

A. Hawkins, L. McCune, R. Whelpton & A .T. Michael-Titus, Department of Neuroscience, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary, University of London, Mile End Road, London E1 4NS.

It has been suggested that excitatory amino acid (EAA) transmission could be a new target of antidepressant treatment (Skolnick, 1999). Glutamate antagonists are effective in animal models of depression. Conversely, antidepressants change the binding characteristics of glutamate receptors, in particular in the cortex. Imipramine and phenelzine decrease EAA release stimulated by 50 mM KCl in rat prefrontal cortex (Michael-Titus *et al.*, 2000). The present study attempts to establish the relative contribution of noradrenaline (NA) and 5-HT to this effect of the two antidepressants.

Prefrontal cortex slices from male Wistar rats (150-200 g) were incubated in Krebs-bicarbonate buffer, and glutamate and aspartate determined by HPLC (Michael-Titus *et al.*, 2000). After collection of basal samples (Fig. 1, B1-B3), slices were exposed (30 s) to buffer with 50 mM KCl in the presence or absence of NA or 5-HT, and returned to fresh buffer (Fig. 1, R1-R2). Analysis of data used ANOVA and *t* test.

Basal glutamate and aspartate outflow was 125 ± 8 and 88 ± 8 pmol/mg protein (n = 30 values). NA (1-100 μM) did not affect basal amino acid release, but it induced a dose-dependent reduction in stimulated amino acid release ($P<0.05$). 5-HT (1-100 μM) did not change the basal or stimulated outflow of the amino acids. In some experiments, a significant *decrease* or *increase* was seen, suggesting a complex response. Therefore, we tested the effects of different stimuli. When the tissue was stimulated with 20 mM KCl, 5-HT 100 μM induced a 29% decrease in glutamate overflow, whereas when the tissue was stimulated with 2 mM 4-aminopyridine, 5-HT 100 μM induced an increase of 25% in glutamate overflow *vs.* controls ($P<0.05$; n=6-8 values).

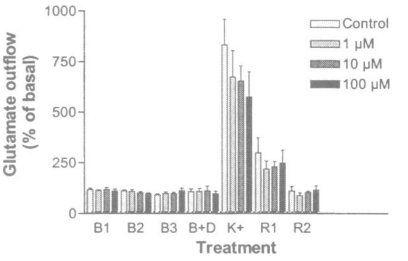


Figure 1. Effect of NA on basal (B+D) and stimulated (K^+) glutamate outflow. Data are means ± s.e.m. of 6–15 values.

Thus, under the conditions used previously (Michael-Titus *et al.*, 2000) NA has an inhibitory effect on EEA release in the prefrontal cortex. However, the contribution of 5-HT to the effects of the two antidepressants is more difficult to establish, as the amine appears to have the potential to decrease *or* increase EAA release, as has been suggested (Marek & Aghajanian, 1998).

Skolnick, P. (1999) *Eur. J. Pharmacol.*, 375, 31-40.
Michael-Titus, A.T. *et al* (2000) *Neuroscience,* 100, 681-684.
Marek, G.J. & Aghajanian, G.K. (1998) *Biol. Psychiatry*, 44, 1118-1127.

373P TROPISETRON (ICS 205-930) INTERACTS WITH HUMAN NICOTINIC ACETYLCHOLINE RECEPTORS

J. Barik, L. M. Houlihan, I. Bermudez & Y. Slater, School of Biological & Molecular Sciences, Oxford Brookes University, Oxford OX3 0BP

The nicotinic acetylcholine (nACh) and 5-hydroxytryptamine type 3 (5HT3) receptors belong to the four-transmembrane family of ligand-gated ion channels, which also includes the γ-aminobutyric-A and glycine receptors. Several studies have shown that the nACh and 5HT3 receptors exhibit a large degree of sequence similarity and structural homology. Analogs of the nACh receptor competitive antagonist D-tubocurarine interact with both receptors in similar fashion and homologous residues have been implicated in agonist binding domains for each receptor, which suggest common structural features in the agonist/antagonist binding domains of these two receptors. The aim of the present work was to further investigate the structural similarities between these two receptors by characterising the interaction of tropisetron, a competitive antagonist of 5HT3 receptors, with human (h) α7 and hα4β2 nACh receptors.

Mature female *Xenopus* toads were sacrificed and the ovary lobes removed. Stage V and VI oocytes were manually defolliculated and microinjected with 50 nl of subunit-specific *in vitro* synthesised cRNAs. Injected oocytes were maintained in Barth's medium, until ready for use. Electrophysiological responses to acetylcholine (ACh) were measured using a standard two-microelectrode voltage-clamp.

Inward currents were observed during superfusion of voltage-clamped oocytes expressing recombinant hα7 nACh receptors with acetylcholine (ACh) or tropisetron. Normalization of the amplitudes of the currents to the maximal ACh response showed tropisetron to be a high-affinity (EC_{50} = 0.6 ± 0.1 μM; n = 4), partial agonist of hα7 nACh receptors. The efficacy of tropisetron at hα7 nACh receptors was 45 ± 7 % (n = 4). Further studies showed that the competitive antagonist of nACh receptors tubocurarine fully inhibited the responses to tropisetron (IC_{50} = 4 ± 0.9 μM; n = 3). Comparison of the voltage dependency of the ACh- and tropisetron-mediated responses revealed identical current-voltage relationships, which were characterised by a marked inward rectification. In contrast, in oocytes expressing hα4β2 nACh receptors tropisetron did not induce inward currents, even at concentrations as high as 1 mM. However, the simultaneous application of tropisetron with an EC_{50} ACh concentration (30 μM), caused inhibition of control ACh responses. The effect of tropisetron was concentration dependent, giving a maximum inhibition of 78 ± 8% (n =3), and gave no further inhibition at concentrations greater than 1 mM. The IC_{50} for tropisetron-mediated inhibition of ACh responses was 100 ± 8 μM (n = 3).

This study shows that tropisetron is a potent partial agonist of hα7 nACh receptors. Given that tropisetron is equally potent at 5HT3 and hα7 nACh receptors (Macor *et al.*, 2001; this study), our results suggest that the effects of tropisetron in emesis and cognition may be partly due to its action on α7 nACh receptors.

Macor, J. E., Gurley, D., Lanthorn, T., Loch, J., Mack, R. A., Mullen, G., Tran, O., Wright, N. & Gordon, J. C. (2001). Chem. Lett. 11, 319-321.

374P CHARACTERISATION OF [^{3}H]ZM-241385 BINDING TO RAT HIPPOCAMPAL MEMBRANES

K. Dhillon[1], A. Lawrence, A. Misra, M.J. Croucher[1], A.R. Knight & K. Quirk. Vernalis Research Ltd, 613 Reading Road, Winnersh, Wokingham, RG41 5UA. Imperial College School of Medicine, Charing Cross Hospital, London, W6 8RF[1].

The neuromodulatory effects of adenosine are mediated in part by the adenosine A_{2A} receptor, which is enriched in striatal tissue. These receptors have been specifically labelled by the agonist [^{3}H]CGS-21680 (Jarvis et al., 1989) and more recently by the antagonist [^{3}H]ZM-241385 (Alexander, 1999), which has also been used to label adenosine A_{2B} receptors (Ji and Jacobson, 1999). Adenosine A_{2A} receptors are also present in the rat hippocampus, but in low abundance in this tissue. This, coupled with the low affinity for [^{3}H]CGS-21680 has hampered the pharmacological characterisation of this receptor. In the present study we have investigated the pharmacology of the binding site for [^{3}H]ZM-241385 in rat hippocampus.

Binding assays were carried out by the method of Jarvis et al. (1989). Rat hippocampal membranes were prepared as described by Ji and Jacobson (1993). Saturation analysis with [^{3}H]ZM-241385 revealed two binding sites in the rat hippocampal membranes with affinities of 5.92 ± 1.46 nM and 39.96 ± 3.23 nM, and Bmax values of 139.93 ± 7.27 and 636 ± 127.04 fmol/mg protein respectively. For displacement studies receptors were labelled with 5nM [^{3}H]ZM-241385 in hippocampal membranes.

Table 1 shows the results of displacement of [^{3}H]ZM-241385 in rat hippocampal membranes by KW-6002, SCH-58261, NECA, DPCPX, CHA, R-PIA and CGS 21680. The xanthine antagonist, DPCPX displaced [^{3}H]ZM-241385 binding with similar affinity in rat hippocampus and striatum (Harvey et al., 1999). However, the antagonists SCH-58261 and KW-6002 were 10 to 100 fold weaker at displacing [^{3}H]ZM-241385 binding in rat hippocampus than other adenosine A_{2A} preparations (Harvey et al., 1999; Palmer et al., 1995). The agonists NECA and CGS 21680 were 2-4 fold weaker at displacing [^{3}H]ZM-241385 binding in rat hippocampus than in rat striatum (Harvey et al., 1999). The pharmacology of [^{3}H]ZM-241385 binding in the hippocampus is therefore unlike that described for the striatal adenosine A_{2A} receptor.

Ji and Jacobson (1999) have previously described [^{3}H]ZM-241385 binding to the A_{2B} receptor in cloned cell lines expressing this receptor. They found DPCPX, NECA and CGS-21680 to have affinities at adenosine A_{2B} receptors of 50nM, 300nM and >100μM, respectively. Thus the binding observed here for [^{3}H]ZM-241385 in rat hippocampus does not reflect the binding of [^{3}H]ZM-241385 to adenosine A_{2B} receptors.

In summary [^{3}H]ZM-241385 labels 2 binding sites in rat hippocampus. Displacement of 5nM [^{3}H]ZM-241385 from rat hippocampus neither represents typical adenosine A_{2A} nor adenosine A_{2B} receptors.

Table1. IC_{50} values (nM) for compounds as displacers of [^{3}H]ZM-241385 in rat hippocampus (mean ± SEM, n=3)

Compound	IC_{50} (nM) in Hippocampus
KW-6002	1250 ± 537
SCH-58261	979 ± 453
NECA	1780 ± 274
DPCPX	4296 ± 2070
CHA	4370 ± 941
CGS 21680	2010 ± 705
R-PIA	3840 ± 170

Alexander S.P.H. et al. (1999) Br.J.Pharmacol **120**(S) 27P
Harvey V. et al. (1999) Br.J.Pharmacol. **128** 289P
Jarvis M.F. et al. (1989) J.Pharmacol.Exp.Ther. **251** 888-893
Ji X.D. & Jacobson K.A.(1993) Arch.Biochem.Biophys. **305**(2) 611-617
Ji X.D. & Jacobson K.A.(1999) Drug Discovery and design. 16 217-226
Palmer T.M. et al. (1995) Mol.Pharm. **48** 970-974

375P CHARACTERISATION OF α_1 ADRENOCEPTOR SUBTYPES IN RAT CORTEX

C.P. Haslam & S.J. Charlton. Novartis Horsham Research Centre, West Sussex, RH12 5AB.

Pharmacological and molecular studies have demonstrated the existence of three α_1 adrenoceptor subtypes, α_{1A}, α_{1B}, and α_{1D}, present in many tissues that mediate a variety of physiological effects (Michel *et al*, 1994). Rat cortex is frequently used in the study of α_1 adrenoceptors using non-selective radioligands that detect binding to all three subtypes. Although there are examples of subtype expression studies, these have only examined α_{1A} and α_{1B} subtypes (e.g. Han and Minneman, 1991). In the current study selective antagonists were used to assess the presence and relative proportions of all three subtypes in rat cortex.

Rat cortex, obtained from male Sprague-Dawley rats (175-200g), was used to prepare a crude membrane fraction by standard homogenisation and centrifugation techniques (Michel *et al*, 1993). Radioligand filtration binding methods were used to characterise α_1 adrenoceptors utilising the non-selective antagonist [^{125}I]HEAT ((±)-β([^{125}I] Iodo-4-hydroxyphenyl)-ethyl-aminomethyl-tetralone), K_d: 58.9 ± 18.7 pM (n = 3). Subtype selective antagonists included 5-methylurapidil (α_{1A} selective), AH11110A hydrochloride (α_{1B} selective) and BMY7378 dihydrochloride (α_{1D} selective), in the presence or absence of the alkylating agent chloroethylclonidine (CEC). All results are expressed as the mean ± standard deviation (n=3).

Non-linear regression analysis of antagonist binding showed bi-phasic curves that were best fit to a two site model when compared to one site model and three site models (F test $p < 0.05$), indicating a heterogeneous population of receptors (pKi values are summarised in Table 1). Pre-treatment with CEC resulted in a concentration dependent reduction in α_1 adrenoceptor binding sites up to 45 %. The percentage reduction at all sites was equal, even at the lowest concentration of CEC.

Compound	Population 1	Population 2
5 Methylurapidil	9.59 ± 0.25	7.80 ± 0.30
	(56.95 ± 16.43)	(43.05 ± 16.44)
AH11110A HCL	9.68 ± 0.87	7.79 ± 0.48)
	(50.07 ± 8.72)	(49.93 ± 8.72)
BMY7378 HCL	9.49 ± 0.51	6.97 ± 0.30
	(27.49 ± 6.14)	(72.51 ± 5.76)

Table 1. Affinity values (pKi) for inhibition of [^{125}I]HEAT with subtype selective antagonists (percentage fractions are given in parentheses).

These results provide evidence that all three subtypes are present in rat cortex, with no single subtype predominant. CEC, reported to be α_{1B} selective (Morrow & Creese, 1986; Minneman *et al*, 1988), was shown to inactivate all α_1 adrenoceptor subtypes present, indicating a lack of selectivity of this compound.

Morrow, A. & Creese, I. (1986) *Mol. Pharmacol.* **29**, 321-330
Minneman, K.P. *et al.* (1988) *Mol. Pharmacol.* **33**, 509-514
Han, C. & Minneman, K. (1991) *Mol. Pharmacol.* **40**, 531-538
Michel, M.C. *et al.* (1993) *Mol. Pharmacol.* **44**, 1165-1170
Michel, M.C. *et al.* (1994) *Br. J. Pharmacol.* **111**, 533-538

376P PHARMACOLOGY OF 5-HT RECEPTORS IN THE CA1 REGION OF RAT HIPPOCAMPAL BRAIN SLICES *IN VITRO*

H.I. Choudhury, G. McAllister, G.R. Seabrook, A. Guiblin & K. Maubach. Merck Sharp & Dohme Neuroscience Research Centre, Terlings Park, Harlow, Essex. CM20 2QR

The 5-HT_7 receptor is one of the most recent additions to the family of 5-HT receptors. Cloned 5-HT_7 receptors exhibit high interspecies homology (~95%) but low homology with other 5-HT receptor subtypes (<40%). Even though the hippocampus contains a high density of 5-HT_7 receptor binding sites and mRNA (Gustafson *et al.*, 1996), the physiological consequences of 5-HT_7 receptor activation in this region have not been extensively studied. In the present study, we have utilised the selective 5-HT_7 receptor antagonist, SB-258719 (Forbes *et al.*, 1998), to investigate the functional role of 5-HT_7 receptors in the CA1 region of rat hippocampus.

Hippocampal slices (parasagittal, 350 μm thick) were prepared from male Wistar rats (150-200 g) and maintained using methods as described previously (Seabrook *et al.*, 1997). All aspects of animal care and use were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and its associated guidelines. Intracellular current-clamp recordings were made from CA1 neurones using microelectrodes filled with 3 M KCl (60-90 MΩ). Drugs were introduced to the bath for 2-3 minutes by superfusion. Data are expressed as mean ± S.E.M. and $P<0.05$ was considered to be significant using the Student's *t*-test.

5-HT produced a robust sequential concentration dependent hyperpolarisation of the majority of CA1 neurones tested. The rank order potency of agonists was 5-carboxytryptamine (5-CT, pEC_{50} = 7.6 ± 0.1, n=7) = lysergic acid diethylamide (LSD, pEC_{50} = 7.4 ± 0.2, n=5) > 5-HT (pEC_{50} = 5.4 ± 0.1, n=6). A sub-maximal dose for each agonist (5-HT 30μM, 5-CT 100nM, LSD 100nM) was chosen for antagonist studies.

The response to 5-HT (-10.6 ± 0.9 mV at 30 μM, n=11) was significantly reduced to 37% of control by the 5-HT_{1A} receptor antagonist s(-)pindolol (3μM, -3.9 ± 0.5 mV, n=7), but was unaffected by SB-258719 (3μM, -8.5 ± 1.6 mV, n=6). In the presence of SB-258719 the remaining response was significantly reduced to 8% of control by the 5-HT_{1A} receptor antagonist WAY100635 (100nM, -0.8 ± 0.2 mV, n=6). The pharmacology of the responses to 5-CT were similar to that of 5-HT. The hyperpolarisation caused by 5-CT (-9.2 ± 0.9 mV at 100 nM, n=13) was significantly reduced to 22% of control by s(-)pindolol (3μM, -2.0 ± 1.2 mV, n=4) and was unaffected by SB-258719 (3μM, -5.9 ± 0.7 mV, n=7). The remaining response was significantly reduced to 24% of control by WAY100635 (100nM, -1.6 ± 0.6 mV, n=5). In contrast, LSD caused a robust hyperpolarisation in only a subpopulation (35%, 9/26) of cells. The response to LSD (-10.7 ± 2.1 mV at 100 nM, n=5) was unaffected by s(-)pindolol (3μM, 89% of control at -9.6 ± 1.5 mV, n=5) but was significantly reduced to 22% of control by SB-258719 (3μM, -2.0 ± 1.0 mV, n=3).

Taken together these data show that the pharmacology of the hyperpolarising responses to 5-HT and 5-CT in hippocampal neurones is consistent with the activation of 5-HT_{1A} receptors. However, the pharmacology of responses to LSD differed in that robust responses were only seen in a subpopulation of cells and were antagonised by SB-258719 and not s(-)pindolol, suggesting that they were mediated by the 5-HT_7 receptor subtype.

Forbes, I.T., Dabbs, S., Duckworth, D.M. *et al.* (1998) *J.Med.Chem.* **41**, 655-657.
Gustafson, E.L., Durkin, M.M., Bard, J.A. *et al.* (1996) *Br.J.Pharmacol.* **117**, 657-666.
Seabrook, G.R., Easter, A., Dawson, G. *et al.* (1997) *Neuropharmacol.* **36**, 823-830.

377P A NOVEL 5-HT_4 RECEPTOR ASSAY USING A PREPARATION OF PIG STRIATUM: A COMPARISON OF 5-HT_4 RECEPTOR PHARMACOLOGY WITH GUINEA-PIG

A.B. Hawcock, C. Huguet & J.D. Gale, Discovery Biology, Pfizer Global Research and Development, Sandwich, Kent, CT13 9NJ

5-HT_4 receptors have been demonstrated in the striatum of guinea-pig (Grossman *et al.*, 1993), and in caudate nuclei of pig, guinea-pig and human (Schiavi *et al.*, 1993). No interspecies differences in receptor characteristics have been demonstrated. Tissue availability limits the utility of these assays and so we have investigated pig striatum as an alternative source of 5-HT_4 receptors and compared the characteristics of binding with 5-HT_4 receptors in the guinea-pig striatum.

Pig heads were obtained from a local abattoir and transported on ice. Pig and guinea-pig brains were removed, striata dissected into ice-cold 50mM HEPES, membranes prepared and protein determined as described by Grossman *et al.* (1993) and stored at -80°C. Binding of [^{3}H]GR113808 (Grossman *et al.*, 1993) at 22°C was optimised for protein and time, and K_D and B_{max} were determined. Affinity estimates (pK_i) were determined for a range of 5-HT_4 ligands with both sets of membranes. Data were compared using ANOVA.

Saturation analysis indicated that [^{3}H]GR113808 bound in a saturable manner to a single site with similar affinity for both preparations. The density of binding sites (B_{max}, fmol mg^{-1} protein) in pig striatum was approximately half that of guinea-pig striatum (Table 1).

Table 1. Saturation analysis of [^{3}H]GR113808 binding to membranes prepared from striata of guinea-pig (n=2) and pig (mean ±SEM, n=4)

	Guinea-pig	Pig
pK_D	10.8 (10.81-10.86)	10.7 ± 0.13
B_{max} (fmol mg^{-1} protein)	280 (273-288)	137.0 ± 39.78

Competition data demonstrated similar affinity of a range of ligands for 5-HT_4 receptors from pig and guinea-pig (Table 2). Analysis of variance between ligand affinities at pig and guinea-pig striatal 5-HT_4 receptors suggests that the 5-HT_4 receptors are not different (p=0.61). When data from both species were correlated, a slope of 1.01 and correlation coefficient of 0.99 was generated.

Table 2. Affinity estimates (pK_i ±SEM n=3) for a range of agonist and antagonist ligands at guinea-pig (G-pig) and pig striatal 5-HT_4 receptors. MCP = metoclopramide, SDZ = SDZ205557.

Ligand	G-pig	Pig	Ligand	G-pig	Pig
5-HT	7.9±0.13	7.9±0.10	RS23597*	8.7±0.16	8.8±0.07
5-MeOT	7.3±0.14	7.1±0.06	R-Zacopride	6.4±0.13	6.6±0.08
MCP	6.7±0.08	6.7±0.02	S-Zacopride	7.6±0.05	7.7±0.08
SDZ*	8.8±0.12	9.0±0.09	Tegaserod	8.8±0.07	8.8±0.11
RS67333#	9.4±0.05	9.5±0.06	Ondansetron	5.8±0.17	5.9±0.02
RS39604*	10.0±0.16	10.2±0.09	Mosapride	7.4±0.19	7.4±0.12
RS67506#	9.5±0.06	9.6±0.07			

Eglen et al (1995) * Claeysen et al (2000)

The similarity between porcine and guinea-pig 5-HT_4 receptors, combined with increased tissue size and availability, makes pig striatum an attractive assay for profiling putative 5-HT_4 receptor ligands.

Claeysen, S., Sebben, M., Becamel, C. *et al.* (2000) *Mol. Pharmacol.* 58, 136-144.
Eglen, R.M., Bonhaus, D.W., Johnson L.G. *et al.* (1995) *Br. J. Pharmacol.* 115, 1387-1392.
Grossman, C.J., Kilpatrick, G.J. & Bunce, K.T. (1993) *Br. J. Pharmacol.* 109, 618-624.
Schiavi, G.B., Brunet, S., Rizzi, C.A. *et al.* (1993) *Neuropharmacol.* 33, 543-549.

378P BINDING AND EFFICACY OF ANTIPSYCHOTIC DRUGS AT THE HUMAN $5HT_{1A}$ RECEPTOR.

M.D. Kennedy & P.G. Strange, School of Animal and Microbial Sciences, University of Reading, P. O. Box 228, Whiteknights, Reading, RG6 6AJ.

The $5HT_{1A}$ receptor has been shown to modulate mood, cognition and motor behaviour, functions that are disturbed in schizophrenia or by antipsychotic medication (Barnes and Sharp, 1999). However, the affinities and efficacies of reference and newer atypical antipsychotic drugs at the $5HT_{1A}$ receptor have yet to be fully characterised.

Assays were carried out in the presence of increasing concentrations of antipsychotic drugs (300pM-1mM). Efficacies (%5HT) and potencies (EC_{50}) were determined using a [^{35}S]GTPγS binding assay (McLoughlin and Strange, 2000) (15μg membranes, 10mM $MgCl_2$, 100mM NaCl, 1μM GDP, 100pM [^{35}S]GTPγS, pH 7.4, final volume 1ml). Binding of drugs was analysed in competition assays where membranes (5-15μg) were incubated in assay buffer (20mM HEPES, 10mM $MgCl_2$, 100μM DTT, pH 7.4) containing either the antagonist [^{3}H]NAD-199 ((R)-3-*N,N*-dicyclobutylamino-8-fluoro-[6-^{3}H]-3,4-dihydroxy-2 *H*-1-benzopyran-5-carboxamide, 0.55nM) or the agonist [^{3}H]8-OH-DPAT (0.25nM) for 2.5h at 25°C.

Non-specific binding was determined in the presence of 100μM 5HT. Data were fitted to either a one or two site model and analysed using a one-way ANOVA followed by a student Newman-Keuls post hoc test (Prism).

Increasing concentrations of haloperidol or chlorpromazine reduced [^{35}S]GTPγS binding indicating inverse agonist activity, while clozapine, olanzapine, quetiapine and risperidone all stimulated binding suggesting agonism albeit with differing efficacies. (Table 1). Quetiapine exhibited two affinities when competed against [^{3}H]NAD-199, consistent with agonism. The other drugs tested all showed a single affinity when competed against either the agonist or antagonist radioligand. However, clozapine, olanzapine and risperidone all show higher affinities in competition versus the agonist radioligand, further suggesting agonism, while chlorpromazine and haloperidol bind with lower affinities, consistent with inverse agonism.

Barnes, N.M & Sharp, T.(1999) *Neuropharmacology*, **38**, 1083-1152
McLoughlin, D.J. & Strange, P.G. (2000) *Journal of Neurochemistry*, **74** 347-57.

Eli Lilly & Co kindly sponsored this work.

	Haloperidol	Chlorpromazine	Clozapine	Olanzapine	Quetiapine	Risperidone
[^{35}S] GTPγS (pEC$_{50}$ ±s.e.m., %5HT).	4.68±0.04 (-23±4)	5.87±0.01 (-51±9)	5.95±0.04 (63±13)	5.85±0.05 (99±2)	5.56±0.01 (109±9)	6.34±0.05 (64±18)
[^{3}H] NAD199 (pK$_i$ ±s.e.m.)	6.49±0.01	6.90±0.04	6.54±0.03	5.59±0.05	7.06±0.01 (pK$_h$, 20%)*** 5.45±0.03 (pK$_l$)***	6.62±0.01
[^{3}H] 8-OH-DPAT (pK$_h$ ±s.e.m.)	6.39±0.07*	6.17±0.02***	6.89±0.03***	5.87±0.04***	6.75±0.04***	6.80±0.01**

Table 1: Efficacies and affinities of antipsychotic drugs at the $h5HT_{1A}$ receptor expressed in CHO cell membranes. Data represent mean ± s.e.m. of 3-4 separate experiments performed in triplicate. *p<0.05, **p<0.01 and ***p<0.001 pK$_h$ vs. pK$_i$ or pK$_l$ and analysed using a one-way ANOVA followed by a student Newman-Keuls post hoc test.

379P EFFECT OF PAROXETINE IN COMBINATION WITH THE 5-HT_{1A} ANTAGONISTS, NAD-299 AND LY 426965, ON EXPRESSION OF THE IMMEDIATE EARLY GENE ARC IN THE RAT BRAIN

R. Tordera, K. Gray, M. Sprakes, Q. Pei & T. Sharp, University Dept. of Pharmacology, Oxford, OX1 3QT, U.K.

Recent strategies aimed at accelerating the antidepressant effect of selective 5-HT reuptake inhibitors (SSRI) have focused on the co-administration of 5-HT_{1A} antagonists with SSRIs (Artigas et al., 2001). Our preliminary studies have shown that the combined treatment of the 5-HT_{1A} antagonist WAY 100635 with the SSRI, paroxetine significantly enhanced Arc mRNA expression in cortical regions (Castro et al., 1999). Arc (activity-regulated cytoskeletal-associated protein) is an immediate early gene, which expression is linked to increased 5-HT function. Here we test the effect of two other recently reported, selective 5-HT_{1A} antagonists, NAD-299, (*R*)-3-N,N-dicyclobutylamino-8-fluoro-3,4-dihydro-2H-1-benzopyran-5-carboxamide hydrogen (2*R*,3*R*)-tartrate mono hydrate (Johansson et al., 1997) and LY 426965, (2*S*)-(+)-1-cyclohexyl-4-[4-(2-methoxyphenyl)-1-piperazinyl]2 methyl-2-phenyl-1-butanone hydrochloride (Rasmussen et al., 2000).

Male Sprague-Dawley rats (250-270 g) were injected s.c. with NAD-299 (1 or 5 m g/kg), LY 426965 (3 or 10 mg/kg) or saline followed 30 min later by paroxetine (5 mg/kg) or saline. Rats were sacrificed 2 h after the last injection and *in situ* hybridisation was carried out (Pei et al., 2000). Arc abundance was determined by densitometric quantification of auto-radiograms, and expressed as % of control.

The abundance of Arc mRNA was not altered by paroxetine, NAD-299 or LY 426965 when given alone, in any region examined when compared to saline controls. In contrast, 5 mg/kg NAD-299 plus paroxetine significantly enhanced *Arc* mRNA abundance in frontal and parietal cortices and in caudate putamen (Table 1). This pattern was similar to that seen with WAY 100635 (Castro et al., 1999). The combination of 10 mg/kg LY 426965 plus paroxetine also increased *Arc* mRNA but this effect was restricted to the caudate putamen. The effects of NAD-299 and LY 426965 were dose-related. These data support our previous finding that an SSRI/5-HT_{1A} antagonist combination induces Arc expression (Castro et al., 1999). The regional differences in the effects of the antagonists tested (WAY 100635/NAD-299 *vs* LY 426965) may reflect different pharmacological properties of these agents.

Table 1. Effect of SSRI/5-HT_{1A} antagonist combinations on abundance of Arc mRNA in rat brain

	Dose (mg/kg s.c.)	Frontal cortex	Parietal cortex	Caudate Putamen	CA3
Saline	-	100±3	100±9	100±5	100±6
Paroxetine	5	100±7	125±8	112±4	97±4
NAD-299	5	107±6	114±5	100±5	101±7
NAD + Par	5/5	129± 5^b	154±14^b	125±10^a	96±6
Saline	-	100±11	100±8	100±4	100±9
Paroxetine	5	78±2	74±7	102±12	87±9
LY 426965	10	127±10	95±7	98±7	96±8
LY + Par	10/5	107±12	82±4	140±4^b	98±5

Data are mean±s.e.m. values (n=5-6) expressed as % of control.^{a}p<0.05; ^{b}p<0.01 *vs* saline (one-way ANOVA followed by Dunnett's *t*-test).

We are grateful to Astra Zenaca and Eli Lilly for the generous gift of drugs.

Artigas, F., et al., (2001) TIPS, 22, 224-228.
Castro, M.E. et al., (1999) *Br. J. Pharmacol.* 128,165P
Pei Q., et al., (2000) *Neuropharmacology* 39, 463-470.
Johansson L. et al., (1997)*J.Pharmacol.Exp.Ther.*283,216-225.
Rasmussen, K. et al., (2000) *J. Pharmacol. Exp Ther.* 294,688.

380P INHIBITION OF [^{3}H]NPA BINDING BY GPP(NH)P AND SURAMIN AT DOPAMINE D_2 RECEPTORS.

D.J. Roberts, H.L. Lin & P.G. Strange, School of Animal & Microbial Sciences, University of Reading, Whiteknights, Reading, UK.

The aim of this study was to examine the effects of using two different G-protein modulators on the binding of the high affinity agonist, [^{3}H]NPA to D_2 dopamine receptors expressed in CHO cell membranes.

Binding of an agonist to receptor leads to formation of the ternary complex (ARG). This can be detected by the presence of high affinity agonist binding. In this study, we have used two modulators of ternary complex formation to examine their effects on the binding of the D_2 agonist [^{3}H]NPA: 1) Gpp(NH)p, a non-hydrolysable GTP analogue, that has been shown to destabilise ternary complex formation. 2) Suramin, reported to act as an inhibitor of G-protein function, possibly by inhibiting R/G interactions (Waldhoer *et al.*, 1998). The effect of varying [^{3}H]NPA concentration on these inhibitors was also examined.

For inhibition of [^{3}H]NPA binding, D_2-CHO membranes (40μg) were incubated in HEPES buffer (mM: HEPES 20, NaCl 100, $MgCl_2$ 10, EDTA 1, EGTA 1; pH 7.4) together with increasing concentrations of G-protein modulator and at three concentrations of [^{3}H]NPA (0.1, 0.2 and 0.4nM) in a final volume of 1ml at 25°C for 3 hours. 3μM (+)-butaclamol was used to determine non-specific binding. The membranes were then filtered and bound radioactivity determined using liquid scintillation counting.

Inhibition of [^{3}H]NPA binding by Gpp(NH)p was characterised by shallow inhibition curves (Hill slope ~0.65). These curves could be resolved into two sites of ~7nM and 1μM with the greater proportion of [^{3}H]NPA binding inhibited with high affinity (~77%; Table 1). There was no significant effect of [^{3}H]NPA concentration on the IC_{50} of Gpp(NH)p. Suramin, in contrast, produced steep inhibition curves (Hill Slope ~2) of IC_{50} ~120nM (Table 1). Increasing [^{3}H]NPA concentrations produced a small, but significant increase in IC_{50}.

Table 1, Inhibition of [^{3}H]NPA binding by either Gpp(NH)p or suramin at three concentrations of [^{3}H]NPA.

Gpp(NH)p

[[^{3}H]NPA]nM	pIC_{50} High	pIC_{50} Low	$\%R_{high}$	Hill Coefficient
0.1	8.14 ± 0.05	6.00 ± 0.23	82 ± 3	0.70 ± 0.07
0.2	8.18 ± 0.08	6.30 ± 0.08	75 ± 2	0.64 ± 0.04
0.4	8.06 ± 0.13	5.96 ± 0.24	73 ± 3	0.57 ± 0.03

Suramin

[[^{3}H]NPA]nM	pIC_{50}	Hill Coefficient
0.1	6.97 ± 0.02	1.9 ± 0.2
0.2	6.94 ± 0.02	2.0 ± 0.1
0.4	6.88 ± 0.01*	1.9 ± 0.1

Data are expressed as mean ± s.e.m. (*) represents significant difference to pIC_{50} of suramin at 0.1nM [^{3}H]NPA ($p<0.05$; ANOVA).

These results show two very different effects of inhibiting high affinity [^{3}H]NPA binding to the D_2 dopamine receptor by modulation of the G-protein. Also little or no shift in IC_{50} with [^{3}H]NPA concentration suggests that Gpp(NH)p and suramin act non-competitively with [^{3}H]NPA. The two apparent affinity states displayed by the Gpp(NH)p inhibition curves suggest a complex process, possibly representing: 1) the removal of G-protein from the receptor, 2) an allosteric interaction between Gpp(NH)p bound G-protein and receptor. The steep inhibition curves of suramin suggest a co-operative effect.

These results suggest that: 1) guanine nucleotides may produce complex interactions between G-protein and receptor, 2) Suramin inhibition of receptor/G-protein interaction is co-operative.

Waldhoer, M, Bofill-Cardona, E., Milligan, G., Freissmuth, M. & Nanoff, C. (1998). *Mol. Pharmacol.*, **53**, 808-818.

Financial support from the Wellcome Trust is gratefully acknowledged.

381P DIFFERENTIAL CENTRIFUGATION REVEALS REDISTRIBUTION OF A_{2A} ADENOSINE RECEPTOR BINDING IN PORCINE STRIATAL SLICES AFTER EXPOSURE TO ADENOSINE

S.P.H. Alexander, J. Aynge & N. Pocock, School of Biomedical Sciences, University of Nottingham Medical School, Nottingham NG7 2UH.

Although A_{2A} adenosine receptors have been shown to desensitize and internalize in heterologous expression systems (Palmer *et al.*, 1994) and cells in culture (Mundell & Kelly, 1998), the potential for such effects to take place in native tissues has not been explored. Here, we have used differential centrifugation to examine the distribution of [^{3}H]-ZM241385 (an A_{2A} antagonist radioligand, Alexander & Millns, 2001) binding in slices of pig brain caudate or putamen after exposure to the endogenous agonist adenosine.

Cross-chopped slices (350 x 350 μm) of pig caudate or putamen were incubated for 60 min in several changes of Krebs' medium, before exposure to adenosine (100 μM) or theophylline (100 μM) for 120 min in a 37°C shaking water bath. Theophylline, as an adenosine receptor antagonist, was chosen as a comparison rather than the absence of exogenous adenosine due to the potential confounding effects of endogenous adenosine production. After rapid washing with Krebs' medium, tissue was homogenised in ice-cold 10 % (w/v) sucrose in 50 mM Tris, 1 mM EDTA, pH 7.4 (TE) buffer and centrifuged at 4°C (12 000 g for 20 min). The supernatant layer was collected and diluted 1:1 with TE buffer, before centrifugation at 4°C (35 000 g for 20 min). The pellets from the first ("heavy fraction") and second ("light fraction") centrifugation steps were homogenised in TE buffer and examined for [^{3}H]-ZM241385 binding activity as previously described (Alexander & Millns, 2001). Data reported are means ± s.e.m. of results from at least six separate experiments and were compared for statistical significance using a paired t-test.

Binding of 2 nM [^{3}H]-ZM241385 in the "heavy fraction" after theophylline exposure was 641 ± 176 and 530 ± 160 $fmol.mg^{-1}$ protein in the caudate and putamen, respectively. After exposure to adenosine, these values were not significantly altered (610 ± 178 and 475 ± 134 $fmol.mg^{-1}$ protein, respectively). In the "light fraction", however, adenosine caused a significant ($P<0.05$) increase in [^{3}H]-ZM241385 binding in both caudate and putamen slices (321 ± 42 and 301 ± 61, respectively) compared to exposure to theophylline (254 ± 43 and 218 ± 49 $fmol.mg^{-1}$ protein, respectively).

In a parallel series of experiments investigating the use of the agonist radioligand [^{3}H]-CGS21680 (20 nM), binding was less dense (20-40 % of [^{3}H]-ZM241385 binding in the same sample), more variable and not significantly affected by exposure to adenosine or theophylline. For example, in putamen slices, [^{3}H]-CGS21680 binding was 111 ± 20 and 102 ± 29 in the "heavy fraction" and 81 ± 50 and 49 ± 42 $fmol.mg^{-1}$ protein in the "light fraction", after exposure to adenosine or theophylline, respectively.

Taken together, these results indicate a re-distribution of A_{2A} adenosine receptors after exposure to the endogenous agonist, adenosine, compared to the antagonist theophylline. These results are consistent with an internalization of receptors from the cell membrane ("heavy fraction") to an intracellular location ("light fraction").

Alexander, S.P.H. and Millns P.J. (2001) *Eur. J. Pharmacol.*, 411, 205-210

Mundell, S.J. and Kelly, E. (1998) *Br. J. Pharmacol.* 125, 1594-1600

Palmer, T.M., Gettys, T.W., Jacobson, K.A. and Stiles, G. (1994) *Mol. Pharmacol.* 45, 1082-1094

382P COMPARISON OF [^{3}H]DPCPX BINDING TO RAT AND HUMAN CORTICAL MEMBRANES

K. Dhillon[1], A. Lawrence, A. Misra, M.J. Croucher[1], A.M. Palmer, M.Esiri[2], A.R. Knight & K. Quirk. Vernalis Research Ltd, Oakdene Court, 613 Reading Road, Winnersh, Wokingham, RG41 5UA. Imperial College School of Medicine, Charing Cross Hospital, London, W6 8RF[1]. University of Oxford, Dept of Clinical Neurology, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE[2].

Adenosine is a major neuromodulator in the central and peripheral nervous systems. Its actions are mediated by G-protein coupled receptors which are currently divided into 4 subtypes, namely A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 1999). The A_1 receptor is highly expressed in the central nervous system with highest expression being seen in the cortex, cerebellum, hippocampus and thalamus (Stehle et al., 1992). Here we compare the binding of [^{3}H]DPCPX to adenosine A1 receptors in membranes derived from rat and human cerebral cortex.

Binding assays were carried out by the method of Jarvis et al. (1989). Rat and human cortical membranes were prepared as described by Ji and Jacobson (1993). Saturation analysis with [^{3}H]DPCPX revealed one binding site in the rat cortical membranes with an affinity of 1.22 ± 0.11 nM and a Bmax of 1564.25 ± 223.80 fmol/mg protein. In the human cortical membranes [^{3}H]DPCPX saturation analysis also revealed a single saturable binding site with an affinity of 3.80 ± 0.59 nM and a Bmax of 2580.25 ± 482.29 fmol/mg protein.

For displacement studies receptors were labelled with 1nM [^{3}H]DPCPX in rat and human cortical membranes. Table 1 shows the results of displacement of [^{3}H]DPCPX in the rat and human cortical membranes by ZM-241385, KW 6002, SCH 58261, NECA, DPCPX, CHA, CGS 15943 and R-PIA. The rank order of potency for these compounds was DPCPX > CGS 15943 = R-PIA = CHA > NECA > SCH 58261 > KW 6002 = ZM 241385 in rat cortex and DPCPX = CGS 15943 > R-PIA > CHA = NECA > SCH 58261 > ZM 241385 > KW 6002 in human cortex. These data are consistent with an adenosine A_1 profile as seen previously by Klotz *et al.* (1991) in rat brain and Ongini *et al.* (1999) in human recombinant receptors.

[^{3}H]DPCPX bound to a single population of receptors in both human and rat cerebral cortex. The Kd for [^{3}H]DPCPX binding was similar in both species. The compounds examined showed a similar rank order of potency in both species. It is concluded that [^{3}H]DPCPX labels pharmacologically similar receptors in both rat and human cortex. These results suggest that there are little or no differences between the pharmacology of adenosine A1 receptors in rat tissue and human tissue, and that the activity of adenosine A1 receptor antagonists in humans may be predicted from data obtained in rat cortex.

Table1. K_i values (nM) for compounds as displacers of [^{3}H]DPCPX in rat and human cortex (mean ± SEM, n=3).

Compound	Rat Cortex	Human Cortex
ZM 241385	2552 ± 811.1	1105 ± 120.9
KW 6002	1708 ± 530.8	1890 ± 238.4
SCH 58261	620.5 ± 112.5	443.1 ± 126.2
NECA	303.5 ± 38.3	181.8 ± 39.3
DPCPX	5.3 ± 1.3	5.5 ± 1.1
CHA	79.6 ± 5.6	214.4 ± 98.8
CGS 15943	57.3 ± 2.2	28.7 ± 7.3
R-PIA	64.1 ± 16.9	93.9 ± 23.5

Fredholm BB et al. (1999) Eur.J.Pharmacol. 380, 197-202
Jarvis MF et al. (1989) J.Pharmacol.Exp.Ther. 251, 888-893
Ji XD & Jacobson KA (1993) Arch.Biochem.Biophys. 30, 611-617
Klotz KN et al. (1991) Naunyn.Schmeid.Arch.Pharm. 343, 196-201
Ongini E et al (1999) Naunyn.Schmeid.Arch.Pharm. 359, 7-10
Stehle JH et al. (1992) Mol.Endocrinol. 6, 384-393

383P FLIPR®-BASED ASSAY FOR SCREENING INHIBITORS OF THE MULTIDRUG RESISTANCE GENE MDR1 *IN VITRO*

D.E. Owen[1], C.A. Brough[2], J. Chen[3] & D. Smart[1], Neurology CEDD[1] & DMPK[2], GlaxoSmithKline, New Frontiers Science Park, Harlow, Essex. [3]MDC, Sunnyvale CA, USA.

The multidrug resistance (MDR1) gene product, P-glycoprotein, is responsible for the ATP-dependent extrusion of a variety of compounds, including chemotherapautic agents, from cells (Abraham *et al.,* 1993). The role of the ATP-binding cassette (ABC) transporter-facilitated ATP transport and signalling in epithelial cell function is an area of research which could impact on disease pathology (Schwiebert, 1999) as well as the pharmacokinetics of new therapeutic agents. However, a high throughput *in vitro* assay for this target has been elusive. We report here a FLIPR-based assay for human MDR1 based on a new reagent kit from Molecular Devices Corporation (MDC).

Cells with or without recombinant human MDR1, were maintained in Dulbecco's Modified Eagle's Media containing 10 % foetal calf serum (FCS) and 2 mM Glutamax I. Cells were seeded (30,000-50,000 cells per well) in 80µl media, with or without 10% FCS, into Costar 96 well black walled plates, and cultured overnight. The cells were then incubated at room temperature or 37°C for 20 min with Cyclosporin A (300 pM-100 µM), verapamil (300 pM-100 µM), probenecid (150 µM-30 mM) or buffer control. The cells were then placed in a FLIPR and the fluorescence monitored (λ_{EX}=488 nm, λ_{EM}=540 nm; Wood *et al.,* 2000) before and after the addition of the MDC reagent. Data reported are mean±s.e.mean where n=3-9.

Following the addition of the MDC reagent there was an initial transient drop (an addition artefact), followed by a subsequent increase, in fluorescence. This increase in fluorescence was linear for at least 15 min and occurred in both wild type and MDR1-expressing cells, although the rate of increase was greater in the untransfected cells (10.1±0.3 *vs* 0.4±0.2 FIU s^{-1}), confirming the MDC reagent was a MDR1 substrate. The responses were most robust in cells seeded at 50,000 cells per well in the presence of 10% FCS, and therefore these conditions were used for all compound studies. In MDR1-expressing cells the classic MDR1 inhibitors, verapamil and cyclosporin A increased the fluorescence in a concentration-dependent manner (pEC50 values of 5.35±0.03 & 5.68±0.01 respectively), whilst probenecid, which inhibits other common extrusion pumps but not MDR1 (Wood *et al.,* 2000) and buffer control (1% DMSO) were without effect. Furthermore, the effects of verapamil and cyclosporin A were temperature-dependent (pEC50 values at room temperature 4.30±0.14 & 5.46±0.01 and at 37°C 4.72±0.17 & 5.56±0.03 respectively) and none of the compounds had an effect in untranfected cells. This clearly indicates that the assay was specific to MDR1. Comparison of the potencies of cyclosporin A over several assays yielded intra- and inter-assay coefficients of 5.73% and 5.82% respectively, demonstrating the robustness of the assay

In conclusion, these data indicate that the MDC reagent allows the generation of a robust FLIPR-based assay for screening inhibitors of MDR1 *in vitro*.

Abraham, E.H. *et al.*, (1993) Proc. Natl. Acad.Sci. USA **90**, 312-316.
Schwiebert, E.M., (1999) Am. J. Physiology. **276**, C1-8.
Wood, M.D. *et al.*, (2000) "Recent research developments in neurochemistry volume 3" pp135-142.

384P THE EXPRESSION OF DOG HEPATIC CYP2B11, CYP3A12, CYP2C21, CYP2C41 mRNA, PROTEIN AND ENZYMATIC ANALYSIS OF CYP3A4, CYP2C19 AND CYP2C9 SUBSTRATES IN DOG LIVER MICROSOMES

A.R. Bell[1], L. Walker[1], M.J. Graham[1], E. Whittaker[2], C.L. Moorcraft[2], H.K. Crewe[2] and M.S. Lennard[2], [1]SAC AstraZeneca, Loughborough, and [2]Molecular Pharmacology and Pharmacogenetics, University of Sheffield.

Interpretation of novel drug exposure and toxicology data from the dog is tempered by our limited molecular and functional knowledge of dog cytochromes P450 (CYPs). The aim of this work was to characterise the expression and function of dog hepatic CYPs.

Total mRNA and/or microsomes were prepared from the livers of 6 male and 6 female Alderley Park beagle dogs (age 7.5 months, wt 7-14 kg), and from 1 macroscopically normal human liver, the latter with the approval of the South Sheffield Research Ethics Committee. CYP mRNA levels were quantified by TaqMan 'Real time' PCR. CYP protein levels were determined by ELISA using Gentest® anti-dog P450 antibodies. The following activities, which are selective markers for individual human CYP isoforms were determined in the same livers: dextromethorphan N-demethylation (CYP3A), warfarin 7'-hydroxylation (CYP2C9), mephenytoin 4'hydroxylation (CYP2C19), and omeprazole 5'-hydroxylation (CYP2C19).

No significant sex differences (unpaired t-test) in the mRNA or protein expression of CYP2B11 (mean±sd mRNA male vs female = 1.65±0.75 vs 2.2±1.3 arbitrary units), CYP3A14 (1.00±0.50 vs 1.00±0.2), CYP2C21 (2.00±0.75 vs 2.75±1.75), or CYP2C41 (0.90±0.45 vs 0.6±0.05) were found.

CYP2C41 mRNA exhibited a polymorphic pattern in the livers studied, with only 5 out of 12 dogs (3 males) expressing this isoform. All livers showed some expression of the 3 other isoforms. The mean relative ratio of CYPs 2B11:3A12:2C21/41 for mRNA expression was 1.6:1:2.8 (male) and 2.3:1:3.5 (female), and for protein expression was 3.0:1:2.3 (male) and 2.8:1:1.7 (female).

The kinetics of dextromethorphan N-demethylation were best described by a two site Michaelis-Menten function. The mean K_m values for the high and low affinity sites were 30 μM and 200 μM, respectively. The former was approximately one order of magnitude higher than that found in human liver. Both male (9.3±1.6 fmol/min/mg) and female (12.3±2.1) dog microsomes showed a very low capacity to 7'-hydroxlate warfarin compared to human liver microsomes (787 fmol/min/mg). The 4'-hydroxylation of mephenytoin in dog liver microsomes (R- >> S-) showed opposite stereoselectivity to that seen in human liver (T. Yosumori, *J. Pharmacol. Exp. Ther.* 283 (1993) 434). The 5'-hydroxylation of omeprazole was best described by a Hill function in three and a single Michaelis-Menten function in a fourth dog (Mean $K_m/K_{50\%}$ = 46 μM, V_{max} = 33 pmol/min/mg). This behaviour differs from that of human data, where 5'-hydroxyomeprazole formation is consistent with two site Michaelis-Menten kinetics. The only significant correlation within the 12 livers studied was that between R-mephenytoin 4'-hydroxylase activity and CYP2B11 mRNA expression (r_s = 0.78, p<0006).

In conclusion, we have identified a distinct pattern of expression of CYP2C41 genes in the Alderley Park beagle and also determined that this strain of dog metabolises human CYP 2C substrates in a manner different to humans. We were not able to identify a functional CYP2C polymorphism using the substrates tested.

385P THE EPHARNET HYPERTENSION TEACHING RESOURCE

Ian Hughes, Kath Huckbody & [1]Jeffrey Atkinson. School of Biomedical Sciences, Leeds University, UK & [1]Dept.of Pharmacology, Universite Henri Poincare, Nancy, France.

EpharNet (The European Pharmacology Network) was funded from 1998-2001 by the European Commission as a Socrates Thematic Network involving 130 institutions in 28 countries throughout Europe. The objectives of EpharNet are to:

facilitate and maintain communication between European pharmacology teachers;
develop a European dimension to pharmacology teaching;
promote the sharing of teaching resources;
improve the efficiency and quality of teaching;
jointly develop new teaching materials and methods.

There are 2 main outputs from the EpharNet project. First, a web site hosted on www.bps.ac.uk from which are available: examples of pharmacological curricula from across Europe; problem-based learning materials; PowerPoint presentations/ slides; a list of pharmacologically interesting web-sites; a database of the scientific and teaching interests of pharmacologists from across Europe; and a compendium of innovations/good ideas in pharmacology teaching. All this material can be freely used, copied or modified for non-commercial purposes by European pharmacologists.

Second, The EpharNet HYPERTENSION Teaching Resource which can be used as a computer-based learning program and supplied to students for self-directed study but WAS DESIGNED as a Teaching Resource i.e. material which would be integrated into courses by teachers who would provide appropriate guidance and direction on the use of the material by students. The program provides core knowledge at an undergraduate student level though in a considerable number of areas more advanced or detailed material is available (accessed through blue-coloured buttons).

There are the following sections in the teaching resource:
1. Introduction - HYPERTENSION: What is it? How frequent is it? Is it dangerous? What is the danger? Does anti-hypertensive treatment work? Are hypertensives treated satisfactorily?
2. Types of hypertension - primary, secondary and special types.
3. Organs involved - involvement of brain, lungs, heart, eye, arteries and kidney.
4. Decision to treat - factors affecting this decision, algorithm for decision to treat.
5. Treatment - non-pharmacological and pharmacological. Details of drugs used (their structure, indications, mode of action, interactions, dosage, contra-indications, unwanted effects).
6. Clinical cases - two short examples are used.

Each section contains interactive material at different levels of complexity and contains a summary and learning points. A set of multiple choice questions is provided dealing with pharmacological or therapeutic material at easy or average levels of difficulty.

European pharmacologists can obtain a free copy of the CD upon request from their National EpharNet Co-ordinator as detailed on the EpharNet web site. Non-European pharmacologists may, for a small charge, obtain a copy from the British Pharmacological Society.