41P THE EFFECTS OF HYPOXIA AND ANOXIA ON THE ACTIVITY OF NEURONAL LARGE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM (BK_{Ca}) CHANNELS

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Neuronal BK_{Ca} channels may act as oxygen sensors either through a direct membrane delimited mechanism (Lewis *et al.*, 2002) or indirectly through cellular signalling mechanisms (Liu *et al.*, 1999). This conflicting evidence may arise as a result of alternative exon splicing of *Slo* mRNA that provides the variation in BK_{Ca} activity required for different cellular environments (Shipston 2001). The exons determine the intrinsic voltage and calcium sensitivity of BK_{Ca} channels and form sites modulated by phosphorylation (Clark *et al.*, 1999). The aim of this research was to determine whether hypoxia and anoxia differentially regulate the activity of alternative α-subunit splice variants (mbr5 and STREX) of the murine *Slo* gene using single channel patch clamp recording. Hypoxic solutions were obtained by displacing O_2 by bubbling solutions with N_2 (PO_2 = 31 ± 5 mmHg, n = 8). Anoxic solutions were obtained using 1 – 10 mM sodium sulphite (PO_2 in 1 mM Na_2SO_3 = 4 ± 1 mmHg, n = 8).

In excised inside-out patch recordings, with a physiological potassium gradient, from mbr5 expressing HEK-293 cells, hypoxia did not cause a significant change in single channel amplitude or channel open probability (Po) in the presence of either 1 or 10 μM free calcium. For example in 10 μM free calcium at +20 mV the Po was 0.41 ± 0.02 (n = 6) in control recordings and 0.39 ± 0.02 (n = 6) in hypoxic solutions. In a subsequent series of experiments the effect of anoxia was investigated. Under these conditions mbr5 channel activity was once again unchanged. For example, in 10 μM free calcium at +20 mV, the Po in 1mM Na_2SO_3 solutions was 0.33 ± 0.02 (n = 4) in control recordings and 0.36 ± 0.09 (n = 4) in anoxic solutions. In experiments using the STREX splice variant changes in channel activity in response to anoxia but not hypoxia were observed. For example in 1 μM free calcium at +20 mV, the open probability was 0.16 ± 0.02 (n = 5) in control recordings and 0.15 ± 0.02 (n = 5) in hypoxic solutions. In contrast in anoxic solutions Po decreased significantly from control values of 0.21 ± 0.01 (n = 3) to 0.10 ± 0.04 (n=3) in 1mM Na_2SO_3 (Student's paired *t*-test, $P<0.05$). The STREX expressing corticotroph cell line AtT20 showed mixed responses to hypoxic and anoxic conditions. In response to hypoxia, in 1 μM free calcium at +20 mV, Po was reduced from control values of 0.18 ± 0.03 (n = 6) to 0.10 ± 0.02 (n = 6) (Student's paired t-test, $P<0.05$). Similar reductions in Po have been observed with 1mM Na_2SO_3 in 1 μM free calcium (n = 2) but not in 10 μM free calcium (n = 3).

These results indicate that the mbr5 and STREX splice variants exhibit differential sensitivity to changes in oxygen tension.

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Clark, A.G. *et al.* (1999) *J. Physiol.*, **516.1**, 45-53
Lewis, A. *et al.* (2002) *J.Physiol.*, **540**: 771-780.
Liu, H. *et al.* (1999) *J. Clin. Invest.*, **104**, 577-588.
Shipston, M.J. (2001) *Trends in Cell Biology*, **11**:9:353-358

42P MODULATION OF KV3.1B CHANNELS EXPRESSED IN HEK293 CELLS BY REDOX AGENTS AND HYPOXIA

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Kv3.1 potassium channels are expressed mainly in the central nervous system, especially in rapidly firing neurones. They are also expressed in PC12 cells (Conforti & Millhorn, 1997) and pulmonary artery smooth muscle cells (Osipenko *et al.*, 2000), both of which display an oxygen-sensitive K^+ current. Hypoxia was recently shown to inhibit Kv3.1b channel currents, both in whole-cell recording and in excised membrane patches (Osipenko *et al.*, 2000). This study investigated the potential mechanisms underlying the effect of hypoxia by studying the effects of redox agents on Kv3.1b channel currents and their response to hypoxia.

Mouse Kv3.1b (mKv3.1b) DNA was stably expressed in human embryonic kidney (HEK293) cells. Kv3.1b channel currents were recorded using the conventional whole-cell configuration of the patch-clamp technique. Cells were perfused with solution composed of (in mM): 124 NaCl, 5 KCl, 1 $MgCl_2$, 21 HEPES, 1.8 $CaCl_2$, 10 D-glucose (pH 7.3). Hypoxic solutions were prepared by bubbling with 100% nitrogen, which gave PO_2 = 9.0 ± 0.8% (n = 7) compared with 20.5 ± 0.5% (n = 7) in the control. Results are given as mean ± S.E.M. Statistical comparisons between groups of data employed a two-tailed Student's t-test, $P<0.05$ being considered significant.

When stably transfected with mKv3.1b DNA, HEK293 cells displayed a voltage-activated potassium current with an activation threshold lying between -20 and -30 mV (n = 31) and amplitude of 157 ± 12 pA/pF at 20 mV. When exposed to hypoxia for 10 min, the amplitude was reduced by 15 ± 3% (n = 7, $P<0.05$). Inhibition was apparent at potentials of 10 mV and above and occurred with no change in activation threshold.

The reducing agents, dithiothreitol (DTT, 1 mM) and bis(2-mercaptoethylsulfone) (BMS, 0.1 mM) both inhibited the Kv3.1b channel current, by 51 ± 6% (n = 5, $P<0.05$) and 31 ± 2% (n = 5, $P<0.05$) respectively, at 20 mV. The natural redox agent, glutathione, also modulated the current. When the pipette solution contained either the reduced (GSH, 5 mM) or oxidised (GSSG, 5 mM) form of glutathione, the current amplitude measured at 20 mV after 10 min of cell dialysis was, respectively, 71 ± 4% (n = 10, $P<0.05$) and 146 ± 10% (n = 3, $P<0.05$) of the current measured immediately after forming the whole-cell configuration. Thus, reducing agents mimicked the effect of hypoxia while the oxidising agent had the opposite effect. To investigate the potential role of redox modulation in the response to hypoxia, we retested the effect of hypoxia in the presence of redox agents. Hypoxia continued to inhibit the current in the presence of 1 mM BMS (16 ± 3% at 20 mV, n = 4) or 5 mM internal GSH (20 ± 3% at 20 mV, n = 8). In each case the inhibition was not significantly different from control.

In conclusion, mKv3.1b channels are subject to redox regulation. Reducing agents mimic the inhibitory effect of hypoxia, but do not prevent it, implying that oxygen-sensing is independent of redox state.

Conforti, L. & Millhorn, D.E. (1997), *J. Physiol*, 502, 293-305.
Osipenko, O.N., Tate, R.J. & Gurney, A.M. (2000), *Cir. Res.*, 86, 534-540.

43P THE EFFECT OF Cl^- CHANNEL BLOCKERS ON Ca^{2+} SIGNALLING IN RAT PULMONARY ARTERY SMOOTH MUSCLE CELLS

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The sarcoplasmic reticulum (SR) plays an important role in regulating the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in smooth muscle. SR Ca^{2+} uptake and release are both accompanied by 'counter-ion' movements across the SR membrane, which prevent or reduce the generation of SR membrane potentials and balance for electroneutrality in the SR lumen. There is increasing evidence that Cl^- movement plays an important role in charge compensation across the SR in smooth muscle (Pollock *et al.*, 1998; Kargacin *et al.*, 2001). In the present study, we have examined the effect of the chloride channel blockers niflumic acid and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), on Ca^{2+} signalling in rat pulmonary artery smooth muscle cells (PASMC's).

Male Sprague-Dawley rats (200 – 300 g) were killed by cervical dislocation and the heart and lungs were removed *en bloc*. PASMC's were then isolated from intrapulmonary arteries (> 400 μm) as previously described (Drummond & Tuft, 1999). To measure $[Ca^{2+}]_i$, PASMC's were incubated with 5 μM fura-2 AM for 40 minutes at room temperature. Caffeine (20 mM) was applied to a single cell via a pressure ejection pipette. All experiments were conducted at room temperature. Following caffeine application, time constants (τ) were calculated by fitting single exponentials to the declining phase of $[Ca^{2+}]_i$ transients and the decay constants ($1 / \tau$) determined. Results are presented as the mean ± s.e.mean and where n = number of cells studied. Statistical tests of difference were made using Student's t-test.

Under control conditions, application of caffeine increased $[Ca^{2+}]_i$ from 87 ± 4.6 nM to 756 ± 12.4 nM. The time to peak was 0.5 ± 0.1 s, and the Ca^{2+} decay constant was 0.07 ± 0.01 s. Niflumic acid (50 μM) increased resting $[Ca^{2+}]_i$ to 253 ± 25.4 nM (n = 6, P < 0.001), and reduced the caffeine-induced $[Ca^{2+}]_i$ transient by 34 ± 8%. The time to peak was increased to 1.1 ± 0.7 s (n = 6, P < 0.05), and the decay constant for Ca^{2+} removal was decreased by 22 ± 1% (n = 6, P <0.01). NPPB (10 μM) also increased resting $[Ca^{2+}]_i$ to 167 ± 8 nM (n = 5, P < 0.05), and reduced the caffeine-induced $[Ca^{2+}]_i$ transient by 38 ± 11%. The time to peak was increased to 2 ± 0.4 s (n = 5, P < 0.05) and the decay constant for Ca^{2+} removal was decreased by 51 ± 2% (n = 5, P < 0.01).

In summary, both niflumic acid and NPPB were found to increase resting $[Ca^{2+}]_i$ in PASMC's. In addition the time to peak for the caffeine-induced $[Ca^{2+}]_i$ transient was increased, and Ca^{2+} removal following caffeine application was significantly slower. These results suggest that Cl^- channels are important for normal SR function in PASMC's.

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Drummond, R.M. and Tuft, R.A. (1999). *J. Physiol.* **516**: 139-47

Kargacin, G.J., Ali, Z., Zhang, S.J., *et al.* (2001). *Am. J. Physiol.* **280**: H1624-1634.

Pollock, N.S., Kargacin, M.E., and Kargacin, G.J. (1998). *Biophys. J.* **75**: 1759-1766.

44P MOLECULAR DETERMINANTS OF HERG BLOCKADE BY PROPAFENONE DIFFER FROM THOSE OF METHANESULPHONANILIDES

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HERG is a pharmacologically promiscuous cardiac K^+ channel that is widely believed to mediate acquired long QT syndrome, which is associated with a range of clinically used drugs. A model for high potency blockade of the HERG potassium channel has been developed, in which amino acids in the putative vestibule of the channel, including T623, S624, V625, G648, Y652, F656, V659 as well as inactivation are important for channel block by methanesulphonanilides (Mitcheson *et al.*,2000; Lees-Miller *et al.*, 2000). We examined whether these residues are critical for the blockade of HERG by the class IC antiarrhythmic propafenone using alanine substituted HERG mutants heterologously expressed in Xenopus oocytes and electrophysiologically tested using two electrode voltage clamp at ambient temperature. Capped RNA from linear template DNA was made using the mMessage mMachine kit, and between 5 and 30 ng of RNA was injected into each oocyte and allowed to express between 1 and 4 days before making recordings. The electrode solution was 3 M KCl, the extracellular solution was a chloride-reduced (MES substituted) Na^+ based recording solution with 2 mM K^+, except in the case of G648A and T623A, in which a 96 mM K^+ (substituted for Na^+) solution was used. The voltage command protocols used were: all cells held at –90 mV, then a 2 second step to 0 mV, followed by observation of tails for 2 seconds at –70 mV, except in the cases of F656A and V659A (tails observed at –140 mV) and V625A (tails observed at –90 mV).

A steady baseline for HERG tails was attained by repeated stimulation using the voltage command protocol (minimum of 50 sweeps, 8 minutes); thereafter drug-containing extracellular solution was added and allowed to equilibrate while oocytes were at the holding potential for two minutes, and steady state blockade was reached after repeated stimuli. In this system the IC_{50}'s (cumulative) for propafenone's blockade of HERG's peak tail current were 2.16 μM (95% Confidence Interval (CI) 1.98 to 2.34 μM) for 2 mM K^+ and 4.36 μM (95% CI 3.47 to 5.47μM) for 96 K^+. 50 μM propafenone (dissolved directly in extracellular recording solution) – which blocked HERG tail currents 96.4 ± 0.1% (mean ± SEM, 2 mM K^+, tails at -70 mV, n = 5), 79.6 ± 4.9% (2 mM K^+, tails at –140 mV, n = 5) and 88.3% ± 2.5% (96 mM K^+, tails at –70 mV, n = 5) – was chosen as a standard concentration to test the mutants because it caused profound channel blockade. At this concentration propafenone blocked F656A 5.0 ± 9.7% (n = 5), implying that the F656 residue is critical for propafenone's functional blockade of HERG. In addition, S631A (a mutant with attenuated inactivation) was blocked 67.0 ± 7.3% (n = 3). All other mutants tested blocked HERG tails between 68.7% – 92.9% (n = 3-5 for all), not substantially different from wild type. We conclude that, unlike HERG blockade by MK-499, T623, S624, V625, G648, Y652, V659 and functional inactivation are not critical determinants of HERG blockade by propafenone, but that F656 is such a determinant.

Lees-Miller JP, Duan Y, Teng GQ, *et al.*, (2000). *Mol. Pharmacol.* **57**:367-374.
Mitcheson JS, Chen J, Lin M, *et al.*, (2000). *Proc. Natl. Acad. Sci. U. S. A.* **97**:12329-12333.

45P FUNCTIONAL CHARACTERIZATION OF SVT 40776, A NOVEL AND POTENT M_3 RECEPTOR ANTAGONIST, ON MICE DETRUSOR AND ATRIA ISOLATED PREPARATIONS USING *IN VITRO* AND *EX VIVO* PROTOCOLS

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Overactive bladder (OAB) is a highly prevalent condition characterised by an increased urinary frequency, urgency and urge incontinence. Muscarinic receptor antagonists are the most widely used therapeutic agents for OAB, but side effects like xerostomy or tachycardia have been limiting their clinical use. SVT 40776 (SVT) is a novel substituted quinuclidine derivative with high M_3 receptor affinity (Farrerons C et al., 2002).

The present study was designed to characterize the functional *in vitro* and *ex vivo* urinary *vs.* cardiac potency and selectivity of SVT compared with a range of muscarinic antagonist agents in clinical use or development for OAB.

Tolterodine (TOL), darifenacin (DAR), YM-905 (YM) and SVT were studied using male CD-1 mice (25-30g). *In vitro* protocol: Animals were sacrificed by CO_2, tissues were dissected and placed in 25 ml organ baths containing Krebs solution maintained at 37ºC and aerated with 95%O_2/5%CO_2. The Krebs solution routinely contained indomethacin (30 μM). Pure isometric transducers were used for all experiments. Contractile force in isolated longitudinal strips (2x1 mm) of bladder detrusor muscle (DT) and beating frequency on isolated spontaneously beating atria (AT) were measured. The pA_2 was calculated for each antagonist and tissue using carbachol (CCh) as agonist (cumulative curve) with a 60 min antagonist incubation period. *Ex vivo* protocol: Groups of animals (n=4-6/dose) received a single oral dose (0.3 to 50 mg/kg) of vehicle, TOL, DAR, YM and SVT (3-5 doses/compound). Mice were sacrificed 3h later and DT and AT activity studied as described before. A pA_2-equivalent dose (pA_2-ED) was calculated using the oral doses instead of bath concentrations for each agent. Briefly, curves obtained from vehicle-treated animals were assigned as 'control curves' and the EC_{50} of CCh determined. Shifts on the EC_{50} of CCh in tissues coming from treated animals respect to control curves were calculated. Maximal tissue responses were determined using KCl (90 mM).

Table 1. *In vitro* and *ex vivo* antagonist activities of TOL, DAR, YM and SVT on mice isolated DT and AT preparations.

Compound	pA_2 (*in vitro*)		*In vitro* AT/DT ratio	pA_2-ED (*ex vivo*, mg/kg, 3h)		*Ex vivo* AT/DT ratio
	DT	AT		DT	AT	
TOL	8.8 (8.5-9.0)	8.4 (7.6-9.1)	2.5	0.66 (0.44-1.0)	0.15 (0.07-0.3)	0.23
DAR	8.8 (8.3-9.2)	7.4 (7.1-7.7)	25.1	1.6 (0.35-6.7)	3.8 (2.1-7.5)	2.4
YM	8.4 (8.2-8.6)	7.6 (7.3-7.8)	6.3	1.4 (0.46-4.2)	2.0 (1.6-2.6)	1.4
SVT	9.7 (9.2-10.3)	7.3 (7.0-8.1)	251	0.72 (0.4-1.1)	>50	>69

SVT shows the highest *in vitro* functional selectivity amongst the tested compounds, inhibiting DT contractions at concentrations 251 fold lower than those active on AT. A very high selectivity is also obtained (>69 fold) when SVT is orally administered and studied using the *ex vivo* protocol. DAR and YM do not show any relevant bladder selectivity whereas TOL even exhibits a moderate atria selectivity when studied using the *ex vivo* protocol. Differences between TOL and DAR *in vitro* and *ex vivo* respective tissue selectivities suggest that other factors than receptor affinity (i.e. tissue distribution) are able to influence the functional selectivity of these agents. Results support the rationale of exploring the therapeutic potential of SVT in the clinical management of OAB.

Farrerons C *et al., SALVAT S.A.* (2002) *PCT patent application WO 02/00652.*

46P PROSTAGLANDIN INVOLVEMENT IN CARBACHOL-EVOKED SHORT CIRCUIT CURRENT IN RAT DISTAL COLON

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The selective muscarinic receptor agonist carbachol is believed to evoke Cl^- secretion in colonic epithelial cells by elevation of intracellular Ca^{2+} (Greger *et al.*, 1997), with Cl^- ion movement through the cAMP-regulated cystic fibrosis transmembrane conductance regulator channel (CFTR). It has been proposed that cAMP production by prostaglandins may have a role in Cl^- secretory activity in the colon (Ko *et al.*, 2002). In this study, we have investigated the involvement of the prostaglandin (PG) pathway in the carbachol-induced short circuit current in the rat distal colon.

The terminal colon (5 cm) was removed from adult male Wistar rats (250-300g) that had been killed by cervical dislocation. The smooth muscle was removed and the mucosal sheet mounted in an Ussing chamber (area 0.5 cm^2) containing Krebs solution at 37 °C, gassed with 95%O_2 5% CO_2. Short circuit current (I_{SC}) measurements were taken from baseline to peak. Values represent mean±s.e.m and Student's t-test was used to test for significance ($P < 0.05$ considered significant).

The spontaneous short circuit current (I_{SC}) was 18.4 ± 5.2 μA cm^{-2} (n = 17) with a resistance of 102.3 ± 6.8 Ω cm^{-2}. Carbachol (100 μM basolaterally) stimulated a triphasic response consisting of an increase in I_{SC} (phase 1, 15.5 ± 2.8 μA cm^{-2}) followed by a decrease towards baseline (phase 2, 9.6 ± 4.0 μA cm^{-2}) then a large increase (phase 3, 74.4 ± 4.8 μA cm^{-2}, n = 17) that decayed to a stable plateau. Indomethacin, (10 μM basolaterally and apically) an inhibitor of PG production by the cyclooxygenase pathway, caused a decrease in basal I_{SC} of 4.8 ± 1.4 μA cm^{-2} (n = 11). In the presence of indomethacin, the responses to carbachol were markedly altered: phase 1, 2.1 ± 0.9 μA cm^{-2}; phase 2, -5.7 ± 2.5 μA cm^{-2} and phase 3, 6.2 ± 3.6 μA cm^{-2} where phases 1 and 3 were smaller than control (n = 11, $P < 0.05$) and where phase 2 peaked below basal I_{SC} (n = 11). Addition of PGE_2 (50 μM basolaterally) caused a sustained increase in basal I_{SC} of 12.9 ± 1.6 μA cm^{-2} and the carbachol response in the presence of PGE_2 was triphasic although phase 3 was significantly reduced compared with control ($P < 0.05$, n = 5). In the presence of both indomethacin (10 μM basolaterally and apically) and PGE_2 (50 μM basolaterally), the responses to carbachol were triphasic and not significantly different from the responses in the presence of PGE_2 alone but where phases 1 and 3 were significantly larger than the responses in the presence of indomethacin alone.

These results suggest that the PG pathway is involved in carbachol-induced short circuit current in rat distal colon.

Greger, R., Bleich, M., Riedemann, N. *et al.*, (1997) *Comp. Biochem. Physiol.* **118(2),** 271-275
Ko, W.-H., Law, V.W., Yip, W.E. *et al.*, (2002) *Am. J. Physiol.* **282,** G508-G518

47P PHARMACOLOGICAL CHARACTERIZATION OF ENDOTHELIN RECEPTORS IN THE MOUSE ISOLATED PROXIMAL AND DISTAL COLON

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Endothelins (ET) are potent constrictors that are expressed in many tissues including the gut. They mediate their pharmacological effects through the ET_A and the ET_B receptors (Masaki *et al.*, 1994). This study investigated the role of endothelin and its receptors in mediating contraction responses in the mouse proximal and distal colon.

Segments of the proximal and distal colon (approximately 2 cm in length and taken 1 cm and 4 cm, respectively, from the ileo-caecal junction) were obtained from MFI mice of either sex (25-35g). Segments were mounted in 10 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit solution (37 °C) and equilibrated for 30 minutes, under an initial tension of 1g. Endothelin (ET-1), which exhibits a high affinity for both receptor subtypes, was administered cumulatively in concentrations ranging from 0.01 nM - 0.3 μM with a 1 minute contact time. The non-peptide ET_A antagonist BMS 182874 (0.3 μM-30 μM) and the ET_B antagonist IRL 1038 (0.3 μM-1 μM) were added 20 minutes prior to the addition of ET-1. A range of endothelin agonists (ET-2, ET-3, [$Ala^{1,3,11,13}$] endothelin and IRL 1620) were also administered cumulatively in concentrations ranging from 0.01 nM-0.3μM. Results are expressed as the mean ± standard errors of mean and statistical differences were determined using two-way students' *t* test ($P > 0.05$).

The cumulative addition of ET-1 produced a dose- dependent contraction response, where, E_{max} was 1.24 ± 0.14g and 1.36 ± 0.19g at 100 nM in the proximal and distal sections of the colon respectively (n = 8). Addition of the ET_A receptor selective antagonist BMS 182874 (Webb *et al.*, 1995) caused a rightward shift of the concentration response curve to ET-1 in both sections of the colon, without suppression of the maximum response. The ET_B receptor antagonist IRL1038 (0.3 μM-1 μM) (Masaki *et al.*, 1994), however, did not significantly effect the response to ET- 1 in the proximal colon but caused a significant decrease towards higher concentrations ranges (> 30 nM, $P < 0.05$) in the distal colon. Comparison of the cumulative-response curves to ET-1, ET-2, ET-3 showed a rank order of potency, where, ET-1≥ET-2>>ET-3 in the proximal colon and ET-1>ET-2>ET-3 in the distal colon. Moreover, addition of the selective ET_B agonists, [$Ala^{1,3,11,13}$] endothelin (Panek *et al.*, 1992) (n = 4) and IRL 1620 (Masaki *et al.*, 1994) (n = 4) did not produce any response in the proximal sections of the colon but produced a smaller contraction in the distal segments (E_{max}= 0.6 ± 0.09g & 0.62 ± 0.10 g at 10 nM).

The data indicate that endothelin can contract both the proximal and distal tissues of the mouse colon via ET_A receptors. The ability of the two ET_B receptor agonists to produce contractile responses in the distal (but not proximal sections) of the colon and the ET_B receptor antagonist to block the endothelin response at higher concentrations indicates the potential for an additional action for endothelin on ET_B receptors in the distal section.

Masaki T. *et al.*, (1994). *Pharmacol. Rev.*, **46**,137-142.
Panek R. L. *et al.*, (1992). *Biochem. Biophys. Res. Comm*, **183**, 566-571.

48P CONCENTRATION-DEPENDENT POTENTIATION OF ANP-EVOKED RELAXATIONS BY GLYCOSAMINOGLYCANS IN BOVINE BRONCHI

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We have previously demonstrated that glycosaminoglycans (GAGs) such as low molecular weight hyaluronic acid (low HA) (100μg/ml), heparin (60μg/ml) (Gribben *et al*, 2002) and chondroitin sulphate (CS, 100μg/ml) (unpublished observations), each potentiate ANP-evoked relaxations in bovine bronchi. These GAGs appear to protect ANP from degradation by the neutral endopeptidase, neprilysin (NEP). The present study examines whether the protective effect of low HA and CS on ANP is concentration-dependant.

Bovine lungs were obtained from a local abattoir. Responses were measured isometrically in rings of bronchi (3-5mm) in 5ml vertical organ baths containing oxygenated Krebs-Henseleit solution at 37 ± 0.5°C. Tissues were preconstricted with methacholine (10^{-5}M) and cumulative concentration-response curves were constructed for ANP (10^{-9}-$3x10^{-7}$M) alone, in the presence of phosphoramidon (10^{-4}M), or with CS or low HA at 50μg/ml, 100μg/ml and 200μg/ml. Statistical significance was examined by two-way ANOVA.

Phosphoramidon potentiated ANP-evoked relaxation (control relaxation at $3x10^{-7}$M ANP; 87.1 ± 6% compared with 62.7 ± 7% in the presence of phoshoramidon) ANP–mediated relaxations were significantly potentiated in the presence of CS at 50μg/ml ($P<0.001$, $n=5$), 100μg/ml ($P<0.001$, $n=5$) and 200μg/ml ($P<0.01$, $n=5$) (control relaxation at $3x10^{-7}$M ANP; 90.2 ± 3% compared with 55.5 ± 16% in the presence of 50μg/ml CS). No significant difference was observed between each concentration, nor between any concentration of GAG and phosphoramidon. Similar results were obtained with low HA at 50μg/ml ($P<0.01$, $n=6$) and 100μg/ml ($P<0.01$, $n=5$), however low HA at 200μg/ml ($n=6$) did not significantly potentiate the ANP-evoked response. (control relaxation at $3x10^{-7}$M ANP; 87.1 ± 6% compared with 71 ± 7% in the presence of 50μg/ml low HA).

The maximum effect of chondroitin sulphate was already achieved at 50μg/ml since potentiation of ANP-evoked relaxations was of similar magnitude at all three concentrations. Low HA, however, produced no significant effect at the highest concentration. This phenomenon has not been seen with these particular GAGs before but has been observed with the related GAG, heparin, which sometimes shows a bell-shaped concentration-dependant response (Kilfeather & Page, 1997). Biological activity with GAG concentrations up to and including 100μg/ml, are in keeping with other reported observations (reviewed by Kilfeather & Page, 1997). The present results support our previous findings that GAGs may be effective in blocking NEP activity in bovine airways.

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Gribben, E.E., Caldwell, S., Grant, A.W. *et al* (2002), *Br. J. Pharmacol.*, 135, 75P.
Kilfeather, S.A. & Page, C., (1997), in *Airway Wall Remodelling in Asthma*, Stewart, A.G. (ed.), CRC Press, 179-212.

49P BRADYKININ-INDUCED RELAXATION OF BOVINE PULMONARY SUPERNUMERARY ARTERIES: INVOLVEMENT OF GAP JUNCTIONS

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In bovine pulmonary supernumerary arteries bradykinin-induced relaxation can be mediated by nitric oxide or an EDHF-like mechanism when nitric oxide or guanylyl cyclase is inhibited (Tracey *et al.*, 2002). The aim of the present study was to evaluate the possible contribution of gap junctions to the nitric oxide and EDHF-mediated responses elicited by bradykinin.

Bovine lungs were obtained fresh from the local abattoir. Segments of supernumerary arteries (diameter 0.5 - 1 mm) were dissected from the lung and freed of surrounding connective tissue. The vessels were then weighed and suspended between stainless steel hooks in Krebs-Henseleit buffer (37°C) under a tension of 1 g and gassed with a mixture of O_2:CO_2 95%/5% v/v. The tissues were allowed to equilibrate for 1 hour then contracted with U46619 (0.3 μM). Cumulative concentration response curves for bradykinin-induced relaxation were constructed. Paired tissues acted as time controls. Relaxations are expressed as % decrease of the U46619-induced tone. Results are means ± s.e. mean. The significance of differences was determined using Student's t-test.

Bradykinin produced a concentration-dependent relaxation (pEC_{50}, 9.7±0.3; maximum relaxation (R_{max}), 89.7±14.8, n=6). The nitric oxide scavenger hydroxocobalamin (200μM; pEC_{50}, 8.0±0.6, R_{max}, 93.4±12.6, n=6; $P<0.05$) and the nitric oxide synthase inhibitor L-NAME (100μM; pEC_{50}, 8.9±0.2; R_{max}, 85±8.9, n=6, $P<0.05$) each produced a rightward shift in the bradykinin concentration response curve. In contrast the guanylyl cyclase inhibitor ODQ (10μM, pEC_{50}, 9.3±0.3; R_{max}, 119±11.4, n=4) did not significantly alter the tissue sensitivity.

The gap junction inhibitor carbenoxolone (100μM) did not change the bradykinin concentration response curve. In the presence of L-NAME (R_{max}, 54.2±10.5; $P=0.05$), hydroxocobalamin (R_{max}, 35.8±17.3; P=0.03) or ODQ (R_{max} 48.5± 9.2; P=0.003), carbenoxolone significantly reduced the maximum relaxation produced by bradykinin. Additionally, in the presence of ODQ, carbenoxolone produced a reduction in the tissue sensitivity to bradykinin.

The present study suggests that gap junctions are not normally involved in bradykinin–induced relaxation of bovine pulmonary supernumerary arteries *in vitro* but are involved in mediating the EDHF-like response to bradykinin when nitric oxide is removed.

Tracey, A., Irvine, J., Bunton, D., MacDonald, A., Shaw, A.M. (2002) *Br. J. Pharmacol.* **135**, 79P.

50P SUPEROXIDE AND SUPEROXIDE DISMUTASE IN PULMONARY HYPOXIC VASOCONSTRICTION

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Acute hypoxia induces vasoconstriction in perfused rat lungs, through mechanisms that are unique to the pulmonary circulation. NO is essential in maintaining a low pulmonary pressure, and opposes the hypoxic increase in vascular resistance. Superoxide has major actions in the pulmonary circulation, and can cause vascular damage and react rapidly with NO, producing peroxynitrite (Demiryürek & Wadsworth 1999). There are three isoforms of superoxide dismutase (SOD) that are widely distributed in the cells of the vasculature which destroy superoxide, however, it is unknown whether they modify hypoxic constriction. Therefore, the effect of endogenous SOD inhibition and superoxide generation on hypoxic pulmonary vasoconstriction was investigated.

Male Sprague-Dawley rats were anaesthetised with sodium pentobarbitone (0.6ml/Kg, ip) and the trachea and pulmonary artery cannulated. The lungs were removed to a warming jacket and ventilated with 20% O_2, 5% CO_2, 75% N_2 (normoxia). The rat lungs were perfused with 25ml of Krebs solution containing 4% albumin and 1μM flurbiprofen. U46619 was injected directly into the recirculating perfusate to give preconstriction before a 10min hypoxic challenge, which was induced by rapidly changing the gas flow from normoxic to hypoxic (5% CO_2, 95% N_2). The lungs were then returned to normoxia for 10min before changing the perfusate to one containing either 1mM DETCA (high affinity Cu^{2+} chelator that inhibits endogenous SOD) or 10μM LY83583 (superoxide generator). The lungs were allowed to equilibrate for 30min, before repeating the hypoxic and normoxic challenges. The NO donor SNAP ($5x10^{-13}$ – $5x10^{-10}$ moles) was injected into the perfusate immediately before entering the lungs.

Hypoxia induced a sustained monophasic increase in pulmonary pressure, with the recirculating perfusate reaching a pO_2 of 20±1 torr. When the gas source was returned to normoxia there was a sharp decrease in pressure, returning to the baseline pressure within 10mins and a pO_2 of 123±3 torr. The hypoxic vasoconstriction was reproducible (+1.0±0.3mmHg and +1.6±0.6mmHg, when repeated. n=4). LY83583 augmented the hypoxic vasoconstriction (+0.7±0.4mmHg control and +2.7±0.8mmHg with LY83583 present, n=3), and attenuated ($P<0.05$) the SNAP induced vasodilation. These results suggest that superoxide can support or enhance hypoxic vasoconstriction, perhaps by consuming endogenous NO. There was a significant reduction of hypoxic vasoconstriction in the presence of DETCA (+2.3±0.3mmHg control and +1.0±0.3mmHg with DETCA, n=6, $P<0.05$), suggesting endogenous SOD may have an important role involving the action of hypoxia in the pulmonary circulation.

Demiryürek A.T., & Wadsworth R.M. (1999) *Pharmacol. Ther.* 84, 355-365.
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51P INCREASED PULMONARY HYPERTENSION IN MICE OVER-EXPRESSING THE 5-HYDROXYTRYPTAMINE TRANSPORTER GENE

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The 5-hydroxytryptamine transporter (5HTT) may play a key role in pulmonary vascular remodelling in primary pulmonary arterial hypertension (PAH) and secondary hypoxia-related PAH. Attenuated hypoxic pulmonary hypertension has been reported in mice lacking the 5HTT transporter gene (Eddahibi *et al.*, 2000) and there is 5HTT over-expression in patients with PAH (Eddahibi *et al.*, 2001).

Here we examine the development of PAH in mice over-expressing the 5HTT gene (5HTT+ mice) and C57BL/6 x CBA wild-type mice (WT) mice (female, 35-40g) exposed to 2 weeks chronic hypoxia.

Right ventricular (RV): total ventricular (TV) ratio was used as an index of pulmonary hypertension. Pulmonary vascular remodelling was assessed by calculating the percentage of remodelled pulmonary arteries <80μm i.d.. Pulmonary arteries (250-300μm) from all mice were mounted (Krebs, 37^{0}C) on wire myographs and cumulative concentration responses to noradrenaline (NA, 1nM-0.1mM), 5-HT (1nM-0.1mM) and endothelin-1 (ET-1, 1pM-0.1μM) examined. To assess 5HTT binding characteristics, preliminary experiments using ^{3}[H]-β-CIT (1R,2S,3S,5S)-3-(4-Iodophenyl)-8-[^{3}H]-methyl-8-azabicyclo [3.2.1]octane-2-carboxylic acid, methyl ester; high affinity radioligand for 5-HT (& DA) transporters) binding were carried out in triplicate on lung membrane preparations using saturation binding studies with 10 μM citalopram to define non-specific binding.

In the WT mice, RV/TV ratio was not altered after 2 wks of hypoxia (0.205 ± 0.005 cf. 0.238 ± 0.012, n=6-7). In 5HTT+ mice, however, the RV/TV ratio was increased (P<0.001, n=7) from 0.194 ± 0.009 to 0.297 ± 0.018. The percentage of remodelled vessels increased (P<0.001, n=6 lungs) from 2.61 ± 0.7%, (WT hypoxic) to 7.8 ± 0.5% (5HTT+ hypoxic). ET-1, NA and 5HT induced contraction in WT mice vessels (pEC$_{50}$s: 9.8 ± 0.3 > 9.0 ± 0.2 > 6.9 ± 0.2, respectively). NA constriction was abolished in the 5HTT+ mice and the potencies of ET-1 and 5HT were reduced (pEC$_{50}$s: 8.4 ± 0.3 and 5.6 ± 0.3 respectively). In 5HTT+ mice exposed to hypoxia however, there was a marked restoration of the contractile response to NA (pEC$_{50}$: 8.6 ± 0.1) and an increase in the response to ET-1 and 5-HT (pEC$_{50}$s: 8.6 ± 0.3 and 6.9 ± 0.4, respectively). The maximum response to ET-1 was increased by ~40%. In WT mice lung membranes, ^{3}[H]-β-CIT bound with a B_{max} of 1.07 ± 0.02fmol.mg^{-1} and a K_D of 0.87 ± 0.05nM (n=3). Binding was markedly increased in the 5HTT+ mice, with a B_{max} of 13.32 ± 0.25fmol.mg^{-1}and a K_D value of 3.74 ± 0.03nM (n=3).

The results indicate that PAH is accelerated in 5HTT+ mice and this is associated with remodelling, increased lung 5HTT and increased contractile responses to 5-HT, ET-1 and restoration of NA-induced contraction. 5HTT+ mice may provide a novel model of genetically susceptible PAH.

Eddahibi, S., Hanoun, N., Lanfumey, L. *et al.*, (2000) *J Clin Invest*, 105, 1555-1562.

Eddahibi, S., Humbert, M., Fadel, E., *et al.*, (2001) *J Clin Invest*, 108, 1141-1150.

52P ASSESSMENT OF THE VASODILATORY ACTION OF TESTOSTERONE IN ISOLATED HUMAN PULMONARY ARTERIES AND VEINS

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Testosterone therapy has been shown to be of benefit to men with coronary artery disease (English *et al.*, 2000). This is proposed to be due to the vasodilatory effect of testosterone on human coronary arteries (Webb *et al.*, 1999). It has also been shown that testosterone therapy is of benefit to men with heart failure (Pugh *et al.*, 2002). Pulmonary vasodilators are commonly used in the treatment of heart failure but it is unknown if testosterone possesses this effect.

Male patients (n=8, age=68±8) were recruited from cardiothoracic operating lists at the Northern General Hospital and gave full written consent. Following resection pulmonary tissue was transferred to the laboratory in physiological saline solution (PSS). 2mm lengths of pulmonary artery (n=16, diameter = 813 ± 586 um) and pulmonary vein (n=14, diameter = 746 ± 329 um) were carefully dissected, mounted in a wire myograph in PSS at 37°C and bubbled with 95% oxygen, 5% carbon dioxide to maintain pH 7.4. The pulmonary arteries were loaded to a tension equivalent to 17.5±2.4mmHg and the veins to 10.8±1.1mmHg. Vessels were contracted with potassium chloride (KCl, 70mM) to confirm the viability of the smooth muscle. Endothelial integrity was confirmed by dilatation to acetylcholine (ACh, 1μM) following preconstriction with noradrenaline (NA, 10μM). Vessels were then exposed to increasing concentrations of KCl (0.1-100mM) followed by cumulative additions of ethanol vehicle. Vessels were then washed, the addition of KCl (0.1-100mM) repeated and exposed to increasing concentrations of testosterone (T, 1nM-100μM).

Results are shown in the table below as mean (standard deviation). Human pulmonary veins were less sensitive to NA and KCl compared to human pulmonary arteries but this failed to reach statistical significance.

	E_{max} NA (mN)	E_{max} ACh (%)	E_{max} KCl (mN)	E_{max} T (%)	E_{max} Eth (%)
Artery	2.62 (4.50)	-55.5 (34.1)	7.61 (6.1)	-54.7 (42.7)	8.4 (8.3)
Vein	1.82 (1.99)	-56.0 (30.1)	3.66 (2.84)	-55.7 (35.4)	4.9 (6.4)

Testosterone dilated human pulmonary arteries and veins with a similar efficacy as ACh. Testosterone-mediated pulmonary vasodilatation may contribute to the beneficial effect of testosterone therapy in men with chronic heart failure.

English KM, Steeds RP, Jones TH *et al.* (2000) *Circulation* **102**, 1906-1911.

Pugh PJ, Jones RD, West JN *et al.* (2002) *Heart* **87**, p5.

Webb CM, McNeill JG, Hayward CS, *et al.* (1999) *Circulation* **100**, 1690-1696.

53P CHARACTERISATION OF P2Y RECEPTORS MEDIATING CONTRACTION OF THE RAT ISOLATED PULMONARY ARTERY

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We have shown that both P2X and P2Y receptors mediate contraction of rat small (SPA, 300-500 μm i.d.) and large (LPA, 1-1.5 mm i.d.) intrapulmonary arteries (Chootip *et al.*, 2000). Purine nucleotides, such as adenosine 5′-triphosphate (ATP) appeared to act largely via $P2X_1$ receptors and uridine 5′-triphosphate (UTP) acted via P2Y receptors. However, which P2Y receptor subtypes are present in these vessels is not clear. Therefore, the aim of this study was to characterise the effects of uridine 5′-diphosphate (UDP), which has a different P2Y subtype activity profile from UTP, in rat SPA and LPA.

Isolated arterial rings were mounted in 1 ml organ baths filled with HEPES-buffered solution at 37 °C. Changes in isometric force were measured using a MacLab data acquisition system. Cumulative contractile concentration-response curves were constructed for UDP and compared with those for other P2 receptor agonists. As these did not reach a maximum, agonist potency was compared at the level equivalent to 40% of the contraction to 40 mM K^+ (EC_{40K}). The data are expressed as mean (95% confidence limits) and were analysed statistically by 1-way ANOVA and Tukey's comparison.

UDP (1-300 μM) evoked contraction of the endothelium-intact SPA with an EC_{40K} = 204 μM (150-275 μM) (n=6). UDP was 4-5 times less potent than UTP, ATP and 2-methylthioATP (2-meSATP) and 227 times less potent than α,β-methyleneATP (α,β-meATP). Removal of the endothelium significantly potentiated responses to UDP only (EC_{40K} = 43 μM (19-98 μM), n=6, $P<0.01$) and the rank order of agonist potency was α,β-meATP >> UDP = UTP = ATP = 2-meSATP.

In endothelium-intact LPA, all P2 receptor agonists evoked relatively small contractions and only those to UDP reached the EC_{40K} level (167 μM (94-298 μM), n=9). Removal of the endothelium significantly increased the size of contractions ($P<0.05$). The EC_{40K} for UDP was 128 μM (92-178 μM) (n=9) and the rank order of agonist potency was α,β-meATP = UTP ≥ UDP > 2-meSATP, ATP. There was no significant difference in the potency of UDP between SPA and LPA.

In endothelium-denuded vessels the UDP-evoked contractions were potentiated by PPADS (30 - 300 μM) and the EC_{40K} values significantly increased ($P<0.05$, n=4-6). Suramin (30 - 100 μM) shifted the UDP concentration-response curves to the right and significantly increased its EC_{40K} values ($P<0.05$), but higher concentrations of suramin (0.3 - 3 mM produced no further inhibition (n=4-7). Reactive Blue 2 (30 - 100 μM) had no effect on UDP-evoked contractions (n=4-6).

This shows that UDP evokes contraction of the rat SPA and LPA via two receptors, one suramin-sensitive and the other suramin-insensitive. Neither is antagonised by PPADS or Reactive Blue 2 and their identities remain to be confirmed.

Chootip, K., Kennedy, C. & Gurney, G. (2000). *Br. J. Pharmacol.*, **131**, 167P.

54P IMMUNOLOCALISATION OF $P2Y_2$ RECEPTOR SUBTYPE IN HUMAN HYPERHIDROTIC ECCRINE SWEAT GLANDS

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The purinoceptor agonist ATP has been shown to induce sweating in isolated human eccrine glands (Sato *et al.*, 1991) as well as, increase $[Ca^{2+}]_i$ in a human sweat gland cell line (Wilson *et al.*, 1994), in freshly dissociated cells (Clunes *et al.*, 1999) and in cells derived from primary sweat gland cultures (Bovell *et al.* 2000). These findings are indicative of the presence of a P2Y receptor and we have recently localised $P2Y_1$, $P2Y_2$ and $P2Y_4$ subtypes in normal human glands (Lindsay *et al.*, unpublished). Hyperhidrosisis is characterised by the production of an inappropriately large volume of sweat and is a condition that is not fully understood. Immunohistochemistry was therefore employed to investigate the localisation of the $P2Y_2$ receptor subtype in hyperhidrotic human eccrine sweat glands.

Axillary skin biopsies were obtained with informed consent and local medical ethical committee approval, from patients suffering from hyperhidrosis (n=7) and from patients with no apparent skin pathology (n=11. Samples were fixed, processed and sectioned using standard techniques. Immunohistochemical staining was performed using antibodies raised against $P2Y_2$ receptor (Alomone Labs), employing the avidin-biotin complex (ABC) procedure. Sections were haematoxylin counterstained, dehydrated, cleared, mounted and viewed using light microscopy.

The reabsorptive duct of hyperhidrotic glands contained dark $P2Y_2$-like immunoreactivity localised to the apical membrane, which was similar to the staining found in normal glands. However, hyperhidrotic glands exhibited staining on the basolateral membranes, which was not present in normal glands. The secretory coils of hyperhidrotic sweat glands demonstrated staining localised in the clear type secretory cells, which was also not seen in the normal gland. Preabsorption of the antibody with the appropriate control peptide abolished all specific staining.

The presence of apical purinoceptors in the eccrine sweat gland duct of both normal and hyperhidrotic glands is suggestive of apical regulation of absorption. Hyperhidrotic sweat glands showed a wider distribution of the P2Y receptor compared to normal glands. These receptors present on the duct, may alter salt reabsorbtion to prevent drastic loss in hyperhidrosis. The clear cells of the secretory coil are involved in sweat secretion and the localisation of the $P2Y_2$ receptor in hyperhidrotic clear cells would suggest that this receptor is implicated in sweat secretion and could play a role in the condition hyperhidrosis. The myoepithelial cells of the sweat gland are not known to be involved in either secretion or reabsorption of sweat, and the presence of $P2Y_2$ receptors in both normal and hyperhidrotic glands requires further investigation.

Bovell *et al.*, (2000) *Eur .J. Pharmacol* **403,** 45-48.
Clunes *et al.*, (1999) *J. Physiol.* **517P,** 88P
Sato *et al.*, (1991) *J.Am.Acad. Dermatol*, **24**, 1010-1014
Wilson *et al.*, (1994) *J. Exp. Physiol.*, **79(3),** 445-459

55P EFFECTS OF CHRONIC, *IN VIVO*, TREATMENT WITH A NITRIC OXIDE SYNTHASE INHIBITOR ON VASORELAXANT RESPONSES TO ANANDAMIDE IN RAT ISOLATED ARTERIES

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The endocannabinoid, anandamide, causes vasorelaxation *in vitro* via several different mechanisms (Harris *et al.,* 2002). Although nitric oxide (NO) does not appear to mediate these responses, it has been reported that chronic, *in vivo,* treatment of rats with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), potentiates the *in vitro* vasorelaxant effects of anandamide (Mendizabal *et al.,* 2001). In this study we monitored the *in vivo* cardiovascular effects of L-NAME by telemetry in conscious, male, Sprague-Dawley rats, and then investigated responses to anandamide in preparations of the aortae and mesenteric vasculature from these animals.

Animals (300-350 g) were housed in pairs after 1 of the pair had had a telemetry module (Datasciences TA 11PA-C40) implanted i.p. (under anaesthesia, fentanyl and medetomidine, 300 μg kg^{-1} i.p. of each). Cardiovascular data were recorded for 7 days before exposure to L-NAME (0.1 mg ml^{-1} in drinking water), and for 28 days thereafter. Rats were then anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and exsanguinated. The mesenteric arterial bed was isolated (Harris *et al.*, 2002) and perfused with oxygenated Krebs-Henseleit solution containing 10 μM indomethacin and 300 μM L-NAME. Following 30 min equilibration, methoxamine was added to increase perfusion pressure (100-120 mmHg). The vasorelaxant effects of anandamide (10 nM-10 μM) were then assessed. Thoracic aortae were also removed and were mounted as 2-3 mm rings for isometric recording (conditions as above). The rings were then placed under 1 g tension and allowed to equilibrate for 1 h. The rings were contracted with methoxamine (100 μM) and the relaxant effects of anandamide (10 nM-30 μM) were determined. All *in vitro* data were compared by Student's t-test.

In the 3 telemetered rats drinking L-NAME, mean blood pressure rose by 17 ± 3 mmHg (mean ± s.e.mean), compared to 3 mmHg in both the rats drinking water.

In the mesenteric arterial beds from the control rats, anandamide induced concentration-related relaxations (pEC_{50} = 6.13 ± 0.08, maximum relaxation (R_{max}) = 106 ± 5%, n = 5). In preparations from rats treated with L-NAME, anandamide was significantly ($P<0.001$) more potent as a vasorelaxant with pEC_{50} = 6.68 ± 0.07 but R_{max} was unaltered (109 ± 3%, n = 6). However, in the aortic rings the responses to anandamide did not differ between arteries from control rats and those drinking L-NAME (e.g., relaxations obtained at 10 μM were 34.0 ± 6.8 % (controls, n = 5) and 38.6 ± 4.6 % (L-NAME treated, n = 6). There appeared to be no difference between *in vitro* responses of preparations from animals with or without implanted modules.

The results of the present study confirm that chronic, *in vivo* treatment with L-NAME, which is associated with elevation in blood pressure, increases the *in vitro* vasorelaxant potency of anandamide in the mesenteric arterial bed. However, this is not a universal change as responses in aortic rings were unaffected. The mechanisms underlying the increased potency of anandamide following chronic NO synthase inhibition are currently being investigated.

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Harris, D., McCulloch, A.I., Kendall, D.A. & Randall, M.D. (2002). *J. Physiol.*, 539, 893-902.
Mendizabel, V.E., Orliac, M.L. & Adler-Graschinsky, E. (2001). *Eur. J. Pharmacol.*, 427, 251-262.

56P EFFECT OF CONNEXINS 37, 40 AND 43 mRNA OVEREXPRESSION IN RATS AND THE POTENTIAL CONTRIBUTION TO INCREASED ENDOTHELIUM-DEPENDENT RELAXATION DURING PREGNANCY

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Pregnancy is associated with a decrease in systemic vascular resistance, which maintains or reduces maternal blood pressure despite increase in plasma volume and cardiac output. Although the mechanism responsible for these cardiovascular changes during pregnancy is not fully understood, an improvement of endothelium-dependent vasodilation has been proposed. In a study conducted *in vitro*, we have suggested a role for gap junctional communication (GJC) in the control of endothelium-dependent vasodilation during pregnancy (Dantas *et al.*, 1999). In this study we explored the contribution of GJC for the *in vivo* endothelium-dependent relaxation observed in pregnant rats. Moreover, we tested the hypothesis that an increased gene expression of connexins (Cx, proteins that constitute the GJC) might contribute to the augmented endothelium-dependent vasodilation observed during pregnancy.

The influence of heptanol (10^{-7}, 10^{-6} and 10^{-5} M, an uncoupler of GJC) on acetylcholine- (ACh, 17 nM) or sodium nitroprusside- (SNP, 38 nM) induced vasodilation was studied by using intravital microscopy in the mesenteric vascular bed as previous described (Fortes *et al.*, 1984), of anesthetized (choral hydrate, 500 mg kg^{-1}, subcutaneously) pregnant (P, 20-21 days of pregnancy) or non-pregnant (NP) Wistar rats. mRNA expression of Cxs 37, 40 and 43 was evaluated in the uterus, thoracic aorta, mesenteric and uterine arteries by RT-PCR and expressed relative to GAPDH.

Increased Cx 37, Cx 40 and Cx 40 mRNA levels were observed in the uterus, thoracic aorta, mesenteric and uterine arteries in pregnant rats with 21 days of pregnancy (figure 1).

Heptanol 10^{-7} and 10^{-6} M, but not 10^{-5} M, inhibited significantly ACh responses in mesenteric arteries from pregnants rats compared with non pregnant (10^{-7}: P=21.2 ± 11.7 %, NP=51.8± 3.6 %; 10^{-6}: P=36.7 ± 5.05 %, NP= 83.9 ± 4.85 %; 10^{-5}: P=78.1 ± 7.0 %, NP=89 ± 3.93 %). In contrast, heptanol did not change SNP–induced relaxation (10^{-7}: P=7.3 ± 11.3 %, NP=1.4 ± 14.9 %; 10^{-6}: P=5.3 ± 9.0 %, NP=1.0 ± 8.4 %; 10^{-5}: P=-27.5 ± 18.8 %, NP=10.3 ± 5.9 %). The results suggest that increased GJC, possibly due to increased gap junction protein expression, may facilitate the effects of endothelium-derived relaxing factors; thus contributing to the augmented endothelium-dependent relaxation in arteries from pregnant rats.

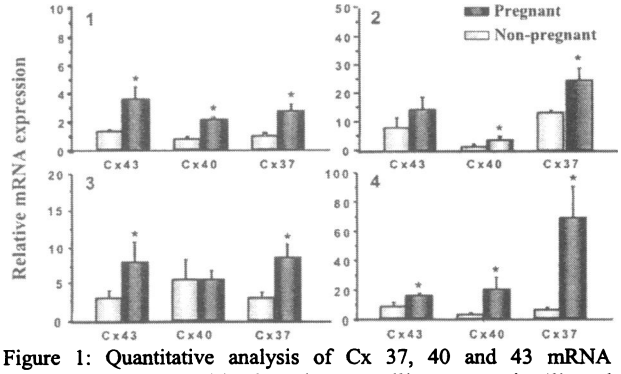

Figure 1: Quantitative analysis of Cx 37, 40 and 43 mRNA expression in uterus (1), thoracic aorta (2), mesenteric (3) and uterine arteries (4) from pregnant (n=10) and non-pregnant (n=12) rats. * $P<0.05$, *vs.* Non-pregnant. Statistical evaluations of the data were carried out by unpaired Student's *t* test.

Dantas MFV; Urban M; Spray D; et al (1999). Hypertension, 34, 937-942.
Fortes, ZB; Garcia Leme J & Scivoletto R (1984). Br. J. Pharmacol., 83, 635-643.

57P THE INVOLVEMENT OF ENDOTHELIUM-DERIVED NITRIC OXIDE IN VASORELAXATION TO BENDROFLUAZIDE IN THE RAT AORTA

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Bendrofluazide is a thiazide diuretic widely used in the management of hypertension. There is evidence that part of the antihypertensive actions of some thiazides is due to vascular smooth muscle relaxation. In this regard Pickkers *et al.* (1998) reported that hydrochlorothiazide, but not indapamide, causes vasorelaxation via potassium channel activation. We have now investigated the vasorelaxant mechanisms of bendrofluazide in rat aortic rings.

Male Wistar rats (250-350g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. Thoracic aortae were removed and placed in Krebs-Henseleit, and were mounted as 2-3mm rings for isometric recording. The rings were then placed under 1g tension and allowed to equilibrate for 1h. The rings were contracted with methoxamine (100μM) and the relaxant effects of bendrofluazide (1nM-1mM) were determined. The role of prostanoids in the relaxant responses to bendrofluazide was investigated by addition of the cyclooxygenase inhibitor, indomethacin (10μM), to some preparations. The role of nitric oxide (NO) in mediating the relaxant responses was determined by carrying out some experiments in the presence of the NO synthase inhibitor, 300μM N^G-nitro-L-arginine methyl ester (L-NAME). The involvement of the endothelium was investigated by rubbing the intimal surface with a wooden stick to remove the endothelium. All data were compared ANOVA.

Bendrofluazide induced concentration-related relaxations (pEC_{50}=3.49±0.28, mean±s.e.mean; maximum relaxation (R_{max})=74.6±14.2%, n=6). The relaxant responses were unaffected by the presence of indomethacin (pEC_{50}=4.29±0.45, R_{max}=58.0±9.34%, n=6). However, in the presence of L-NAME the relaxant responses to bendrofluazide were inhibited (pEC_{50}=3.85±0.14; R_{max}=31.7±2.8%, P<0.05; n=6). In 7 preparations removal of the endothelium similarly opposed the relaxations to bendrofluazide, such that the responses at the highest concentration used (1mM) was 18.1±18.2%.

The results of the present study have confirmed that bendrofluazide is a vasorelaxant. Furthermore, we have demonstrated that these relaxant responses are endothelium-dependent and largely mediated via NO.

Pickkers, P., Hughes, A.D., Russel, F.G.M., *et al.* (1998). *Hypertension*, 32, 1071-1076.

58P COMPARATIVE EFFECTS OF S-METHYL-L-THIOCITRULLINE (SMTC) AND N^G-NITRO-L-ARGININE METHYL ESTER (L-NAME) ON SALBUTAMOL-INDUCED HINDQUARTERS VASODILATATION IN CONSCIOUS RATS

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β-adrenoceptor stimulation can increase neuronal NO release in some vascular beds (see Ferrer & Balfagón, 2001). Here, we tested the hypothesis that β_2-adrenoceptor-mediated hindquarters vasodilatation is suppressed by inhibition of nNOS, by comparing the effects of SMTC, a putative nNOS inhibitor (Furfine *et al.*, 1994) and L-NAME, on the hindquarters vasodilator effect of salbutamol. Under anaesthesia, (fentanyl and medetomidine, 300 μg kg^{-1} of each i.p.), male, Sprague-Dawley rats (350-450g) had renal (R), mesenteric (M), and hindquarters (H) Doppler flow probes chronically implanted, and, at least 14 days later, intravascular catheters for recording blood pressure (BP) and heart rate (HR), and for i.v. drug administrations. The following day, two groups of animals were infused with saline (vehicle) for 90 min (1 ml kg^{-1} h^{-1}), before infusion of salbutamol (0.6 μg kg^{-1} min^{-1} for 3 min). Two days later, animals received SMTC (n = 8) or L-NAME (n = 9) at 3 mg kg^{-1} h^{-1} for 90 min, before salbutamol.

The cardiovascular changes after SMTC or L-NAME (Table 1) were qualitatively similar to those reported previously (Wakefield *et al.*, 2002). The hindquarters vasodilatation caused by salbutamol was markedly attenuated by L-NAME, but not by SMTC (Table 2).

The failure of SMTC to inhibit the hindquarters vasodilator response to salbutamol indicates that this process does not involve nNOS, and hence the inhibitory effect of L-NAME on this response (see also Gardiner *et al.*, 1991), is likely due to suppression of eNOS.

Table 1. Baseline cardiovascular variables (mean ± s.e.mean) in each group immediately prior to infusion of salbutamol in the presence of vehicle, SMTC or L-NAME. Units for vascular conductance (VC) are [kHz $mmHg^{-1}$]10^3. * P < 0.05 vs corresponding vehicle (Wilcoxon's test).

	Vehicle	SMTC	Vehicle	L-NAME
HR (beats min^{-1})	338±10	311±13*	340±13	289±13*
BP (mmHg)	105±1	113±4	101±2	138±4*
RVC (units)	93±7	84±9	86±7	53±6*
MVC (units)	94±8	72±8*	98±11	48±6*
HVC (units)	42±3	34±3	41±3	19±2*

Table 2. Integrated (mean ± s.e.mean, 0-3 min areas under or over curves (AUC, AOC)) changes in HR (beats), BP (mmHg min) and HVC ([kHz $mmHg^{-1}$]10^3 min) in response to salbutamol in the presence of Vehicle, SMTC or L-NAME. * P < 0.05 vs corresponding vehicle (Wilcoxon's test).

	Vehicle	SMTC	Vehicle	L-NAME
HR (AUC)	+189±14	+176±14	+154±26	+171±25
BP (AOC)	-27±4	-36±3	-26±2	-47±10
HVC (AUC)	+113±12	+121±17	+112±10	+49±5*

Ferrer, M. & Balfagón, G. (2001). *Clin. Sci.*, 101, 321-328.
Furfine, E.S. *et al.* (1994). *J. Biol. Chem.*, 269, 26677-26683.
Gardiner, S.M. *et al.* (1991). *Br. J. Pharmacol.*, 103, 1725-732.
Wakefield, I.D. *et al.* (2002). *Br. J. Pharmacol.*, 135, 296P.

59P THE ROLE OF THE ENDOTHELIUM, CYCLOOXYGENASE, LIPOXYGENASE AND CYTOCHROME P450 IN THE VASORELAXANT ACTIONS OF ARACHIDONIC ACID IN SHEEP CORONARY ARTERIES

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The vascular effects of arachidonic acid appear to be species- and vascular tissue-specific, depending on which one of 3 metabolic pathways (cyclooxygenase, lipoxygenase or cytochrome P450) predominates (Miura & Gutterman, 1998; Sobey *et al.*, 1998). We have recently reported that the vasodilator effects of anandamide in sheep coronary arteries result from its conversion, *via* arachidonic acid, to a prostanoid (Grainger & Boachie-Ansah, 2001). However, the vascular effects of arachidonic acid itself have not been fully characterised in sheep coronary arteries (Cornish *et al.*, 1983). We sought, therefore, to determine the role of the endothelium and the oxygenases (cyclooxygenase, lipoxygenase or P450) in the vascular effects of arachidonic acid in sheep coronary arteries.

Paired circumflex coronary artery rings were removed from sheep hearts (obtained from local abattoir) and mounted under 2 g resting tension in a 10 ml tissue bath containing Krebs-Henseleit solution equilibrated with 95% O_2/5% CO_2 at 37°C. Isometric tension was monitored *via* a force displacement transducer coupled to a Grass 79D polygraph. One of each pair of rings was left endothelium intact and untreated (control), while the other ring was either endothelium denuded or pre-treated for 30 min with either the analogue inhibitor of arachidonic acid metabolism, eicosatetraynoic acid (ETYA; 1 & 3 μM), the cyclooxygenase inhibitor, indomethacin (3 μM), the lipoxygenase inhibitors, baicalein and nordihydroguaiaretic acid (NDGA; 10 μM), the cytochrome P450 inhibitors, 17-octadecynoic acid (17-ODYA; 3 & 10 μM) and 1-aminobenzotriazole (1-ABT; 1 & 3 mM), or the NO synthase inhibitor, L-NAME (100 μM). Rings were precontracted to the thromboxane-A_2 mimetic, U46619 (0.1-0.6 μM) and relaxed by cumulative addition of arachidonic acid (0.01-10 μM). Mean sensitivity (pEC_{50} values) and % maximal relaxation (R_{max}) to arachidonic acid in control and treated rings were compared using Student's *t*-test or ANOVA and Dunnett's *post-hoc* test.

Arachidonic acid (0.01-10 μM) induced concentration-dependent relaxation of U46619-evoked contractions in endothelium-intact rings, with a mean pEC_{50} of 6.68±0.07 and R_{max} of 99.8±2.5% (n=6). Endothelium denudation only partially attenuated this effect, decreasing the mean pEC_{50} value for arachidonic acid to 6.18±0.13 (P<0.01; n=6) without modifying R_{max} (102.5±2.9; P>0.05). Pre-treatment with the inhibitor of arachidonic acid metabolism, ETYA (1 & 3 μM), virtually abolished the arachidonic acid effect, reducing the R_{max} from 93.6±3.7% in control rings to 35.8±20% and 5.8± 3.9%, respectively (P<0.001; n=4-9). Similarly, pre-treatment with the cyclooxygenase inhibitor, indomethacin (3 μM), also markedly attenuated arachidonic acid-induced relaxations (R_{max} reduced from 94.6±2.5% in control rings to 7.8±10.7%; P<0.001; n=7).

By contrast, pre-treatment with the lipoxygenase inhibitors, baicalein or NDGA (10 μM), did not modify arachidonic acid-induced relaxations. For example, mean pEC_{50} and R_{max} values for arachidonic acid in baicalein (10 μM)-treated rings were 7.0±0.17 and 101.7±5.0%, compared with 6.86±0.04 and 94.5±2.8%, respectively, in control rings (P>0.05; n=3). Likewise, pre-treatment with the cytochrome P450 inhibitors, 17-ODYA (3 & 10 μM) and 1-ABT (1 & 3 mM), or NO synthase inhibitor, L-NAME (100 μM), also failed to modify the relaxant responses to arachidonic acid.

These findings suggest that the relaxant effects of exogenous arachidonic acid in the sheep coronary artery are (i) mediated, at least in part, through the endothelium, and (ii) depend on its prior cellular uptake and conversion to a vasodilatory prostanoid(s).

Cornish EJ *et al.* (1983) *Clin Exp Pharmacol Physiol* **10**: 171-174.
Grainger J & Boachie-Ansah G (2001) *Br J Pharmacol* **134**:1003-1012.
Miura H & Gutterman DD (1998) *Circ Res* **83**: 501-507.
Sobey CG *et al.*, (1998) *Am J Physiol* **275**: H1606-H1612.

60P THE INVOLVEMENT OF K^+ CHANNELS IN THE VASORELAXANT RESPONSES TO TESTOSTERONE IN THE RAT AORTA

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Previous studies have shown that testosterone causes vasorelaxation via activation of K^+ channels (Yue *et al.*, 1995; Honda *et al.*, 1999; Tep-areenan *et al.*, 2002). However, the exact types of K^+ channels, which mediate the testosterone-induced responses are still unclear. Honda *et al.* (1999) demonstrated that vasorelaxation to testosterone was mediated by activating ATP-sensitive K^+ (K_{ATP}) channels. In contrast, we have recently reported that testosterone induces vasorelaxation by increasing K^+ efflux through large-conductance calcium-activated K^+ (BK_{Ca}) channels, but not via K_{ATP} channels (Tep-areenan *et al.*, 2002). In the present study, we now investigate the role of K^+ channels in testosterone-induced vasorelaxation in rat aortic rings.

Male Wistar rats (250-300g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. The thoracic aortae were dissected from the rats and cut into 5mm lengths. Each ring was bathed with oxygenated Krebs-Henseleit solution. The aortic rings were stretched to optimal passive tension of 10mN. Following a 1-hour equilibration period, methoxamine (80-150μM) was added to increase tension by 5-10mN. Testosterone was added cumulatively to the perfusion fluid (1nM-1mM). The vasorelaxant effects of testosterone were assessed in the presence of 10μM indomethacin, a cyclooxygenase (COX) inhibitor, 100nM charybdotoxin (ChTx), a K_{Ca} channel and voltage-sensitive K^+ (K_V) inhibitor, 1mM 4-aminopyridine (4-AP), a K_V channel inhibitor, 10μM glibenclamide, a K_{ATP} channel inhibitor, or 30μM barium chloride ($BaCl_2$), a voltage-dependent inward rectifier K^+ (K_{IR}) channel inhibitor. To examine the effects of high extracellular K^+ on responses to testosterone, 60mM KCl was added to induce tone by substituting an equimolar concentration of NaCl with KCl (McCulloch *et al.*, 1997). Maximal responses (R_{max}) are expressed as mean±s.e.mean and pEC_{50} values are expressed as mean with 95% confidence intervals (CI). Data were compared by the Student's *t*-test for unpaired values or ANOVA.

In the absence of indomethacin, testosterone (1nM-1mM) induced concentration-dependent vasorelaxation, and the data were best fitted (r^2=0.999) to a 1-site model (pEC_{50} = 4.39(4.25-4.54, 95%CI), with R_{max} = 168±5%, n=5). However, in the presence of indomethacin, a high potency vasorelaxant site was uncovered, and the data were best fitted (r^2=0.999) to a 2-site model ($pEC_{50\text{-}1}$ = 7.69(6.84-8.56), $pEC_{50\text{-}2}$ = 4.35(4.14-4.55), with R_{max} = 181±6%, n=7). High extracellular K^+ or pre-treatment with ChTx significantly (p<0.001) reduced the potency of testosterone-induced vasorelaxation at its high potency site, but had no effects on R_{max} (R_{max}: indomethacin = 181±6%, n=7; indomethacin+KCl = 178±10%, n=7; indomethacin+ChTx = 184±0%, n=4). The inhibitory effects of high KCl were found only in the presence of indomethacin, but not in the absence of indomethacin. Addition of 4-AP, glibenclamide, or $BaCl_2$ with indomethacin did not affect testosterone-induced vasorelaxation in the presence of indomethacin.

In the present study we have demonstrated that testosterone induces vasorelaxation in the rat aortae. Following blockade of the COX pathway we also uncovered a high potency vasorelaxant action and relaxation at this site was mediated via K_{Ca} channels. However, at the low potency site, K^+ channels are not involved in the vasorelaxation.

Honda, H., Unemoto, T. & Kogo, H. (1999) *Hypertension*, **34**, 1232-1236.
McCulloch, A.I., Bottrill, F.E., Randall, M.D., *et al.* (1997) *Br. J. Pharmacol.*, **120**, 1431-1438.
Tep-areenan, P., Kendall, D.A. & Randall, M.D. (2002) *Br. J. Pharmacol.*, **135**, 735-740.
Yue, P., Chatterjee, K., Beale, C., *et al.* (1995) *Circulation*, **91**, 1154-1160.

61P MECHANISMS OF ACUTE VASORELAXANT EFFECTS OF TESTOSTERONE IN THE RAT AORTA

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Chou *et al.* (1996) reported that testosterone induced endothelium-dependent relaxation in the canine coronary artery. However, Yue *et al.* (1995) showed in the rabbit coronary artery that vasorelaxation to testosterone was independent of both the endothelium and testosterone receptors. The present study aimed to investigate the role of the endothelium, prostanoids, nitric oxide (NO), and steroid receptors in testosterone-induced vasorelaxation in rat aorta.

Male Wistar rats (250-300g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. The thoracic aortae were dissected from the rats and cut into 5mm lengths. Each ring was bathed with oxygenated Krebs-Henseleit solution. The aortic rings were stretched to optimal passive tension of about 10mN. Following a 1-hour equilibration period, methoxamine (20-100μM) was added to increase tension by 5.0-10mN. Testosterone was added cumulatively to the perfusion fluid (1nM-1mM). The vasorelaxant effects of testosterone were investigated in the presence of 10μM indomethacin or 10μM flurbiprofen, which are both cyclooxygenase (COX) inhibitors, 300μM N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, 10μM flutamide, a testosterone receptor antagonist, or 30μM mifepristone, a steroid receptor antagonist. To examine the role of the endothelium, some aortae were denuded by gently rubbing the luminal surface with a cocktail stick before mounting. The preparation was considered to be endothelium-denuded when the response to 10μM carbachol was less than 10%. Maximal responses (R_{max}) are expressed as mean±s.e.mean and pEC_{50} values are expressed as mean with 95% confidence intervals (CI). Data were compared by the Student's *t*-test for unpaired values or ANOVA.

Testosterone (1nM-1mM) induced acute vasorelaxations in a concentration-dependent manner (pEC_{50} = 4.39(4.25-4.54, 95%CI), with R_{max} = 168±5%, n=5). Pre-treatment with either indomethacin or flurbiprofen significantly ($p<0.01$) enhanced the potency of testosterone-induced vasorelaxation and the data were now best fitted (r^2=0.999) to a 2-site model, but had no changes in R_{max} (indomethacin: $pEC_{50\text{-}1}$ = 7.69(6.84-8.56), $pEC_{50\text{-}2}$ = 4.35(4.14-4.55), n=7; flurbiprofen: $pEC_{50\text{-}1}$= 7.86(7.00-8.71), $pEC_{50\text{-}2}$ = 4.47(4.34-4.59), n=6). In the presence of indomethacin, removal of the endothelium significantly inhibited R_{max} (non-rubbed: R_{max}= 181±6%, n=7; rubbed: R_{max}= 158±7%, n=6, $p<0.05$), and inhibited testosterone-induced responses at its high potency site. However, in the absence of indomethacin, the R_{max}, but not the potency, of testosterone-induced responses was significantly ($p<0.01$) decreased in denuded-aortic rings (non-rubbed: R_{max}= 168±5%, n=5; rubbed: R_{max}= 142±5%, n=5). Addition of both indomethacin and L-NAME, or L-NAME alone had no effects. Similarly, neither flutamide nor mifepristone inhibited testosterone-induced vasorelaxation.

Our results have shown that, in rat aortic rings, testosterone causes acute and potent vasorelaxations, which are independent of steroid receptors. We have also identified an additional vasorelaxant action of testosterone, which is uncovered only in the presence of blockade of the COX pathway. Our results suggest that endothelium-derived prostanoids may modulate the responses to testosterone at the high potency site.

Yue, P., Chatterjee, K., Beale, C., *et al.* (1995) *Circulation*, **91**, 1154-1160.

62P SLV306, A NOVEL ENDOTHELIN CONVERTING ENZYME (ECE) AND NEUTRAL ENDOPEPTIDASE (NEP) INHIBITOR PREVENTS SYSTEMIC CONVERSION OF INFUSED BIG ENDOTHELIN-1 IN HUMANS

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The aim of this study was to determine whether SLV306 (Gray *et al.*, 2000) inhibited the systemic conversion of big ET-1 to mature ET-1 and the C-terminal fragment (CTF). On four separate occasions, following the oral administration of one of three increasing doses of SLV306 (to reach an average target concentration of 75, 300, 1200 ng ml^{-1} of the active metabolite KC12615) or a placebo, in a randomised, double blinded regime, big ET-1 was infused into 13 healthy male volunteers (mean age 23 years). Big ET-1 was administered at a rate of 8 and 12 pmol kg^{-1} min^{-1} (20min each). Plasma samples were collected pre, during and post big ET-1 infusion. Following selective solid-phase extraction, immunoreactive (IR) mature ET, big ET-1 and CTF was measured by RIA (Plumpton *et al.*, 1995).

The infusion of big ET-1 resulted in an increase in systemic IR big ET-1 by two orders of magnitude above basal levels in the placebo group. SLV306 dose dependently caused a significant rise in circulating big ET-1 levels (compared with placebo) indicating that at the two highest doses, SLV306 was inhibiting an increasing proportion of endogenous conversion activity (Figure 1). In the placebo group, levels of the CTF increased an order of magnitude above basal confirming a

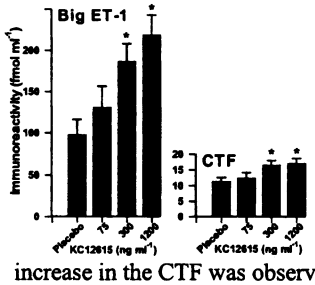

Figure 1. Dose dependent increase in plasma big ET-1 and CTF (*p<0.005,ANOVA. n=13, mean± s.e.mean).

proportion of the infused big ET-1 was being selectively converted as expected. In the presence of the highest two doses of SLV306, a small increase in the CTF was observed, consistent with our previous studies with the dual NEP/ECE inhibitor, phosphoramidon since CTF is also a substrate for metabolism by NEP and inhibition of this enzyme by SLV306 may reduce proteolysis. NEP is also thought to metabolise ET-1 to biologically inactive fragments. Intriguingly, despite inhibition of NEP activity, there was no increase in levels of mature ET. These results suggest that in the presence of SLV306, the biologically active peptide may continue to be removed beneficially from the circulation by clearing receptors. Overall, the results clearly show that SLV306 can inhibit the conversion of big ET-1 and may be of benefit in cardiovascular disease where big ET-1 levels are elevated, particularly in human atherosclerosis where enzymatic conversion of big ET-1 is significantly upregulated (Maguire & Davenport, 1998).

Gray, G.A., Battistini, B., & Webb, D.J. (2000) *Trends Pharmacol. Sci.* **21**, 38-40

Maguire, J.J. & Davenport, A. P. (1998) *Br. J. Pharmacol.* **125**, 238-240.

Plumpton, C., Haynes, W.G., Webb, D. J. & Davenport, A. P. (1995) *Br. J. Pharmacol.* **116**, 1821-1828.

63P RADIOSYNTHESIS AND *IN VIVO* BIODISTRIBUTION OF [^{18}F]-BIG ET-1, A POTENTIAL PET RADIOLIGAND FOR THE STUDY OF ENZYME CONVERSION OF BIG ET-1 TO ET-1

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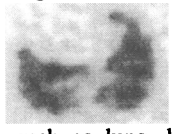
Molecular imaging with positron emission tomography (PET) is a sensitive and informative means to identify and study biological processes in normal and diseased tissue *in vivo*. The very potent vasoconstrictor endothelin-1 (ET-1) plays an important role in maintaining vascular tone by action on its two receptors ET_A and ET_B. Alteration in ET function has been suggested to play a role in a number of human vascular diseases. ET-1 is produced in endothelial cells from its precursor peptide Big ET-1 by the action of endothelin-converting enzymes (ECE). ECE's are also present on smooth muscle cells in the vasculature. We have previously shown that endothelin receptors can be imaged *in vivo* using PET and [^{18}F]-ET-1 (Johnström *et al.*, 2002). The aim of this work was to label the precursor peptide Big ET-1 with ^{18}F as a potential tool to follow enzyme conversion and subsequent binding to ET receptors by the formed [^{18}F]-ET-1 *in vivo*.

Big ET-1 was labelled according to the method previously described (Johnström *et al.*, 2002) using the Bolton-Hunter type reagent *N*-succinimidyl 4-[^{18}F]fluorobenzoate. Radiochemical yields were 12-18%.

[^{18}F]-Big ET-1 kinetics and distribution was studied using PET (microPET, Concord Microsystems). [^{18}F]-Big ET-1 was injected intravenously into anaesthetised Sprague-Dawley rats (isofluorane). From the acquired PET data images were reconstructed showing the distribution of [^{18}F]-Big ET-1. Regions-of-interest for various organs were drawn in the PET images and time-radioactivity curves were constructed. At end of acquisition the animal was killed and organs were removed for autoradiographic analysis of tissue sections (30μm) using storage phosphor imaging.

Figure 1. PET image showing localisation of radioactivity to vasculature in lung (transverse section).



High uptake of radioactivity was found in the bladder. Accumulation was also found in organs with high density of ET receptors such as lung, liver and kidney. In kidney the distribution kinetic showed accumulation to the glomeruli region at early time points which at later time points was redistributed to the papilla/pelvis region. A subsequent increase in radioactivity in the bladder was observed. In lung an initial decrease of radioactivity was observed, most likely reflecting blood flow, which then levelled out with a terminal $t_{½}$ of 7.4 h.

Ex vivo analysis of tissue sections of lung and liver using phosphor imaging showed localisation of radioactivity to the vasculature most likely reflecting local conversion of [^{18}F]-Big ET-1 and subsequent binding to ET_A receptors.

In conclusion, we have labelled Big ET-1 with ^{18}F and initial *in vivo* experiments indicate that [^{18}F]-Big ET-1 can be used for studying ECE activity with PET.

Johnström P., Harris N.G., Fryer T.D. *et al.* (2002). *Clinical Science.* **103** (Suppl. 48), 4S-8S.

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64P INOTROPIC ACTIONS OF ENDOTHELIN AND PHENYLEPHRINE ARE SELECTIVELY DEPRESSED IN RIGHT, BUT NOT LEFT, VENTRICULAR MYOCYTES FROM RATS WITH HYPOXIA-INDUCED RIGHT VENTRICULAR HYPERTROPHY

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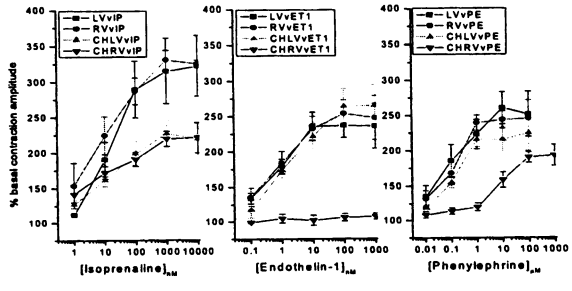
AIMS. Chronic hypoxia (CH) increases plasma noradrenaline and leads to down-regulation of cardiac β-adrenoceptors (Mardon *et al.*, 1998). Plasma levels of endothelin-1 (ET) are also increased in CH (DiCarlo *et al.*, 1995). Therefore we were interested to see if a down-regulation of ET-induced responses occurred in the right and left sides of the CH heart.

METHODS. Male Wistar rats were subjected to ± 3 weeks normobaric CH (10% O_2). Animals were anaesthetised with Na pentobarbitone (100mg.kg^{-1} i.p.) and cardiac myocytes isolated from the right (RV) and left (LV) ventricle (Yew *et al.*, 1998). Myocytes were plated onto laminin, paced (1Hz), and superfused (25°C) with Krebs-Henseleit solution (2ml.min^{-1}). Cell shortening was assessed using video-edge detection. The inotropic actions of cumulative additions of ET, phenylephrine (PE), and isoprenaline (IP) were assessed. Differences between groups (n≥5 per group) were assessed using an unpaired Student t Test.

RESULTS. In control myocytes all three agents produced increases in contractility (Figure 1). For each agonist there was no significant difference ($p>0.05$) between RV and LV. In CH myocytes there was a bilateral decrease in the maximal positive inotropic effect of IP when compared with its effect in control cells ($p<0.05$). In contrast, the inotropic response to the PE was shifted to the right in CHRV myocytes ($p<0.05$) but not in CHLV myocytes ($p>0.05$). When ET was examined in RV CH myocytes its inotropic action was completely inhibited ($p<0.05$). In contrast, in LV cells from CH hearts the inotropic action of ET was not significantly different from control responses seen in normoxic cells ($p>0.05$).

Figure1. Effects of IP, ET, and PE on cell shortening in RV and LV cardiac myocytes from control and CH rat hearts.

Means ± s.e.m n≥5/group.



SUMMARY. This shows that CH causes a selective downregulation of PE- and ET-induced cell shortening in RV myocytes. This suggests that local changes are responsible for these effects rather than elevated circulating levels of catecholamines and ET. This contrast to the bilateral desenstisation seen with β-adrenergic responses elicited by IP.

REFERENCES

DiCarlo,VS, Chen,S-J, Meng,QC., *et al* (1995) *Am.J.Physiol.* 269: L690-L697.
Mardon K., Merlet,P., Syrota,A., *et al* (1998) *J.Appl.Physiol,* 85:890-897.
Yew,S-F., Reeves,KR., Woodward,B. (1998) *Cardiovasc.Res.* 40:538-545.

65P SEASONAL VARIATION OF MYOCARDIAL ENDOTHELIN RECEPTOR mRNA EXPRESSION IN RATS

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Both endothelin ET_A and ET_B receptors are found in the myocardium although their role, if any, in regulation of cardiac contractility is not clear. Recent functional studies in our laboratory have indicated variation in the effects of endothelin receptor antagonists on contraction of papillary muscle when investigated at different time points in the year. The aim of the present study was to investigate seasonal differences of endothelin system expression in different regions of the heart using semi-quantitative RT-PCR.

Hearts were removed from male Wistar rats (age range 15-17 weeks in both studies, weight range 457 ± 23 g in study 1, 507 ± 23 g in study 2.). The right ventricular papillary muscle was carefully removed for functional studies. The remaining heart was then split into the left ventricle (LV), septum and right ventricle (RV) and these segments were rapidly frozen and stored at -70°C. Total RNA was extracted from frozen samples using the guanidinum thiocyanate-phenol-chloroform method. Reverse transcription (RT) was then carried out using 2µg of RNA in a standard protocol. Amplification of cDNA was carried out using specific primers designed for pre-pro ET-1; ET_A receptor, and ET_B receptor. PCR products were electrophoresed on a 1% agarose gel, visualised by ethidium bromide staining and quantified by densitometry. ppET-1, ET_A and ET_B receptors mRNA expression was calculated as a ratio of GAPDH mRNA expression in the same tissue samples as previously described (Serneri *et al.*, 2000).

Figure 1 shows that ET receptor expression levels were increased during study period 2 (July/ August) relative to study period 1 (Jan/ Feb) in right ventricular tissue. In contrast, expression levels of the ET-1 precursor pre pro ET-1 were unchanged over the same period.

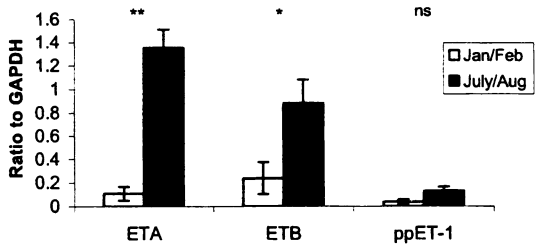

Figure 1. ET system mRNA expression in right ventricle. Means ± s.e.m, n=5, *P<0.05, **P<0.01, Student's unpaired t-test.

Similar patterns of expression were seen in LV and septal tissue, although there was evidence for minor elevation of pre-pro ET-1 expression in the septum during study period 2. Changes in RNA expression can be explained by variation in RNA stability and do not necessarily reflect protein synthesis. However, together with the functional data from our previous studies, these data support the hypothesis that there is regulation of ET system expression by seasonal factors in the rat.

Reference: Serneri GG, Cecioni I, Paniccia R *et al.* (2000) *Circ. Res.*, 86, 377-385

AFW is supported by the British Heart Foundation FS/99037

66P SERUM CYTOKINE LEVELS IN MALE PATIENTS WITH STABLE CORONARY ARTERY DISEASE

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Coronary artery disease is an inflammatory condition, with numerous cytokines being proposed to be involved in the initiation of plaque development, the progression of the lesion, and the rupture of the mature atherosclerotic plaque (Ross, 1999). The aim of this study was to measure the serum levels of the pro-inflammatory cytokines tumour necrosis factor α, interleukin-1β and interleukin-6, and the anti-inflammatory cytokine interleukin-10 in a cohort of 78 men with proven coronary artery disease.

Early morning fasting blood samples were taken from male patients (n=78, mean age = 59.7 years) who had previously undergone coronary angiography and had a diagnosis of coronary artery disease with >75% occlusion of at least one major coronary artery. Patients were then divided into groups according to the number of coronary arteries occluded; either 1,2 or 3 vessel coronary artery disease. After collection the blood was centrifuged at 2000rpm for 5min, the serum was removed and frozen at minus 80°C until cytokine analysis was undertaken. Serum levels of the pro-inflammatory cytokines tumour necrosis factor-α, interleukin-1β and interleukin-6 and the anti-inflammatory cytokine interleukin-10 were subsequently measured via high sensitivity solid phase sandwich ELISA (R&D Systems, UK). Results are expressed as mean (SEM), and analysed via Student's unpaired *t* test.

A significant step-wise increase in serum levels of interleukin-1β and interleukin-10 were seen in patients with either one, two or three vessel coronary artery disease:
Interleukin-1β; 0.18 (0.04), 0.28 (0.08) and 0.45 (0.08) pg/ml respectively (P=0.03, ANOVA).
Interleukin-10; 0.88 (0.11), 1.32 (0.14) and 2.94 (0.58) pg/ml respectively (P=0.004, ANOVA).
No differences were seen in serum levels of tumour necrosis factor α or interleukin-6.

This study supports the involvement of interleukin-1β in the development of coronary artery disease and suggests that interleukin-10 may have a protective role, dampening the effects of this pro-inflammatory response.

Ross R (1999) *New England Journal of Med.* **340**, 115-126.

67P 5-HYDROXYTRYPTAMINE INCREASES CALCIUM CURRENT BUT NOT REFRACTORINESS IN HUMAN SINGLE ATRIAL MYOCYTES

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5-hydroxytryptamine (5-HT) has a positive inotropic and chronotropic effect in human atrium associated with an increase in the L-type Ca^{2+} current (I_{CaL}) (Ouadid et al., 1992). However, its effects on functional electrophysiological properties including refractoriness have not been studied. The aim of this study was to characterise and compare the actions of 5-HT on I_{CaL}, action potentials and the effective refractory period (ERP) in human atrial isolated myocytes.

The whole cell patch clamp technique was used in single myocytes isolated enzymatically from the right atrial appendage of patients undergoing cardiac surgery. All patients were in sinus rhythm. The perforated-patch clamp technique (Nystatin, 184 μM) was used to minimise current "run-down". Cells were continuously superfused with a physiological salt solution at 37±1 °C. Electrodes were filled with a caesium-based solution to eliminate outward potassium currents during I_{CaL} recordings, and a potassium-based solution was used for action potential recordings. I_{CaL} voltage-dependent activation was measured from a holding potential of –40 mV, with voltage pulses of 250 ms duration, increasing in amplitude from –30 mV to +50 mV in steps of 10 mV. Actions potentials were stimulated at 75 beats per min with current pulses of 5 ms duration and 1.2x threshold strength, after current clamping resting cells at -75 to -80 mV and keeping the holding current constant thereafter. The ERP was measured using a standard S_1-S_2 stimulation protocol, and was defined as the longest S_1-S_2 interval which failed to elicit an S_2 action potential of amplitude >80% of the preceding S_1 action potential. Data are expressed as mean ± standard error of the mean (SEM). Values were compared using a two-tailed paired Student's *t* test, with $P<0.05$ taken as statistically significant.

5-HT (0.001-100 μM, *n*=7-24 cells, 4-12 patients) elicited a concentration-dependent increase in the amplitude of I_{CaL} with a calculated EC_{50} of 35±2 nM and a Hill coefficient of 1.6±0.8. The maximum effect was observed with 1 μM 5-HT, which produced a 210% increase in peak I_{CaL} from –3.1±0.5 to –9.6±1.8 pA/pF at +10 mV (*n*=11 cells, 6 patients; $P<0.05$), without any change in the voltage dependency of the current. This effect of 5-HT was reversible on its washout (-2.6 ± 0.8 pA/pF). With respect to action potential characteristics, 5-HT at 10 μM (*n*=12 cells, 9 patients) caused an increase in the action potential duration measured at 50% repolarisation (APD_{50}) from 15.0±7.0 ms to 33.1±12.4 ms ($P<0.05$), that was fully reversible after 3 min washout of 5-HT (11.8±3.7 ms). However, the APD_{75} (137±18 vs 142±22 ms), APD_{90} (222±23 vs 209±25 ms) and the ERP (251±27 vs 242±25 ms; *n*=9 cells, 6 patients) were unaffected by superfusion with 10 μM 5-HT.

In conclusion, 5-HT increased the magnitude of the L-type Ca^{2+} current in human atrial myocytes and this was associated with a significant prolongation of the plateau phase (APD_{50}) of the action potential, but with no change in late repolarisation or refractoriness.

Ouadid, H., Seguin, J., Dumuis, A. *et al.* (1992) *Mol. Pharmacol.* 41, 346-351.

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68P A COMPARISON OF TWO RABBIT MODELS OF DRUG INDUCED POLYMORPHIC VENTRICULAR TACHYCARDIA

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Torsade de pointes (TdP) is a rare form of polymorphic ventricular tachycardia (PVT) that exhibits a characteristic twisting of QRS complexes around the isoelectric axis. The mechanism of onset is unknown but class III antiarrhythmic drugs such as sotalol that prolong QT intervals and induce bradycardia are associated with TdP. Aim of this study is to compare the effects of two rabbit models for the assessment of pro-arrhythmic potential.

Male New Zealand White rabbits (2.0 – 3.0 kg) were purchased from Statens Serum Institut (SSI) Denmark. For the in vivo studies general anaesthesia was induced by i.v. administration of sodium pentobarbital followed by α-chloralose, via a marginal ear vein. The trachea was cannulated, and the animal ventilated with room air. A vascular catheter was implanted in the left carotid artery for blood pressure and heart rate monitoring. Needle electrodes were placed subcutaneously to record the standard bipolar lead II; the negative electrode was placed in front of the right shoulder, the positive electrode close to the left loin for measuring QTc. Rabbits were bolus dosed with increasing doses of sotalol (0.16, 0.32, 0.64, 1.25, 2.5, 5.0, and 10.0 mg/kg) (n=5) or saline vehicle (n=5).

For the in vitro studies the rabbits were anaesthetised with pentobarbital and the hearts were rapidly removed and retrogradely perfused via the aorta at a constant flow of 50 ml/min with Krebs-Henseleit solution. The atrioventricular node (AV) was ablated mechanically slowing the heart rate (HR). Initially the hearts were electrically stimulated at a cycle length (CL) of 300 ms. Following an equilibration period sotalol (3 μM) was administered via the perfusate for 30 min. Following 30 min infusion the effects of CL variation (between 300 and 1300 ms) was measured on QT (in vitro) and compared to a vehicle. All data is presented as mean ± S.E.M.

In the rabbits sotalol significantly increased QTc and decreased heart rate (HR) at doses of 0.64 ($p<0.03$) and 1.25 mg/kg ($p<0.02$), respectively (Student t test). At 10 mg/kg QTc was maximally increased to 282.8 ± 7.5 ms (n=5) compared to the vehicle (166.9 ± 3.8, n=5, $p<0.001$ (Student t-test)). At 10 mg/kg the corresponding HR was 154.3 ± 6.0 (n=5) and 244.6 ± 5.3 (n=5) in the vehicle group, $p<0.001$ (Student t-test). Ventricular premature beats occurred in 3 out of 5 rabbits at doses ranging form 2.5 to 10.0 mg/kg. PVT occurred in one rabbit at the highest dose tested.

In the isolated AV ablated hearts sotalol caused PVT in 2 of the 4 hearts when changing from 500 ms CL. The last measurable QT intervals when switching CL prior to PVT were; 239 to 234 and 253 to 271 ms. The time matched vehicle QT interval was 246.8 ± 4.9 (n=4) at a CL of 500 ms.

In conclusion, sotalol induced PVT in both rabbit models. In the in vivo rabbit this was associated with an increase in ventricular repolarisation time and bradycardia. Likewise in the isolated ablated Langendorff heart PVT was induced when shifting to a lower frequency of ventricular pacing.

69P THE POSITIVE INOTROPIC ACTION OF PHENYLEPHRINE IN RAT ISOLATED CARDIAC MYOCYTES, PLATED ON LAMININ, IS CONVERTED TO A NEGATIVE EFFECT WHEN CELLS ARE PLATED ON FIBRONECTIN

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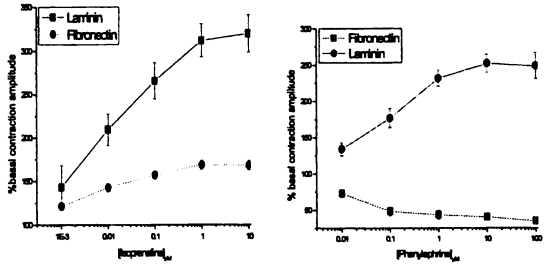
AIMS. Cardiac hypertrophy is associated with changes in extracellular matrix (ECM) protein deposition (Boluyt, *et al.* 1994), which compromises contractility. As cardiac myocytes interact, via integrins, with the ECM and these interactions trigger intracellular signalling, we thought this might affect G-protein-coupled receptor activity. Therefore, in this study we have compared the actions of isoprenaline (IP), and phenylephrine (PE) on cell shortening in rat isolated cardiac myocytes plated on laminin (LN) and fibronectin (FN).

METHODS. Male Wister rats were anaesthetised (Na pentobarbitone 100mg.kg^{-1} i.p.). Cardiac myocytes were isolated from right (RV) and left (LV) ventricles (Yew *et al* 1998). Cells were plated on LN or FN coated coverslips (0.15μg.ml^{-1}), superfused (25°C, 2ml.min^{-1}) with Krebs-Henseleit solution and paced (1Hz). Cell shortening was monitored using a video edge detector. Drugs were added cumulatively. Data represents mean ±s.e.m. of the % control cell shortening in the absence of drug. Statistical differences ($p<0.05$) were determined using an unpaired Student t Test. In all experiments $n\geq5$.

RESULTS. Responses of RV and LV myocytes to PE and IP were not significantly different ($p>0.05$). Therefore pooled data for RV and LV cells is presented. When cells were plated on LN, IP and PE both caused concentration-dependent positive inotropic actions (Figure 1). The maximal concentration of IP and PE increased cell shortening by 331± 16 % and 245±12% respectively. When cells were plated on FN, the maximal effect of IP increased cell shortening by 170±6%. This was significantly reduced when compared with LN plated cells ($p<0.05$). There was no significant difference in the threshold concentration of IP. In contrast to IP, the positive inotropic action of PE was completely inhibited when cells were plated on FN ($p<0.05$), and converted to a small negative inotropic response (Figure 1).

Figure 1.Inotropic effects of IP and PE on cardiac myocytes plated on laminin or fibronection. $n\geq5$/group.



SUMMARY. When compared with LN, FN has the ability to suppress IP-induced cardiac contractility and it reverses PE-induced inotropy. The precise cause of this change in response to α-adrenoreceptor stimulation is not known but it could have important implications in the setting of heart failure where the expression of FN is increased and β_1-adrenoceptors are downregulated.

Boluyt, MO., O'Neill, L., Meredith, AL. (1994). *Circ.Res.* 75:23-32.

Yew,S-F., Reeves,KR., Woodward,B. (1998) *Cardiovasc.Res.* 40:538-545

70P EFFECT OF ASPIRIN AND DEXAMETHASONE ON PROLIFERATION OF HUMAN AORTIC SMOOTH MUSCLE CELLS

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Intimal hyperplasia (IH) is a major contributor to restenosis associated with vascular injury and involves abnormal proliferation of medial smooth muscle cells in the intima of affected vessels. This study investigates the inhibitory affect of aspirin and dexamethasone on the *in vitro* proliferation of human aortic smooth muscle cells (HA-VSMC) to determine their potential to control IH *in vivo*.

HA-VSMC cells obtained from the American Tissue Culture Collection were seeded on 96-well plates at 10^4 cells/cm^2 in Ham's F12-K medium with 10% (v/v) foetal calf serum. After allowing the cells to adhere for 24h, aspirin (1-1000μM), dexamethasone (0.1-100μM), or a combination of both drugs were added. Medium and drug solutions were replenished daily, and after 3 or 7 days cell proliferation was measured both by the Neutral Red (NR) assay and the MTT assay.

The NR and the MTT assays gave equivalent results, and figure 1 shows the results obtained with the MTT assay after treating the cells for 7 days. Both drugs inhibited cell proliferation at high concentrations, and the extent of inhibition was similar after 3 and 7 days treatment in all cases. Their combined effect was significantly more potent in terms of inhibition. At concentrations above 1μM dexamethasone and 10μM aspirin the combination caused significantly greater inhibition than either drug alone.

On its own, at concentrations between 10 and 250μM aspirin consistently stimulated cell growth after 7 days exposure; this effect was not observed after 3 days treatment.

In conclusion, high local concentrations of aspirin and dexamethasone may inhibit development of IH, but low concentrations of aspirin may increase proliferation of the smooth muscle cells.

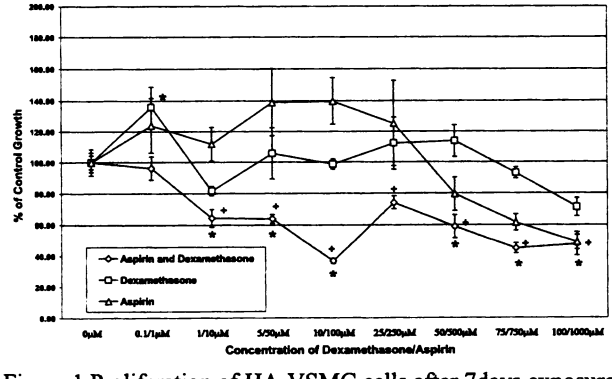

Figure 1 Proliferation of HA-VSMC cells after 7days exposure to aspirin and dexamethasone, alone and in combination. Growth was measured by the MTT assay, and the growth of control cells growing in the absence of drugs was taken as 100%. Results are mean +/- SEM, of 8 experiments. + $P<0.05$, comparing combination treatment with either of the single treatment groups; and * $P<0.05$, comparing each treatment with the control value. Comparisons were made by ANOVA followed by Dunnett's test.

71P ARE VASOCONSTRICTOR RESPONSES TO TYRAMINE IN HUMAN BLOOD VESSELS, IN VITRO, MEDIATED BY THE ORPHAN TRACE AMINE RECEPTOR, TA_1?

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In vertebrates, the trace amines such as tyramine and octopamine are thought to act as indirect sympathomimetics or false transmitters whilst a transmitter role has been established for these amines in invertebrates. Recently it has been shown in rat aorta that vasoactive responses to tyramine occur in the presence of antagonists of the known adrenoreceptors, suggesting activation of a novel tyramine receptor (Varma *et al.*, 1995). This has been confirmed by the discovery of an orphan G-protein coupled receptor that is selective for the trace amines, tyramine and β-phenylethylamine, and has been designated the TA_1 receptor (Borowsky *et al.*, 2001). mRNA for the TA_1 receptor has been localised to cardiovascular tissue (Bunzow *et al.*, 2001) and we have therefore investigated the vasoactive effects of tyramine on human blood vessels *in vitro*.

Human mammary artery (LIMA), saphenous (SV) and umbilical vein (UV) were obtained with local ethical approval. Endothelium-denuded rings (4mm) were set up in organ baths, with oxygenated Krebs' solution containing pargyline (100μM) and EDTA (40μM) (37°C) for isometric force recordings. Cumulative concentration-response curves were constructed to tyramine (10^{-8}-10^{-3}M) and the experiments terminated by addition of 100mM KCl to determine the maximum possible contractile response for each tissue. Tyramine responses were expressed as a percentage of this. Data were analysed using the iterative curve fitting program Fig P (Biosoft, Cambridge UK). Data are mean±s.e.mean, n- values are the number of patients from whom tissues were obtained.

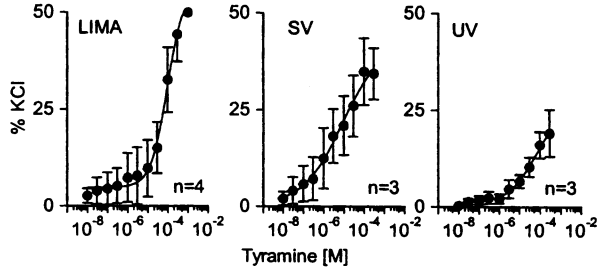

Fig. 1 Responses to tyramine in human blood vessels

Vasoconstrictor responses to tyramine were observed in LIMA (pD_2=4.81±0.6), SV (pD_2=5.29±0.5) and UV (curve incomplete at 10^{-3}M) (Fig. 1). Preliminary experiments in UV suggest that these responses persist in the presence of α- and β-adrenoreceptor blockade (data not shown). The lack of innervation in UV suggests that these responses, at least in this tissue, are independent of release of endogenous transmitters and may therefore be the result of direct activation of smooth muscle receptors. Further experiments are required to confirm whether the observed vasoconstriction to tyramine is mediated via activation of the novel trace amine TA_1 receptor.

Supported by grants from the British Heart Foundation

Borowsky, B., Adham , N., Jones, K.A. *et al.*, (2001). Proc. Natl. Acad. Sci., 98, 8966-8971.
Bunzow, J.R., Sonders, M.S., Arttamangkul, S. *et al.*, (2001). Mol. Pharmacol., 60, 1181-1188.
Varma, D.R., Deng, X.F., Chemtob, S., *et al.*, (1995). Can. J. Physiol. Pharmacol., 73, 742-746.

72P ANALYSIS OF α_2 ADRENOCEPTOR AND ANGIOTENSIN MEDIATED RESPONSES IN MOUSE AORTA

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α_2 adrenoceptor (AR) and angiotensin (Ang II) mediated contractile responses in the mouse aorta were studied with the original intention of studying their contractile synergy as exemplified in rabbit saphenous artery (Dunn *et al* 1991). In other species both agents are known to act via multiple subtypes of receptors directly on smooth muscle and indirectly via endothelium. The pharmacological interactions are very complicated so we sought simplification using mice with KOs of α-adrenoceptors: controls C57Bl; D79N (functional $\alpha_{2A/D}$ knockout) and α_{1d}-KO mice. We started with the aorta as the simplest preparation of a mouse blood vessel.

Aortas were cut into 2mm rings, mounted on a wire myograph (Mulvany *et al* 1976) and used to acquire cumulative concentration response curves to UK14304 or angiotensin, alone, in combination and with or without prior tone to 5HT (10^{-7}M). Endothelial involvement was tested with acetylcholine and challenged with L-NAME (10^{-4}M) or physical removal.

UK14304 acted as a partial agonist at α_{1d}-AR, causing weak contraction that was absent in the α_{1d}-KO and antagonising α_{1d}-mediated contraction to phenylephrine. In the presence of tone UK produced relaxation, abolished by L-NAME or removal of the endothelium. Thus UK14304 contracts smooth muscle directly via α_{1d}-AR and releases endothelial NO to relax smooth muscle. In the D79N mouse, the relaxant effect of UK14304 was absent and its contractile response was present. Thus the endothelium-mediated relaxant effect of UK14304 is mediated by $\alpha_{2A/D}$-AR -receptors and the direct contractile response is not $\alpha_{2A/D}$-AR but is at least partially α_{1d} AR-mediated.

Ang II had no effect on UK14304-mediated relaxation. Preincubation with Ang II (3 x 10^{-8}M) significantly reduced the maximum obtained to cumulative addition of NA ($p<0.0002$). Ang II had a dual effect in the presence of elevated tone (5HT), initially causing contraction, followed by a slow relaxant effect. These actions were partly attenuated by Losartan or PD123319 (AT_1 and AT_2 antagonist respectively). Thus Angiotensin II, like UK14304 also produced direct contraction and endothelium-mediated relaxation, both actions being partially attenuated by both AT1 and AT2 blockers.

This indicates overall that the mouse aorta has a multiple population of α-adrenoceptor and angiotensin II receptors capable of initiating contraction or relaxation and thus it can be used to analyse mechanisms involved with such receptors. However there was little sign of synergism between the two systems under the conditions employed. In particular the AT_2 receptor and $\alpha 2_{A/D}$ AR are working independently of each other. As in other species it seems that synergistic interaction should be sought in resistance and distributing arteries or in veins.

Mulvany M.J *et al.*, *Nature*, 1976 260: 659-665
Dunn W. R. *et al.*, *Br.J.Pharmacol* 1991 102: 10-12

MMS is funded by the Iranian Islamic Republic Embassy, London. This lab. is a member of EC project QLG1-CT-1999-00084.

73P VASOACTIVE INTESTINAL POLYPEPTIDE RECEPTOR SUBTYPES INVOLVED IN VASODILATION OF PORCINE BASILAR ARTERY

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Vasoactive Intestinal Polypeptide (VIP), a 28 amino acid peptide was originally isolated from porcine duodenum and belongs to the glucagon-secretin peptide family. VIP binds with high affinity to $VPAC_1$ and $VPAC_2$ receptors (Kd=1nM), which are coupled to adenylate cyclase via Gs (Harmar *et al.*,1998). VIP can also bind to natriuretic peptide clearance receptor (NPR-C), which is coupled to G_{i-1} and G_{i-2} and causes Ca^{2+} influx with subsequent NOS activation. VIP is a major cerebrovasodilator neurotransmitter present in cerebral intramural nerves, however the receptors through which it acts in cerebral arteries are unknown and were therefore investigated.

Pig heads were obtained from a local slaughterhouse and the basilar artery excised out and connective tissue removed. The basilar arteries were analysed by RT-PCR for expression of VIP receptors. Specific primers were designed based on homologous human, mouse and rat $VPAC_1$, $VPAC_2$ and NPR-C sequences. Reversibly transcribed cDNA was amplified by PCR under standard conditions and the products separated by electrophoresis in 0.7% agarose gel in the presence of ethidium bromide and visualised by UV fluorescence. The PCR products were purified with the use of a QIAquick Gel Extraction kit (Qiagen) and then sequenced. Using a wire myograph functional responses of the artery were also studied. 2mm rings of artery were mounted on a wire myograph in Krebs' solution bubbled with 95%O_2, 5%CO_2 and 1.5g pre-tension applied. 0.1μM U-46619 was applied to attain a stable sub-maximal contraction before addition of VIP.

$VPAC_1$, $VPAC_2$ and NPR-C receptors were found to be expressed in the pig basilar artery, giving the expected PCR products of 550, 580 and 410 bases pairs respectively. In the functional studies, concentration-dependent relaxations were obtained to VIP (n=8, $EC_{50}=2.4\times10^{-8}$M), with complete relaxation at 3×10^{-7}M. Ro-25-1553 (n=7, $EC_{50}=2.2\times10^{-9}$M), a selective $VPAC_2$ agonist, also caused relaxation and was more than 10x more potent than VIP. Pre-incubation with the NO synthase inhibitor, L-NAME (100 μM) significantly reduced VIP-induced relaxation ($P<0.05$, n=6) at all concentrations, but had no effect on Ro-25-1553 (n=7).

This work has shown, for the first time, $VPAC_1$, $VPAC_2$ and NPR-C receptors are all expressed in porcine basilar arteries. The functional data suggests that $VPAC_2$ receptors are involved in VIP-mediated cerebral vasodilation through a pathway independent of NO. However, other VIP receptors present in these arteries appear to mediate a NO-dependent component of the vasodilation. This is relevant to states of altered cerebral blood flow, such as stroke and cerebral vasospasm, which are associated with reduced NO bioavalability.

Harmar *et al.*, Pharmacol Rev 50:265-269,1998.
S. Grant is funded by a BHF studentship.

74P QUANTIFICATION OF LUMEN OCCLUSION DUE TO NEOINTIMAL CELL GROWTH IN CULTURED THORACIC AORTAE FROM TESTICULAR FEMINIZED AND CONTROL MICE

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Current research indicates that low levels of testosterone are associated with the development of coronary artery disease (CAD) in men. Testosterone replacement therapy improves myocardial ischaemia in men with CAD (English *et al.*, 2000) and reduces atherosclerosis in experimental animal models (Alexandersen *et al.*, 1999). Finking *et al.* (1999) describe an in vitro model of atheroma formation. Following endothelial denudation and tissue culture, vessels develop neointimal growth arising from the proliferation of the smooth muscle cells in the vessel wall. The testicular feminised (Tfm) mouse exhibits a mutation in the gene encoding the androgen receptor, resulting in the expression of a truncated, non-functional receptor protein. Previous work by our group demonstrated that Tfm males have reduced levels of testosterone compared to littermate controls. The aim of this study was to determine whether this reduced circulating testosterone profile and androgen receptor deficiency are associated with alterations in atheroma formation in vitro.

Tfm mice (n=22) and XY littermate controls (n=20) were killed by an approved schedule 1 method at 10 weeks of age. Thoracic aortae were dissected, then threaded onto a sterile needle and gently rubbed to denude the endothelium. The aortae were then cultured in six well plates in phenol red-free Dulbecco's modified Eagle medium with Ham's F12 containing 4.5g/L D-glucose, 15% foetal calf serum, 5ml penicillin streptomycin, 2.5ml fungizone, 1% isopropanol and 1% dimethyl sulfoxide at 37°C. After 28 days in culture, the aortae were immersion fixed in 10% formalin solution, embedded in paraffin wax, and 8x5μm sections taken at 1mm intervals along each vessel. Each section was then stained with haematoxylin and eosin, and the % occlusion measured via digital image analysis. The mean value of the 8 sections per vessel was calculated and used for statistical evaluation.

All vessels developed significant neointimal growth. In thoracic aortae (n=20) harvested from control mice this constituted 16.4 ± 2.9 % occlusion. In thoracic aortae (n=22) harvested from Tfm mice this constituted 11.8 ± 2.1 % occlusion. The amount of neointimal growth was statistically similar in Tfm and control mice ($P=0.2$, Student's unpaired *t* test).

The reduced circulating testosterone profile and androgen receptor deficiency has no detrimental influence upon atheroma formation in vitro.

Alexandersen P., Haarvo J., Byrjalsen I. *et al.*, (1999) *Circulation Research* **84**, 813-819.

English K.M., Steeds R.P., Jones T.H. *et al.*, (2000) *Circulation* **102**, 1906-1911.

Finking G., Wohlfrom M., Lenz C. *et al.*, (1999) *Coronary Artery Disease* **10**, 607-615.

75P CHRONIC NIFEDIPINE TREATMENT DECREASES THE FUNCTIONALITY OF α_{1D}-ADRENOCEPTORS IN SPONTANEOUSLY HYPERTENSIVE RATS

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The constitutively active population of α_{1D} adrenoceptors seems to play a modulatory role in the contractile response of the vessels to an adrenergic stimulation (Ziani et al, 2002). An imbalance in this modulating mechanism could give rise to pathologies such as hypertension (Villalobos-Molina et al.,1999). An increase in the functionality of the constitutively active population of α_{1D}-adrenoceptors has been evidenced in adult spontaneously hypertensive rats (SHR 16 weeks old) respect to normotensive Wistar Kyoto (WKY) animals and this increase is avoided by captopril treatment (Gisbert et al, 2002). We have analyzed the functional role of α_{1D}-adrenoceptor in arteries from adults rats (SHR and WKY) that have been treated with nifedipine.

Methods: Male normotensive (WKY) and spontaneously hypertensive (SHR) rats (6 weeks old) received nifedipine 50mg/kg per day in drinking water until the age of 16 weeks. The experimental procedures are the same as those described by Gisbert et al, 2002.

Results: Values of systolic blood pressure (SBP) and heart rate determined 24h before the animals were sacrificed are summarized in table 1. The relaxant potency (pIC50) of BMY 7378 (a selective α_{1D}-AR antagonist) against noradrenaline induced contraction obtained in each artery is interpreted as an indicator of the functionality of the α_{1D} subtype in that artery and it is summarized in table 2.The increase in the functionality of the constitutively active population of α_{1D}-adrenoceptors observed in SHR animals was normalized after nifedipine treatment.

TABLE 1

	Adult rats		Pretreated adult rats	
	WKY	SHR	WKY	SHR
SBP,mmHg	124 ±8	176± 4***	121± 4	119± 5
Heart rate,beats/min	290± 14	329 ±6*	214± 53	214 ±41

TABLE 2

	Adult rats		Pretreated adult rats	
	WKY	SHR	WKY	SHR
Aorta	8.17±0.16	8.86±0.21*	7.03±0.11***	6.97±0.13***
Iliac	7.37±0.15	7.75±0.22	6.12±0.08***	6.29±0.11***
Mesenteric	7.27±0.10	8.07±0.12***	6.59±0.20**	6.24±0.09***
SMA-1	5.67±0.12	6.56±0.26**	5.54±0.09	5.75±0.12
SMA-2	5.49±0.15	6.12±0.11**	5.67±0.12	5.6±0.13
Tail	6.33±0.20	6.69±0.12	6.26±0.22	6.07±0.16

SMA-1 and SMA-2 are first and second small mesenteric branches respectively. Values are expressed as the mean±s.e.m of n=4-9 animals. *P<0.05, **P<0.01, ***P<0.001 *vs* untreated WKY rats.

Conclusion: In nifedipine treated adult rats, where the hypertensive state has been avoided, no differences between the WKY and SHR strains with respect to BMY7378 potency were observed. These results show that the increase in the functional role of α_{1D}-adrenoceptors and their constitutive activity that were found in untreated SHR rats were prevented by nifedipine treatment.

Gisbert, R. et al. *Br .J. Pharmacol.*, **135**, 206-216(2002) Villalobos-Molina, R. et al *Br. J. Pharmacol.*, **126**, 1534-1536(1999). Ziani, K. et al. *Am. J. Physiol. Heart. Circ. Physiol,* **282**, H475-81 (2002)

76P REGIONAL HAEMODYNAMIC EFFECTS OF THE 5-HT AND NORADRENALINE RE-UPTAKE INHIBITOR, SIBUTRAMINE, IN CONSCIOUS RATS: EFFECTS OF α-OR β-ADRENOCEPTOR ANTAGONISM

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We have assessed the haemodynamic effects of sibutramine (Luscombe *et al.*, 1989) in the absence and presence of α- or β- adrenoceptor antagonism.

Under anaesthesia (fentanyl and medetomidine, 300 μg kg^{-1} of each i.p.), male Sprague-Dawley rats (350 – 450 g) had pulsed Doppler flow probes implanted to monitor coeliac (C), mesenteric (M), and hindquarters (H) vascular compliance (VC), and, two weeks later, i.a., i.v. and i.p. catheters implanted for measurement of mean arterial blood pressure (MAP) and heart rate (HR), and administration of drugs. Experiments began on the day after catheterisation. Rats were randomised to receive saline (0.1 ml, i.v. bolus, 0.4 ml h^{-1}, infusion; n=8), or phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg kg^{-1} h^{-1}, infusion; n=9), or propranolol (1 mg kg^{-1}, i.v. bolus, 0.5 mg kg^{-1} h^{-1}, infusion; n=8) starting 30 min prior to bolus injection of sibutramine (9 mg kg^{-1} i.p.).

Tables 1 and 2 summarise some of the results. Phentolamine caused an increase in HR and HVC, with a fall in MAP, whereas propranolol caused bradycardia associated with a small increase in MAP and a fall in MVC (Table 1). In the presence of saline, sibutramine caused rises in HR, MAP and HVC accompanied by falls in CVC and MVC. Phentolamine blocked the pressor and vasoconstrictor actions of sibutramine and unmasked vasodilatations. Propranolol blocked the sibutramine-induced increase in HR and HVC (Table 2).

Table 1. Cardiovascular variables before and 30 min after administration of saline, phentolamine (Phent) or propranolol (Prop). Values are mean ± s.e.mean. Units are beats min^{-1} (HR), mmHg (MAP) and (kHz $mmHg^{-1}$) 10^3 (VC). * $P \leq 0.05$ vs before (Friedman's test)

	HR	MAP	CVC	MVC	HVC
Before	344±9	104±1	111±10	94±6	39±4
Saline	338±11	102±2	109±9	93±7	42±4
Before	351±8	106±1	110±10	92±6	37±2
Phent	477±16*	77±5*	116±11	80±6	115±10*
Before	342±7	104±3	101±13	99±6	46±6
Prop	329±7*	110±4*	90±9	90±5*	44±4

Table 2 Integrated (0-10 min) cardiovascular responses to sibutramine in the presence of saline (Sal), phentolamine (Phent) or propranolol (Prop). Values are mean ± s.e.mean. Units are beats (HR), mmHg min (MAP) and % min (VC). * $P \leq 0.05$ vs saline (Kruskal-Wallis test).

	HR	MAP	CVC	MVC	HVC
Sal	+390±108	+150±11	-231±38	-231±27	+281±43
Phent	+197±78*	-87±7*	+140±40*	+525±77*	+148±89
Prop	-155±52*	+152±28	-232±37	-213±43	-92±41*

This study demonstrates an important role for adrenoceptor activation in the haemodynamic responses to sibutramine. The mediator(s) of the C and M vasodilator effects of sibutramine in the presence of phentolamine is (are) unknown.

Luscombe, G. *et al.* (1989). *Neuropharmacology*, 28, 129-134

77P EFFECTS OF DIET ON THE REGIONAL HAEMODYNAMIC RESPONSES TO SIBUTRAMINE IN CONSCIOUS RATS

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The effects sibutramine, a novel anti-obesity drug, are likely attributable to its actions as a 5-HT and noradrenaline reuptake inhibitor (Luscombe *et al.*, 1989). Since adrenoceptors appear to be involved in the haemodynamic effects of sibutramine (Woolard *et al.*, this meeting), we hypothesised that feeding rats a palatable diet (Brown *et al.*, 2001), might influence sympathoadrenal mechanisms and so alter the haemodynamic responses to sibutramine.

Male Sprague-Dawley rats (175 – 200 g) were fed either a normal diet or a modified diet (33 % milk protein, 7 % sucrose; Brown *et al.*, 2001) for 4 weeks. Then, rats were anaesthetised (fentanyl and medetomidine, 300 μg kg^{-1} of each i.p.) and had pulsed Doppler flow probes implanted to monitor coeliac (C), mesenteric (M) and hindquarters (H) blood flows, and, at least 10 days later, i.a. and i.p. catheters implanted, to measure heart rate (HR) and mean arterial pressure (MAP) and for drug administration. Experiments were started 24 h after catheterisation, and were conducted over the subsequent 3 days. Rats received sibutramine (3 or 9 mg kg^{-1} i.p.) on Day 1, vehicle on Day 2, and the other dose of sibutramine on Day 3. Baseline values (mean ± s.e.mean) for the normal diet (body weight 492±18 g; n=8) and modified diet (body weight 461±11 g; n=9) groups, respectively were: HR (beats min^{-1}) 335±8, 322±9; MAP (mm Hg) 99±2, 104±2; vascular conductance (VC) ([kHz mm Hg^{-1}]10^3), CVC 96±8, 106±13; MVC 94±4, 107±16; HVC 48±2, 40±4.

Table 1 shows some responses to sibutramine. In both groups, sibutramine caused increases in HR, MAP and HVC accompanied by reductions in CVC and MVC. Responses to sibutramine were similar in the two groups, with the exception of the fall in MVC, which was greater in the group fed the modified diet.

Table 1: Integrated (0-10 min) cardiovascular changes (Δ) after sibutramine (9 mg kg^{-1}) in rats fed either a normal diet (n=8) or a modified diet (n=9). Values are mean ± s.e. mean $^*P<0.05$ for integrated response vs. normal diet (Mann Whitney U test)

	Normal diet	**Modified diet**
ΔHR (beats)	+640±123	+590±148
ΔMAP (mmHg min)	+126±18	+164±27
ΔCVC (% min)	-126±43	-160±50
ΔMVC (% min)	-189±24	-267±37*
ΔHVC (% min)	+341±55	+372±86

If the mesenteric vascoconstrictor response to sibutramine is due to α-adrenoceptor activation (see Woolard *et al.*, this meeting), then the results of this study indicate that, in rats fed a modified diet, there is a selective modulation of this process in the mesenteric vascular bed.

Brown, M. *et al.* (2001). *Br. J. Pharmacol.*, 132, 1898-1904
Luscombe, G. *et al.* (1989). *Neuropharmacology*, 28, 129-134

78P PRELIMINARY INVESTIGATIONS INTO THE PHARMACOKINETIC INTERACTION BETWEEN CYCLOPHOSPHAMIDE AND THALIDOMIDE IN THE MOUSE

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Concurrent administration of racemic thalidomide (TH) potentiated the antitumour effect of cyclophosphamide (CP) with little increase in toxicity in the murine Colon 38 tumour model. This was accompanied by a > 2-fold increase in the area under the plasma concentration-time curve for CP and 4-hydroxycyclophosphamide (4-OH-CP) (Ding *et al.*, 2002). CP is activated by CYP isozymes to 4-OH-CP, which decomposes to yield the cytotoxic mustard. Other CYP-mediated deactivation pathways also exist for CP (Clarke & Waxman, 1989). In contrast, spontaneous hydrolysis is the major degradation pathway for TH, with CYP-mediated metabolism playing only a minor role. The aim was to investigate whether TH or a TH-derived product was responsible for this pharmacokinetic interaction, and whether inhibition of CP metabolism was involved.

CP and 4-OH-CP were measured using a LC-MS method (Ding *et al.*, 2002). For *in vivo* studies, C57B1/6 male mice (30 – 35 g) received i.p. injections of CP (220 mg/kg) followed by TH (20 mg/kg), with 3 mice used for each time point. Having established the concentration–time profile for 4-OH-CP with and without TH, a 60 min blood collection time point was selected to study the effects of 20 mg/kg of 5-hydroxy-thalidomide (5-OH-TH) (the major CYP-mediated metabolite), phthaloylisoglutamine (PG) (the major TH hydrolysis product), and *N*-phenethyltetrafluorophthalidomide (PEFP) (a more potent immunomodulator than thalidomide). At 60 min, blood was collected for 4-OH-CP measurement. *In vitro* studies of CP oxidation were undertaken with mouse liver microsomes (0.5 mg/ml protein, 5 mM $MgCl_2$, 1 mM NADPH, CP [20 – 2000 μM] in pH 7.4 phosphate buffer) incubated for 30 min at 37°. This was followed by the addition of 500 μL methanol and 200 μL semi-carbazide, and the formation of 4-OH-CP measured. The effects of TH, 5-OH-TH, and PG (all at 50 and 500 μM) on the formation rate of 4-OH-CP were investigated. A reduction of > 30% was considered significant.

Plasma 4-OH-CP concentrations (mean ± s.d.) were increased 2-fold by both 5-OH-TH (186 ± 29 *vs* 92 ± 36 μM control) and PEFP (147 ± 24 *vs* 74 ± 10 μM control), considerably less than the 4-fold increase observed with TH. In contrast, PG had no significant effect on 4-OH-CP concentrations. The formation rate of 4-OH-CP in mouse microsomes followed typical Michaelis-Menten kinetics with an apparent K_m, 970 ± 242 μM, and V_{max}, 113 ± 13 nmol/min/mg protein. Using 1 mM CP as substrate, the rate of formation of 4-OH-CP was not influenced by co-incubation with TH, 5-OH-TH or PG.

These results indicate that 5-OH-TH may play a part in this pharmacokinetic interaction, but that TH hydrolysis products are probably not involved. Interestingly, the more potent PEFP had a smaller effect on 4-OH-CP's pharmacokinetics. Direct competitive inhibition of CYP-mediated CP oxidation does not appear to play a part in this interaction in the mouse.

Clarke L, Waxman DL. (1989) *Cancer Res.*, 49: 2344-2350
Ding Q, Kestell P, Baguley BC *et al.* (2002) *Cancer Chemother. Pharmacol. (in press)*

79P METABOLISM AND DISTRIBUTION OF ISOMETAMIDIUM IN ISOLATED RAT HEPATOCYTES

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Isometamidium (ISM) chloride hydrochloride is the drug of choice for chemoprophylaxis of African animal trypanosomiasis. However, resistance to the drug limits its efficacy in several countries. The marketed drug, Samorin, is a mixture of related compounds with varying degrees of trypanocidal activity, ISM being most active. Little is known about the intracellular metabolism and distribution of ISM, or the contribution of metabolites to its pharmacological properties and the development of resistance.

In this study, isolated hepatocytes were obtained from male Sprague-Dawley rats (160-260g, n=3) by perfusion of the liver with collagenase. 5×10^6 cells/ml were incubated with the ISM, (purity 90% w/w,100μM) for 4h at 37°C under an atmosphere of 95% O_2/5% CO_2. Measurement and identification of metabolites was by high performance liquid chromatography (HPLC) and LC-electrospray ionisation mass spectrometry (LC/ESI-MS). Intracellular distribution of ISM was detected by confocal laser scanning microscopy using the innate fluorescence of the drug at 590nm.

ISM was rapidly taken up into cells, and within 5 min was localised mainly in the nuclear membrane, endoplasmic reticulum and nucleolus. Incubation of ISM with isolated rat hepatocytes led to the formation of two metabolites MI and MII (Figure. 1). LC/ESI-MS analysis yielded an ion of m/z=460 for ISM, one of m/z=502 for MI, and one of m/z=477 or 476 for MII.

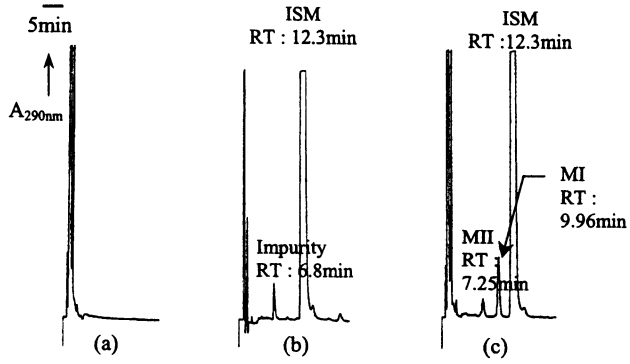

Figure 1: HPLC chromatograms of 4h incubations of (a) hepatocytes in buffer, (b) ISM 100μM in buffer, and (c) hepatocytes with ISM 100μM.

Comparing the masses of the metabolites to that of the parent, MI corresponds to formation of an acetyl metabolite, and MII to an addition of a hydroxyl group or an oxygen atom. ISM was metabolised only to a small extent (2.29 ± 0.41%, n=3) in the isolated hepatocytes compared to ethidium bromide, another phenanthridine trypanocide which undergoes extensive metabolism (Tettey et al., 1999). ISM binds strongly to DNA and macromolecules and this may limit its metabolism.

Tettey J.N.A et al., 1999, *Xenobiotica* 29, 349-360.

80P TOXICITY OF CODEINE, CODEINONE AND OXYCODONE IN HEPG2 CELLS: IDENTIFICATION OF A CODEINONE-GLUTATHIONE CONJUGATE

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Codeine, a naturally occurring opiate, and oxycodone, its structural analogue, are widely used analgesics. Codeinone, previously identified as a metabolite of codeine (Ishida et al, 1998), is linked with the toxicity of codeine but the mechanism is as yet unknown.

In this study, human HepG2 hepatoma cells were seeded at a density of 10^5 cells/cm^2 into 24-well plates and cultured until confluent (3 days) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) foetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml). Incubations of the confluent cells were performed with oxycodone, codeine or codeinone (each substrate 200 μM) in DMEM/0.1 % dimethylsulphoxide (incubation time 24 h). Reduced glutathione (GSH) content was determined fluorimetrically by derivatisation with o-phthaldehyde and cell viability by the leakage of lactate dehydrogenase (LDH) through the membrane. Liquid chromatography/tandem mass spectrometric (LC/MS/MS) analysis was performed using a TSQ 7000 MS/MS instrument (Thermo Finnigan, UK).

Hep G2 cells incubated with codeine or oxycodone showed no significant decrease in GSH content or leakage of LDH over 24h compared with control incubations. In contrast, Figure 1 shows that codeinone caused rapid depletion of GSH which was evident after 5 min.

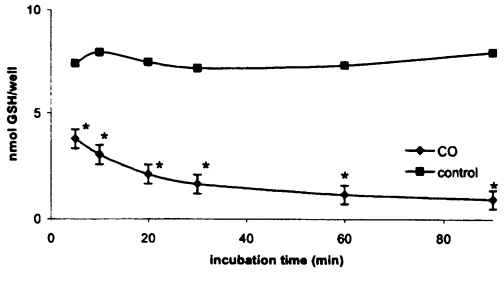

Figure 1: Reduced glutathione (GSH) content after incubations of HepG2 cells with 200 μM codeinone (CO). The results are means ± S.E. of three incubations, * $p < 0.05$, compared with control incubations by Student's t-test.

This was accompanied by leakage of cytosolic LDH through damaged cell membranes, which represented 100% cell death after 12 h. GSH depletion preceded loss in viability and may be partly responsible for it. It could be due to oxidation to the dimer, GSSG, and/or to conjugation with codeinone. Using LC/MS/MS, a codeinone-GSH conjugate was identified in the incubations (m/z = 605 [M^+], with a major fragment ion at m/z = 298 corresponding to the loss of a GSH moiety), and quantified using a synthesised standard. At the 90 min time point, the conjugate formation accounted for 30 % of the GSH depletion. The remainder will be partly due to oxidation.

Ishida, T., Yano, M., Toki, S., 1998, *J.Anal.Toxicol.*, 22, 567-572

81P MEMBRANE TRANSPORT IS A DETERMINANT OF ARABINOSYLCYTOSINE CYTOTOXICITY IN LEUKAEMIC MYELOBLASTS FROM PATIENTS WITH ACUTE MYELOID LEUKAEMIA

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Arabinosylcytosine (araC) is a nucleoside drug that is used in the treatment of acute myeloid leukaemia (AML). Membrane transport of araC is a determinant of araC accumulation in leukaemia cells. The *es* nucleoside transporter, a facilitated diffusion process that is inhibited by nanomolar concentrations of nitrobenzylthioinosine (NBMPR), accounts largely for araC uptake in AML cells. Thus, we hypothesize that a deficiency in *es* nucleoside transport is a mechanism of araC resistance in AML. Other studies have shown that cellular *es* nucleoside transporter abundance varies among AML patients, and that *es* nucleoside transporter abundance and araC sensitivity are correlated in fresh AML blasts from patients (Gati *et al.*, 1997). The present study investigated the role of *es* nucleoside transporter activity in araC cytotoxicity in fresh AML blasts from patients by using NBMPR to pharmacologically block transporter activity and, consequently, araC uptake.

Mononuclear cells were isolated from peripheral blood samples by using Ficoll-Paque centrifugation (30 min, 400x*g*). Cells were incubated (37°C, 5% CO_2, 96 hr) in 1.5-ml cultures ($5x10^5$ cells/ml, Hepes-buffered RPMI 1640 medium / 10% fetal bovine serum) in the absence or presence of graded concentrations of araC ± NBMPR (1 μM). Surviving cells were stained with Cy-Chrome™-labelled anti-CD45, and analyzed by flow cytometry to identify and enumerate leukaemic blasts in control and drug-treated samples. The effect of NBMPR on araC cytotoxicity was measured similarly in the human leukaemic myeloblast cell line, KG1. GraphPad Prism® 3.0 software was used to calculate IC_{50} values and to perform statistical comparisons (Student's *t*-test).

The IC_{50} value (95% confidence interval) for araC cytotoxicity in KG1 cells was 0.012 μM (0.0095-0.016), n=4. In the presence of NBMPR, the IC_{50} value was increased to 0.051 μM (0.026-0.100), n=4. In all AML blast samples examined (Table 1), IC_{50} values for araC cytotoxicity were higher (24- to 230-fold) than in KG1 cells, and NBMPR consistently protected cells against araC cytotoxicity, significantly ($P<0.05$) increasing IC_{50} values several-fold, as in KG1 cells. NBMPR, alone, did not affect cell viability.

Table 1. Modulation of araC cytotoxicity by NBMPR in fresh leukaemic blasts from patients with AML

Patient No.	IC_{50}(araC), μM (95% confidence interval), n=2	
	− NBMPR	+ NBMPR
1	2.1 (0.89-5.2)	>10
2	2.2 (0.67-7.1)	>10
3	2.7 (2.0-3.6)	>10
4	0.29 (0.25-0.33)	1.2 (0.76-2.1)
5	0.33 (0.27-0.39)	1.5 (0.76-2.8)

These data show that araC sensitivity in KG1 cells and in fresh AML blasts was reduced by the presence of an *es* nucleoside transport inhibitor, supporting the view that nucleoside transport deficiency may contribute to araC resistance in AML.

Gati, W.P., Paterson, A.R.P., Belch, A.R., *et al.* (1997) *Blood*, 90, 346-353

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82P EXTRACELLULAR PRODUCTS OF THE MASTITIS-INDUCING PATHOGENS, ESCHERICHIA COLI (EC) AND STAPHYLOCOCCUS AUREUS (SA), ACTIVATE BOVINE MILK KALLIKREINS

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Levels of bradykinin (BK), a potent inducer of pain and oedema, are greatly raised in bovine milk, during infectious mastitis (Eshraghi *et al*, 1999). These raised levels are not secondary to increased blood BK, nor to the presence of inflammatory cells in the milk. Both in vitro (Zeitlin *et al*, 2000) and in vivo (Eshraghi *et al*, 1999), the presence in milk of the pathogenic bacteria, EC and SA, is accompanied by raised BK levels. The mechanism of this increase is unknown. Healthy Holstein-Friesian milk (47ml.) was incubated (37°C) with peptone (3ml, 1%) inoculated with EC or SA (Suspension turbidity = McFarland Standard 1). Sequential samples were tested for cleavage of synthetic tissue- and plasma-kallikrein substrates (TKSub=V.L.R.pNA; PKSub=ArCO.P.F.R.pNA) at pH 8.5 (Tris 0.1M), or extracted in ethanol, dried and RIA'd for BK (Eshraghi *et al*, 1999). Controls contained no bacterial inoculum. Additionally, EC LPS or SA-derived V8 protease (an extracellular virulence factor) were incubated (3h) with milk (1:10) in Tris (0.1M, pH 8.5, 37°C) in the presence of TKSub or PKSub (0.1M). Incubation was stopped by adding TCA (1.2%), boiling and centrifuging. Substrate cleavage was detected as absorbance increase at 405nm. A unit (U) of enzymic activity will be 1 $nMol.h^{-1}$ pNA released. Data are evaluated using regression analysis or ANOVA and mean±sem. In milk incubated with SA, BK concentration increased from 36.2±36.2 $pg.ml^{-1}$, peaking at 4h (514.9±153.0 $pg.ml^{-1}$) compared with milk incubated with medium alone ($P<0.01$, n=4-5). With EC, BK increased up to 6h, reaching 600.7±167.7 $pg.ml^{-1}$. Medium alone caused no BK increase ($P>0.05$). Both SA and EC caused an increase in rate of cleavage of both TKSub and PKSub, ranging between 314% and 704% compared with the control ($P<0.001$, n=6), peaking at 1h and remaining raised up to 6h. EC LPS and SA-derived V8 protease (V8) were examined as possible activators of TK and PK. Incubating (4h) V8 (0-40$\mu g.ml^{-1}$) with TKSub in the presence of buffer plus milk caused an increase ($P<0.01$, n=3) in substrate cleavage, reaching a plateau (9.9±0.8 $U.ml^{-1}$) at 10 $\mu g.ml^{-1}$ of V8 protease. Incubation of V8 with TKSub in the absence of milk caused no detectable increase in cleavage. Incubation of V8 (0-40$\mu g.ml^{-1}$) with PKSub produced a linear ($r^2=0.86$, $P<0.01$, n=3) increase in cleavage in the presence of milk, but showed negligible direct action on the substrate in the absence of milk. LPS (0-1$mg.ml^{-1}$) similarly increased ($P<0.01$) TKSub cleavage in the presence of milk, reaching a plateau (14.9±0.9 $U.ml^{-1}$) at 250 $\mu g.ml^{-1}$ LPS. With PKSub, LPS concentration was also linearly correlated with cleavage rate in the presence of milk ($P<0.01$). LPS had no direct effect on either substrate. Thus, both EC and SA directly activate BK formation in milk. They appear to do this by activating TK and PK-like activity in the milk. The results indicate EC LPS and SA V8 as possible mediators of this action.

Eshraghi H.R, Zeitlin I.J., Fitzpatrick J.L., *et al* (1999). Life Sciences, 64, 1675-1687

Zeitlin I. J., Ng R. S. K., Eshraghi H. R., *et al* (2000). J. Physiol. 527, 144P

83P MAST CELL INDEPENDENT MECHANISMS OF IMMEDIATE HYPERSENSITIVITY: A ROLE FOR PLATELETS

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Data from studies in the skin have implicated the mast cell as the central effector cell in leukocyte recruitment during allergic responses (Wershil *et al*, 1991). However, a fatal anaphylactic response can still be induced in mast cell-deficient mice suggesting other important allergic mechanisms exist (Jacoby *et al*, 1984). In this study we examined the immediate hypersensitivity response (vascular permeability and leukocyte kinetics), in skeletal muscle microvasculature, in vivo, using male mast cell deficient WBB6F1-KitW/KitW-v (W/W^v) or wild type (WT) mice (20-30 g). These data were compared to allergic responses in the skin. Mice were sensitized i.p. with 10 μg chicken ovalbumin (OVA) in 10 mg aluminium hydroxide. Two weeks later postcapillary venules (25-40 μm diameter) in the skin or creamaster muscle were studied in anesthetised (ketamine 200 mg/kg, xylazine 10 mg/kg) mice using intravital microscopy. Venules were studied before, and for 1 h following immediate challenge with OVA (50 μg/mL) in buffer or 4 h after local challenge with OVA (intrascrotal 10 μg; intradermal 100 μg). Vascular permeability was assessed by FITC-albumin leakage (25 mg/kg i.v.) from intravascular to extravascular space. Leukocyte kinetics (rolling, adhesion and emigration) were determined using intravital microscopy (Hickey et al, 1999). Data were expressed as mean ± S.E.M. of $n \geq 5$ mice. Statistical analysis was performed using a Student's t test with Bonferroni correction where necessary (significance was taken at $p < 0.05$).

Immediate challenge in skin induced a significant increase in vascular permeability (75 ± 7.0 from 15 ± 10 %) within 30 minutes which was accompanied by increased leukocyte adhesion 4 h post challenge (5 ± 0.5 from 0.5 ± 0.5 cells/100 μm). In the absence of mast cells no changes in vascular permeability (10 ± 8 %) or leukocyte recruitment (1.0 ± 0.8 cells/100 μm) were observed in skin. In the cremaster, immediate challenge induced a rapid significant increase in vascular permeability within 5 min (80 ± 13 %). In contrast to the skin, immediate challenge in W/W^v mice induced a gradual increase in vascular permeability, reaching a maximum (50 ± 14 %; $p < 0.05$) within 30 minutes. Allergen-induced leukocyte recruitment (emigration) was significantly increased within 1 h post challenge (4.5 ± 1.5 from 1.0 ± 0.5 cells/field) and had tripled (15.5 ± 3.5 cells/field) by 4 h post challenge. Although leukocyte recruitment was delayed in W/W^v mice initially, by 4 h, cells were recruited in similar numbers to WT mice (13.5 ± 4.5 cells/field), despite the absence of mast cells. Pretreatment with rabbit anti-mouse platelet serum (0.5 mL/Kg i.v., Accurate chemical) returned the antigen-induced increase in vascular permeability and leukocyte recruitment to baseline in W/W^v mice (25 ± 5.0 % and 3.5 ± 0.5 cells/field respectively), but did not affect the response in WT (85 ± 10.0 % and 13.8 ± 1.0 cells/field respectively). These data illustrate that, unlike the skin, vascular permeability and leukocyte recruitment changes induced by allergen can occur in the absence of mast cells in skeletal muscle and are mediated, in part, by the presence of platelets.

Hickey, M.J. *et al.* (1999) *J. Immunol.*, 162:1137-1143
Jacoby, W., *et al* (1984) *J. Invest. Dermatol.*, 83: 302-304
Wershil, B.K., *et al.* (1991) *J. Clin. Invest.*, 87: 446-453

84P EFFECTS OF ANTI-INFLAMMATORY DRUGS ON ESTABLISHED LUNG INFLAMMATION INDUCED BY ALLERGEN IN ACTIVELY SENSITISED BROWN NORWAY RATS ASSESSED NON-INVASIVELY BY MRI

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Magnetic resonance imaging (MRI) can be used to follow non-invasively the development of an oedematous signal, a key parameter of airway inflammation, in the lungs of actively sensitised Brown Norway (BN) rat challenged with ovalbumin (OA). This approach provides a reliable means of profiling anti-inflammatory drugs *in vivo* (Beckmann et al., 2001). Formerly, anti-inflammatory drugs have been given in pretreatment regimen. The aim of the present study was to compare the effects of Compound 1, [4-(4-Fluorophenyl)-2-(1-methylpiperidin-4-yl)-5-(2-(1-(S) phenylethyl) amino-4-pyridinil) thiazole fumarate, a p38α mitogen activated protein kinase inhibitor, NVP-ABE171, a phosphodiesterase-4 inhibitor (Trifilieff et al., 2002), and budesonide, a glucocorticosteroid on established allergic inflammation.

Male BN rats (250-300 g) were sensitised to OA and challenged with OA (Beckmann et al., 2001). Compound 1 (10 mg kg^{-1}, p.o.), NVP-ABE171 (1 mg kg^{-1}, p.o), budesonide (1 mg kg^{-1}, i.t) or vehicle were administered 24 h post OA challenge. A gradient echo MRI sequence was used to define the oedematous signal in the OA-challended BN rats (Beckmann et al., 2001). Images were acquired before OA challenge and 24 (before drug administration), 27, 30, 48, 72 and 96 h after challenge. In separate animals, differential leukocyte cell counts, eosinophil peroxidase (EPO) and myeloperoxidase (MPO), and protein concentration were determined in bronchoalveolar lavage (BAL) fluid immediately after MRI acquisition at 30 and 72 h.

At the doses used, drugs have shown anti-inflammatory effects when administered prior to OA-challenge. Oedematous signals were significantly reduced 6 to 72 h for NVP-ABE171 and budesonide applied 24 h after OA. In contrast, Compound 1 had minimum effects on oedema (Figure 1). No inhibition in BAL fluid markers of inflammation was observed with any of the drugs tested when examined 30 h post OA challenge. However, at 48 h post treatment `EPO, MPO and protein levels were significantly reduced by both budesonide and NVP-ABE171. Compound 1 had no inhibitory effects on any of the parameters determined in BAL fluid. Our data demonstrate differences in anti-inflammatory effects of Compound 1, NVP-ABE171 and budesonide when administered 24 h post OA challenge. They also demonstrate that resolution of oedematous signal occurs independently of inhibition of key inflammatory parameters from BAL fluid. The animal model described in the present study brings an important new perspective for the analysis of effects of anti-inflammatory drugs.

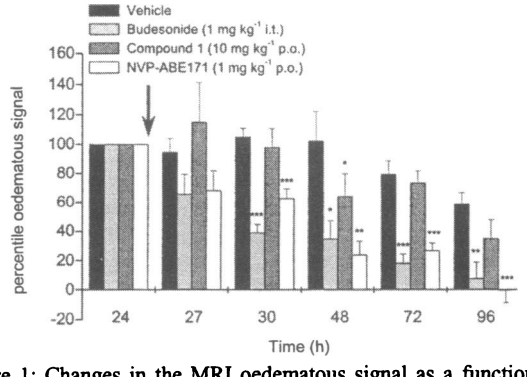

Figure 1: Changes in the MRI oedematous signal as a function of time starting 24 h after challenge with OA (0.3 mg kg^{-1} i.t.). Actively sensitised BN rats were treated with budesonide, compound 1, NVP-ABE171, or vehicle immediately after the 24 h MRI acquisition. The value at 24 h is taken as 100 %. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ indicates significant difference by comparison with equivalent value in vehicle-treated group using ANOVA test.

Beckmann, N., et al. (2001). Magn. Res. Med., 45, 88-95.
Trifilieff, A., et al. (2002). J. Pharmacol. Exp. Ther., 301, 241-248.

85P THE EFFECT OF THE p38 KINASE INHIBITOR SB203580 ON TUMOUR NECROSIS FACTOR-α PRODUCTION BY HUMAN MONOCYTES IN RESPONSE TO *E. COLI* O157:H7 SUPERNATANT AND PURIFIED VEROTOXINS

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Escherichia coli O157:H7 infection can lead to diseases such as haemorrhagic colitis and haemolytic uraemic syndrome, mediated in part via the production and release of toxins, termed verotoxins (VTs). In addition, both *E.coli* O157:H7 and the VTs can up-regulate the production of inflammatory cytokines such as tumour necrosis factor-α (TNFα), which play a pivotal role in the development of hypotensive shock (Cairns *et al.*, 2000). The p38 mitogen-activated protein (MAP) kinase has been shown to play a crucial role in cytokine production (Carter *et al.*, 1999). Therefore, the aim of the present study was to determine whether the production of TNF-α from monocytes in response to stimulation with supernatant from *E. coli* O157:H7 (O157sup) and purified VT1 & 2 could be modulated by the p38 Kinase Inhibitor SB203580.

Human peripheral blood monocytes (PBMϕ) were isolated from blood obtained from healthy volunteers, by Ficoll density centrifugation, followed by differential plating. Adherent monocytes were incubated with O157sup, VT1 or VT2 in 12-well tissue culture plates for 20 hrs and the level of TNFα in the supernatants were measured by sandwich ELISA. MAPKAP kinase-2 activity, in solubilised cell extracts, was assayed by measuring the incorporation of radiolabelled phosphate into a peptide substrate, after incubation with [γ-^{32}P]-ATP, followed by blotting and subsequent liquid scintillation counting.

A concentration-dependant reduction in the TNFα released from monocytes in response to O157sup, was observed in the presence of SB203580. SB203580 (20 μM) reduced the TNFα levels to near basal levels (control = 43.1 ± 2.6 pg.ml^{-1}, O157sup = 943.4 ± 334.4 pg.ml^{-1} and O157:H7 + SB203580 = 68.8 ± 5.5 pg.ml^{-1}, means ± s.d, n = 3). VT1-induced TNFα release was also decreased when monocytes were pre-incubated with increasing concentrations of SB203580 (control = 29.4 ± 14.3 pg.ml^{-1}, 2 μg/ ml VT1 = 1031.5 ± 81.1 pg.ml^{-1} and VT1 + 20 μM SB203580 = 595.3 ± 84.0 pg.ml^{-1}, means ± s.d, n = 3). Similarly, the VT2-induced TNFα release was reduced in a concentration-dependant manner when monocytes were pre-incubated with SB203580 (control = 29.4 ± 14.3 pg.ml^{-1}, 2 μg/ ml VT2 = 914.3 ± 8.6 pg.ml^{-1} and VT2 + 20 μM SB203580 = 265.76 ± 67.85 pg.ml^{-1}, means ± s.d, n≥3). No significant increase in TNFα levels above that of control was observed with SB203580 alone. Treatment with SB203580, O157:H7 supernatant, VT1 or VT2 had no detectable effect on cell viability, as assessed by trypan blue dye exclusion indicating that the reduction in TNFα was not due to cell toxicity. SB203580 (20 μM) was also shown to inhibit the O157sup– and VT1-stimulated activity of MAPKAP kinase-2 (O157:H7 = 14.6 ± 4.6, O157:H7 + SB203580 = 0.7 ± 0.1, VT1 = 10.0 ± 1.9, VT1 + SB203580 = 0.8 ± 0.1 fold stimulation, means ± s.d, n >3).

These data indicate that both O157sup, containing verotoxins, and purified VT1 and VT2 can upregulate the production of TNFα from monocytes and constitute a pro-inflammatory signal. Additionally, it would appear that this enhancement of TNFα release is dependent on p38 MAP kinase activity..

Carter, A. B., Monick, M. M., and Hunninghake, G. W. (1999). *Am. J. Resp. Cell Mol. Biol.* **20**, 751-758.
Cairns, C. B., Panacek, E. A., Harken, A. H., and Banerjee, A. (2000). *Academic Emergency Medicine* **7**, 930-941.

86P REGULATION OF NK-CELL INTERFERON-γ SYNTHESIS BY PROSTAGLANDIN E2 AND EP-RECEPTOR ANALOGUES

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IFN-γ production by NK cells is an important process in innate host resistance particularly in response to monocyte-derived IL-12 which is amplified by the additional presence of IL-18. However, despite its recognised importance little is known about how this process is regulated, particularly during acute inflammatory responses which ultimately result in the release of PGE2. To address this issue we analysed the effects of the inflammatory lipid mediator PGE2 on the ability of NK cells to produce IFN-γ.

IFN-γ synthesis by the murine NK cell line LNK5E3 was induced by stimulation with IL-12 / 18 for 24 hours in the absence or presence of PGE2. IFN-γ protein in culture supernatants and cellular IFN-γ mRNA expression was subsequently determined by ELISA and ribonuclease protection assay (RPA) respectively. Radioligand binding analysis was carried out by incubating intact cells at 4 °C with [^{3}H]-PGE2 in the presence of excess (3 μM) unlabelled PGE2 or EP receptor analogues.

PGE2 suppressed NK cell IFN-γ production in a concentration-dependent manner even in the combined presence of the potent IFN-γ inducing cytokines, IL-12 and IL-18. PGE2 was effective at concentrations of 0.01 μM (all values $P < 0.05$, n = 3) with a maximal effect between 0.1-1 μM. At 1 μM PGE2 reduced the IL-12/ IL-18-stimulated level of IFN-γ from 69 ± 2.3 ng/ ml to 55 ± 4.6- in the order of a 20 % reduction. The reduction of IFN levels with IL-12 alone was more profound from 24.9 ± 5.4 to 1.5 ± 0.6 ng/ ml i.e. a 94 % reduction.

PGE2 did not have any significant effect on NK cell viability as measured by trypan blue exclusion and crystal violet staining. The EP2/EP4 receptor agonists butaprost and misoprostol (1 μM) both suppressed IL-12/ IL-18-stimulated IFN-γ synthesis by 26.5 ± 3.2 % and 34.6 ± 5.2 respectively whereas the EP1/EP3 agonist sulprostone did not have any significant effect. The suppressive actions of both butaprost and misoprostol were further decreased ($P < 0.05$) in presence of the phosphodiesterase inhibitor IBMX. Indicating that suppression of IFN-γ by these ligands is cyclic AMP-mediated. Binding data indicated that total binding (100 ± 7.2 %) was reduced to 53.4 ± 7.3 % by PGE2 and to 48.6 ± 5.5 % by butaprost (all 3 μM, n = 3) whereas sulprostone did not have any significant effect (107 ± 8.9 %). Analysis of mRNA levels showed that PGE2 reduced IFN-γ mRNA levels 2.5-fold. However, PGE_2 did not modulate expression of mRNA for cytokine receptors capable of transducing IFN-γ-inducing signals. Specifically, RPA analysis showed that expression of the IL-12R or IL-2R subunits were not affected by PGE2.

These data indicate that PGE2 predominantly mediates its suppressive effects on NK cell IFN-γ synthesis via a mechanism that involves the EP2 receptor and which is cyclic AMP-dependent. Furthermore, PGE2 appears to directly affect IFN-γ gene expression and is not due to the down-regulation of cell-surface receptors which relay IFN-γ-inducing signals. These findings indicate that PGE2 may play an important role in limiting innate inflammatory processes by directly inhibiting cytokine-induced NK cell IFN-γ synthesis.

87P SUPPRESSION OF TUMOUR NECROSIS FACTOR-α PRODUCTION IN HUMAN BLOOD BY INTERACTIONS BETWEEN PROSTAGLANDIN E2, INTERLEUKIN-10 AND TRANSFORMING GROWTH FACTOR-β

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The production of pro-inflammatory cytokines especially tumour necrosis factor-α (TNF- α) and interleukin-1 (IL-1) is an important process in innate host resistance particularly in response to bacterial challenge. It is now well established that these cytokines can induce their own production, thus providing a positive feedback loop. The restraint of this cascade becomes of critical importance in preventing host inflammatory reactions from overactivity which could result in severe host damage. The cascade of pro-inflammatory cytokines results in the production of prostaglandins (PGs) predominantly PGE2 which can downregulate both TNF-α and IL-1 release. More recently it has been recognised that both IL-10 and TGF-β can also downregulate inflammatory cytokine release. However, it is not clear whether these 3 inhibitory signals are completely independent or whether there is crossover between the pathways. The aim of this study was to ascertain the effect of bacterial stimulation on the production of IL-10 and TGF-β and the effect of these 2 cytokines along with PGE2 on TNF-α levels in human blood.

Human blood obtained from the Scottish National Blood Transfusion Service was incubated in the absence and presence of *E.coli* O153:H7 (1×10^7.ml^{-1}) at 37 °C for 20 hrs. The blood samples were then centrifuged (10,000 x g for 30 s at 22 °C) and the cytokine levels in the resultant plasma were measured by sandwich ELISA and PGE2 by competitive ELISA.

PGE2, IL-10 and TGF-β all reduced TNF-α production by human blood in response to bacterial stimulation in a concentration-dependent manner. The threshold concentration for each inhibitor was 1 nM PGE2, 5.5 pM IL-10 and 0.4 pM TGF-β. Maximal reductions were 100 ± 1.2 % (control stimulated TNF-α level = 1.4 ng.ml^{-1}) for PGE2 (100 nM), 94.3 ± 2.2 for IL-10 (55 pM) and 82.2 ± 2.4 % for TGF-β (4 pM) all values are means of n =3 ± s.d. and are representative of at least 3 separate experiments. Qualitatively identical data was obtained for IL-1β levels which were measured from the same samples. The PGE2 synthesis inhibitor ketoprofen (KP, 50 μM) enhanced the bacterial-stimulated increase in TNF-α levels from 100 ± 3.4 % to 136.5 ± 5.3 % indicating the suppressive action of endogenously formed PGs. This concentration of KP also inhibited PGE2 production to 22.4 ± 7.4 % over the control bacterial-stimulated level (100 ± 4.2 %). The effect of KP on levels of IL-10 and TGF-β in response to bacteria was also measured. The levels of IL-10 and TGF-b increased from 100 ± 3.2 % to 131 ± 4.4 % and from 100 ± 2.1 % to 137 ± 3.2 % respectively. KP further increased the levels of IL-10 to 158 ± 12.3 % ($P < 0.05$) but did not affect TGF-β. Neither TNF-α nor IL-1β over a wide concentration range had any effect on either IL-10 or TGF-β. However, TGF-β (10 ng.ml^{-1}) increased IL-10 to 160 ± 17 pg. ml^{-1} of IL-10. from 111 ± 2 pg. ml^{-1}(n = 3 ± s.d. $P < 0.05$).

The data indicate that all 3 inhibitors can suppress inflammatory cytokines and that they are also all produced during these activation responses. It would appear that PGE2 has the greatest efficacy and it can endogenously control IL-10 levels, however, their interactions all overlap.

88P PRIMING OF EQUINE EOSINOPHIL SUPEROXIDE PRODUCTION

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Eosinophil responses can be increased by exposure to agents such as platelet activating factor (PAF) or interleukin (IL)-5 which alone cause little or no response, a process known as priming (Koenderman *et al.*, 1996). Priming of equine eosinophil adherence to fibronectin-coated plastic has been demonstrated following pre-treatment with PAF or leukotriene B_4 (Foster *et al.*, 1997). In the present study the effects of pre-treatment with PAF or IL-5 on superoxide production by equine eosinophils has been examined.

Eosinophils from normal ponies were purified using Percoll density gradients and superoxide production measured in a colorimetric assay, as described previously (Foster & Cunningham, 1997). Initial experiments using the potential priming agents alone (PAF (10^{-8}M-10^{-4}M) and human recombinant (hr) IL-5 (10^{-11}M -10^{-9}M)) established that 10^{-6}M PAF and 5 x 10^{-11}M hrIL-5 did not induce significant superoxide production (repeated measures ANOVA; $p>0.05$; n=3). Eosinophils were then pre-incubated with PAF or hrIL-5 at these concentrations for 5min or 30min, respectively at 37°C prior to addition of histamine (His) (10^{-6}M-10^{-4}M) or serum-treated zymosan (STZ) (0.3 – 3333.3 μg/ml; PAF only). As previously reported (Foster & Cunningham, 1997), concentration-dependent superoxide production occurred over this range of His and STZ concentrations.

Equine eosinophils produced significantly more superoxide in response to His or STZ after pre-treatment with PAF or hrIL-5 when compared to cells that had been pre-treated with vehicle ($p < 0.001$; 3-way ANOVA; Table 1).

Table 1: Effect of pre-treatment with PAF or hrIL-5 on His and STZ-induced superoxide production

	Superoxide (nmol reduced cytochrome C/10^6 cells)				
His (M)	Control	10^{-6}M	10^{-5}M	3.3x10^{-5}M	10^{-4}M
Pre-treatment (n=5 (PAF) or 3 (hrIL-5))					
Vehicle	-0.8±0.6	-1.1±0.7	2.5±1.4	8.4±1.9	11.3±1.6
PAF	-1.0±0.7	0.6±0.8	6.1±2.1	10.5±1.8	12.9±1.6
Vehicle	-1.2±0.5		-1.0±0.6	1.8±0.5	3.0±0.5
hrIL-5	0.1±0.8		2.5±0.4	5.9±0.9	8.7±0.9

STZ(μg/ml)	Control	0.3	3.3	33.3	333.3	3333.3
Pre-treatment (n=5)						
Vehicle	-7.4±8.0	-9.9±9.6	-3.6±8.2	7.2±9	51.9±3.5	99.5±10.7
PAF	-3.4±2.0	-2.1±1.7	4.5±1.8	17.4±3.9	69.4±6.4	116.3±8.0

Values are means±SEM

Priming of equine eosinophils may, by increasing generation of superoxide, enhance the beneficial effects of these cells in host defence or increase the local tissue damage that can occur as a result of inappropriate cell activation in allergic disease.

Foster AP & Cunningham FM (1997). *Vet. Immunol. Immunopathol.* 59, 225-237.

Foster AP, McCabe P, Sanjar S & Cunningham FM (1997). *Vet. Immunol. Immunopathol.* 56, 205-220.

Koenderman L, van der Bruggen T, Schweizer RC *et al.* (1996). *Eur. Respir. Suppl.* 22, 119s-125s.

89P REDUCED PLATELET AGGREGATION BUT NO CHANGE IN VASCULAR RESPONSIVENESS WITH CHRONIC ADMINISTRATION OF 17β-OESTRADIOL

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We have recently shown that chronic administration of 17β-oestradiol significantly reduced the incidence of reperfusion-induced ventricular fibrillation (VF) but not ischaemia-induced VF (Byrne *et al.*, 2002; Byrne & Coker, 2002). As platelet aggregation has been implicated in the generation of reperfusion-induced arrhythmias (Shaw *et al.*, 1997) the aim of this work was to look at the effects of 17β-oestradiol on platelet aggregation and vascular responsiveness.

Female Wistar rats (200-310g) were ovariectomized under Hypnorm/diazepam anaesthesia. After 12 to 16 days, rats (240-320g) were treated with 17β-oestradiol (10, 30 & 100 μg kg^{-1}) or vehicle (sesame oil) s.c. daily for 7 days. Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and blood was removed from a cannulated carotid artery and diluted 1:1 with saline containing 10 Units ml^{-1} heparin. Aliquots (1ml) of diluted blood were placed in cuvettes, incubated at 37°C for 5 min and platelet aggregation responses to ADP or collagen were measured by impedance aggregometry. Aortic ring preparations were set up in organ baths in a Krebs-bicarbonate solution maintained at 37°C and gassed with 95% O_2/5% CO_2. The preparations were suspended under a resting tension of 10 mN and allowed to equilibrate for 1hr. Cumulative constrictor concentration response curves to U46619, KCl, noradrenaline or 5-HT were obtained. Tissues were then washed, pre-constricted with 5-HT (10μM) and a cumulative dilator curve constructed to iloprost, pinacidil, sodium nitroprusside, or isoprenaline.

In the vascular experiments 17β-oestradiol did not significantly alter the maximum responses or EC_{50} values for any of the agonists. Platelet aggregation responses to ADP (0.1 to 10μM) were reduced by 17β-estradiol, whereas there were no significant alterations in the responses to collagen (1μg ml^{-1}). The amplification of the response to ADP (0.1μM) by the thromboxane-mimetic U44619 (1μM) was markedly attenuated by 17β-oestradiol in a dose-dependent manner (Figure 1).

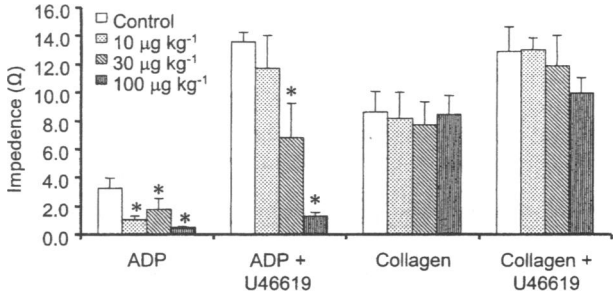

Figure 1. Aggregation responses (Ω) in blood taken from rats given vehicle or 17β-estradiol. Values are mean ± s.e. mean, n=5-6 per group. *P<0.05 vs. control, Kruskal-Wallis test.

These result suggest that the effects of 17β-estradiol on platelet aggregation may contribute to the reduced incidence of lethal reperfusion-induced arrhythmias.

Byrne, N.F. *et al.* (2002). *J. Mol. Cell. Cardiol.* in press
Byrne, N.F. & Coker, S. J. (2002). This meeting
Shaw, L.A. *et al.* (1997). *Br. J. Pharmacol.* 121, 875-882.

90P NEURALLY EVOKED RESPONSES OF RAT FEMORAL SMALL ARTERIES ARE PREDOMINANTLY MEDIATED BY α_{1A}-ADRENOCEPTORS

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Previous studies have shown that the predominant α-adrenoceptor subtype that mediates contraction to exogenous noradrenaline in rat femoral resistance arteries is the α_{1A}-subtype (Jarajapu *et al.*, 2001). The aim of the present study was to characterise α_1-adrenoceptor subtype(s) involved in response to electrical field stimulation (EFS) using the antagonists prazosin (α_1-selective), 5-methyl-urapidil (5MU, α_{1A}-selective), BMY 7378 (α_{1D}-selective) and the preferential α_{1B}- alkylating agent, chloroethylclonidine (CEC).

Second and third order branches of femoral artery (normalised diameter $L_{0.9}$ = 219±5 μm, n =47) were dissected out from male Wistar rats (200 - 250 g) and mounted on a small vessel wire myograph in physiological salt solution at 37°C continuously bubbled with carbogen for isometric recording. The vessels were activated by 123 mM KCl twice followed by 10 μM noradrenaline. Vessels were stimulated using platinum electrodes. EFS at 20 V and 0.05 ms pulse width was applied at 5 min intervals for 10 sec at frequencies of 5-30 Hz. Antagonists were present for 15 minutes before EFS. For CEC treatment, arterial segments were incubated with CEC (10 μM) for 30 min followed by washing for 60 min (each wash every 15 min).

Tetrodotoxin (1 μM) significantly reduced, but did not completely abolish responses (e.g. 30 Hz responses reduced to 27 ± 7% of control response, n =4, P <0.01). Likewise, prazosin (1 μM) partially blocked the response (e.g. 30 Hz responses reduced to 28 ± 7% of control response, n=5, P<0.001). Subsequent addition of 10 μM α,β-methylene ATP produced an additional small blockade (responses, 21±8% of control at 30 Hz; n=5, P<0.05). The α_2-adrenoceptor antagonist RS 79448 (0.1 μM) potentiated responses at all frequencies, particularly 10 Hz.

Prazosin and 5MU inhibited the nerve-evoked contractions with pIC_{50}s of 9.23±0.13 (n=8) and 8.17±0.17 (n=5) respectively at 20 Hz. There was no significant difference in the sensitivity to prazosin or 5MU at different frequencies. BMY 7378 inhibited responses at 5 Hz with a pIC_{50} of 8.06±0.07 (n=5). Responses at higher frequencies were less sensitive to BMY 7378 (e.g. pIC_{50} at 10 Hz was 6.6±0.05, n=5), significantly greater than that at 5 Hz (P<0.001). CEC (10 μM, n=4) had no significant effect on responses at all frequencies.

In conclusion, nerve-mediated vasoconstriction in rat femoral resistance arteries is predominantly noradrenergic, with a small purinergic component. The responses are sensitive to low concentrations of prazosin and 5-MU in agreement with an action at α_{1A}-adrenoceptors. The low potency of BMY 7378 and the lack of effect of CEC at frequencies of 10-30 Hz rule out a contribution of α_{1D}- and α_{1B}-adrenoceptors at these frequencies. Responses at 5 Hz were more sensitive to BMY 7378, raising the possibility of a contribution of α_{1D}-adrenoceptors at low frequencies.

Jarajapu, Y.P.R., Hillier, C. & MacDonald, A. (2001) *Eur. J. Pharmacol.*, **422**, 127-135.

91P CONTRACTILE RESPONSES TO UK14304 ARE INHIBITED BY LOW CONCENTRATIONS OF RAUWOLSCINE AND ARE UNAFFECTED BY PRAZOSIN IN THE MOUSE TAIL ARTERY

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The aim was to analyse the effect of rauwolscine and prazosin on contractions to the α_2-adrenoceptor-selective agonist UK14304 in mouse tail artery. Previous studies in the rat (Templeton et al, 1991) and mouse (McBride et al, 2002) tail artery have shown that the presence of a synergist is required to investigate α_2-adrenoceptor mediated contractions. In the D79N mouse (a functional knockout of the $\alpha_{2A/D}$ receptor) only part of the control response to UK14304 was eliminated. Having established suitable conditions for studying UK14304 mediated contractions in the mouse tail artery we aimed to assess if these responses could be inhibited by the α_2-adrenoceptor-selective antagonist rauwolscine.

Arteries from C57Bl6v (30.3-34.2g) and D79N (29.3-32.3g) four month old, male mice were dissected from the tail and cut into 2mm rings. These were mounted in Krebs at 37°C on a Mulvany/Halpern myograph. UK14304 was administered in a non-cumulative manner (10^{-9} – 10^{-5}M). Contractile responses to UK14304 were obtained in the presence of the synergist U46619 (with nifedipine 1 x 10^{-7}M to suppress rhythmic activity), shown to be optimal for UK14304 contractions (McBride et al, 2002). Rauwolscine (3 x 10^{-8}M) and prazosin (3 x 10^{-8}M) were given 30mins prior to the first addition of UK14304. Prazosin (3 x 10^{-8}M), n=6) had little effect on the absolute size of contraction or on the pEC_{50} obtained to UK14304 (Table1).

Rauwolscine (3x10^{-8}M, n=6) caused a rightward shift in the concentration response curve to UK14304 (Table 1).

This data indicates that the contractile effect of UK14304 is due to α_2-adrenoceptor stimulation. The lack of an inhibitory effect of prazosin suggests that even at relatively high concentrations of agonist (10^{-5}M), UK14304 still appears to be acting selectively to stimulate contractions by α_2 stimulation. Overall this data suggests that the response remaining in the D79N mouse is attributable to stimulation of an α_2 subtype other than the $\alpha_{2A/D}$.

UK14304 vs drug	Max (gms force)	pEC_{50}	pK_b
Control (n=12)	0.32±0.03	8.0±0.3	N/A
prazosin(n=6)	0.28±0.01	8.3±0.1	N/A
rauwolscine(n=6)	0.29±0.03	*6.6±0.2	8.5±0.2
D79N + rauwolscine(n=6)	0.38±0.02	*6.5±0.2	8.8±0.3

Table 1: The effect of prazosin and rauwolscine on UK14304 induced contractions of the mouse tail artery
* $p<0.05$ indicates significance in pEC_{50}'s for control vs rauwolscine following an unpaired Student's t test

McBride M, Daly C.J., McGrath J.C. (2002) *Br.J.Pharmacol.* 135, 304P.
Templeton A.G.B, *et al.*, (1989) *Br.J.Pharmacol.* 97, 563-567

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92P CHARACTERISATION OF THE CONTRACTILE α_1 ADRENOCEPTOR RESPONSE IN THE MOUSE THORACIC AORTA

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We have examined the adrenergic component of contractile responses in mouse thoracic aorta using a transgenic approach. Earlier Cavalli *et al.* (1997) who generated mice lacking the α_{1B} adrenoceptor (AR) suggested that the α_{1B} AR contributes to aortic vasoconstriction. Daly *et al.* (2002) and Tanoue *et al.* (2002) suggested the α_{1D} AR as the main adrenergic vasoconstrictor in mouse aorta with α_{1B} ARs playing a minor contractile role. Our aim was to determine α_1 AR profile in old mice since pathology of conducting arteries is potentially of interest. We also tested younger mice for comparison.

4 and 16 month (m) male (35-45g) 129/Sv/C57BL/6J control (WT) and mice lacking either α_{1B} (α_{1B}-KO) or α_{1D} (α_{1D}-KO) ARs were euthanised by CO_2 and their aortae isolated. 2mm rings were mounted on a Mulvany/Halpern myograph in Krebs at 37°C. After initial challenges to 62.5mM KCl and 10μM phenylephrine (PE) cumulative curves were constructed for PE and then 5-hydroxytryptamine (5-HT.)

Assessment of the α_{1B}-KO and WT responses showed generally larger contractions at 16m than at 4m. However, α_{1B}-KO mice were not significantly different in PE, KCl or 5-HT responses from WT.

We then assessed responses in the α_{1D}-KO at 16m. The maximum response was significantly reduced (56.0%) and the pEC_{50} shifted tenfold compared with the 16m WT. Maximal response to 5-HT was also reduced but maximal KCl response and 5-HT sensitivity remained similar to the WT.

		KCl	PE	5-HT
4m WT	max(g)	0.54±0.04*	0.75±0.08*	0.68±0.04***
	pEC_{50}		5.93± 0.31	6.93± 0.13
4m α_{1B}-KO	max(g)	0.51± 0.05*	0.72± 0.10*	0.61± 0.04***
	pEC_{50}		6.53± 0.25	7.20± 0.12 *
16m WT	max(g)	0.71± 0.08	0.91± 0.05	1.39± 0.13
	pEC_{50}		6.28± 0.13	6.65± 0.13
16m α_{1B}-KO	max(g)	0.86± 0.15	1.16± 0.14	1.58± 0.16
	pEC_{50}		6.51± 0.27	6.72± 0.13
16m α_{1D}-KO	max(g)	0.63± 0.05	0.40± 0.03**	0.77± 0.07**
	pEC_{50}		5.35± 0.14*	7.23± 0.17*

Table 1. Maximal responses and pEC_{50} values WT, α_{1B}-KO and α_{1D}-KO mice. Statistical analysis carried out against 16m WT (*$p<0.05$, ** $p<0.01$, ***$p<0.001$).

Thus in 16m old α_{1D}-KO mice, where sensitivity and maximal response to PE are decreased, α_{1D} ARs are the major ARs contributing to vasoconstriction in the aorta. This is consistent with the findings of Tanoue *et al.* (2002) in 3-5m old mice but in our study 16m old mice showed a marked reduction in maximal response. There was no evidence of a major contribution of α_{1B} ARs to vasoconstriction at 4 or 16m counter to the interpretation of Cavalli *et al.* (1997). However the response of the aorta from young mice is more reduced in the double α_{1D}-α_{1B}-KO (G. Tsujimoto, unpublished findings) suggesting that α_{1B} ARs may play a modulatory role

Cavalli *et al.* (1997) *Proc.Natl.Acad.Sci.* USA **94** 11589-11594
Daly *et al.* (2002) *Physiol.Genomics* **9** 85-91
Tanoue *et al.* (2002). *J.Clin.Invest.* **109** 765-75.

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93P MEASUREMENT OF CELLULAR AND TISSUE DISTRIBUTION OF BETA-ADRENOCEPTORS IN RAT THORACIC AORTA USING BODIPY-CGP 12177

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In the present study we examined the distribution of β-adrenoceptors in segments of rat thoracic aorta using a fluorescent derivative of the β_1/β_2-adrenoceptor antagonist CGP 12177 (BODIPY-CGP 12177, Molecular Probes). Fluorescent-ligand binding was assessed by quantitative confocal microscopy (Daly *et al.*, 1998). The affinity of BODIPY-CGP 12177 was assessed in functional studies.

In functional studies, relaxations of preconstricted aortic ring preparations (Brahmadevara *et al.*, 2001) to isoprenaline were obtained in the presence (30min incubation) or absence of antagonists. In fluorescence studies paraformaldehyde fixed (or unfixed) aortic segments were incubated in 30 -100 nM BODIPY-CGP 12177 for 30 min at room temperature (protected from light), in the absence or presence of CGP 20712 (0.3 μM), propranolol (10 μM) or ICI 118551 (0.3 μM). Tissue segments were cut open and slide mounted, endothelial side up, on an Odyssey CLSM. The 529 nm line of an argon ion laser was used to collect a z-series of images in 1 μm steps from the endothelium to ~60 μm within the media. Three random areas from each vessel segment were scanned. Samples were imaged under identical conditions of laser intensity, gain (contrast) and offset (brightness). Average intensity projections (AIPs) were created for each z- series. The average fluorescence of each projection was then used for comparison purposes.

Pre-incubation with CGP12177, BODIPY-CGP 12177 and propranolol (all 30 nM) produced 32 fold, 3.5 fold and 10 fold shifts, respectively, of the isoprenaline induced CRC, giving pK_bs of 8.99±0.09, 7.88±0.12, 8.64±0.09, n=5.

In unfixed tissues, fluorescence (BODIPY-CGP 12177 100 nM) was detected on endothelial cells (ECs) and on smooth muscle cells (SMCs). Following fixation, fluorescence (BODIPY-CGP 12177 30 nM) was also observed in perinuclear regions in both ECs and SMCs. Fluorescence was inhibited (%, n=4) by propranolol (53±1), CGP 20712 (33±1) and ICI 118551 (53±1).

In conclusion, BODIPY-CGP 12177 exhibited 10-fold lower affinity at β-adrenoceptors compared with CGP 12177. The fluorescence inhibition by ICI 118551 and CGP 20712 indicates the presence of β_2 and β_1-adrenoceptors as previously reported (Brawley *et al.*, 2000). The binding of BODIPY-CGP 12177 to perinuclear regions of ECs and SMCs is similar to that observed for the fluorescent α_1-adrenoceptor ligand, QAPB (Daly *et al.*, 1998), and suggests a possible intracellular location of β-adrenoceptors.

Brahmadevara, N., Shaw, A.M. & MacDonald, A. (2001) *Br J Pharmacol.*, **134,** 110P
Brawley L, Shaw, A.M. & MacDonald, A. (2000) *Br. J. Pharmacol.*, **129,** 637-644
Daly, C.J., et al., (1998). *J. Pharmacol., Exp. Ther.* **286** 984-990

CD & JCM are members of VASCAN-2000 (QLT1-00084-1999)

94P ROLE OF POTASSIUM CHANNELS IN β-ADRENOCEPTOR-MEDIATED VASORELAXATION IN RAT AORTA AND SUPERIOR MESENTERIC ARTERY

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Previous studies have suggested a role for potassium channels in β-adrenoceptor-mediated relaxation in blood vessels e.g. (Satake *et al.*, 1996). As the level of pre-constriction is an important factor in β-adrenoceptor-mediated relaxation (Brahmadevara *et al.*, 2002), we have examined the effects of potassium channel inhibitors on both phenylephrine-induced pre-constriction and isoprenaline-induced relaxations in rat aorta and superior mesenteric artery (SMA).

Male Wistar rats (200 - 250 g) were stunned and killed by cervical dislocation before removal of the thoracic aorta and SMA. Ring preparations were suspended in Krebs physiological saline solution (PSS) gassed with 95/5 % O_2/CO_2 at 37 °C for isometric recording. Artery rings were pre-constricted with phenylephrine (aorta, PE, 0.5 μM; SMA, PE, 1 μM) and the integrity of endothelium was checked with acetylcholine (1 μM) before carrying out cumulative concentration-response curves (CRCs) to isoprenaline. In inhibitor studies, tissues were incubated with inhibitor for 30 min. before constricting. Values are mean±s.e.mean. Statistical analysis was carried out using ANOVA followed by post tests.

Pre-treating aortic rings with iberiotoxin (IBTX, 100 nM), an inhibitor of large conductance Ca^{+2}-activated K^+channels (BK_{Ca}), tetraethylammonium chloride (TEA, 1 mM), an inhibitor of Ca^{+2} activated K^+channels, glibenclamide (10 μM) an inhibitor of ATP-sensitive K^+ (K_{ATP}) channels, or 4-aminopyridine (4-AP 10 μM), an inhibitor of voltage-dependent K^+ (K_v) channels had no effect on isoprenaline-induced relaxations (pEC_{50}s: control, 7.71±0.01; IBTX, 7.57±0.02; TEA, 7.61±0.02; glibenclamide, 7.75±0.06; 4-AP, 7.54±0.02, n=7, p>0.05). A Similar lack of effect was observed in SMA with IBTX, glibenclamide and 4-AP. In contrast relaxations to levcromakalim, an activator of KATP channels, were inhibited by glibenclamide (10 μM) in aorta (pEC_{50}s: control, 7.20±0.06; glibenclamide, 5.35±0.05, n=5, p<0.05) and in SMA (pEC50s: control, 7.13±0.03; glibenclamide, 5.30±0.04, n=5, p<0.05). Relaxations to NS1619, a putative activator of BK_{Ca}, were unaffected by IBTX (100 nM) in aorta (pEC50s: control, 5.39±0.06; IBTX 5.43±0.05, n=5, p>0.05) and in SMA (pEC50s: control, 5.08±0.03; IBTX, 5.06±0.03, n=5, p>0.05).

In SMA, but not in aorta, pre-incubation with IBTX (100 nM) significantly increased the PE pre-constriction (g tension: control, 0.11±0.008; IBTX, 0.16±0.01 n=6, p<0.05) and shifted the PE CRC to the left without affecting the maximum response (pEC_{50}s: control, 6.26±0.03; IBTX 6.97±0.02, n=7, p<0.05). None of the other potassium channel inhibitors affected the PE pre-constriction in aorta or SMA.

In conclusion, these results provide no evidence for the involvement of BK_{Ca}, K_{ATP} channels or K_v channels in beta-adrenoceptor mediated relaxation in rat aorta and in SMA.

Brahmadevara,N., Shaw, A.M. & MacDonald, A. (2002) *Br J Pharmacol.*, in press.
Satake, N., Shibata, M. & Shibata, S. (1996) *Br. J. Pharmacol.*, **119,** 505-510.

95P AGONIST STIMULATED GENE TRANSCRIPTION MAY DISCRIMINATE TWO CONFORMATIONS OF THE HUMAN β_1-ADRENOCEPTOR

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CGP 12177 has recently been proposed to activate a low affinity conformation of the β_1-adrenoceptor that is different from that utilised by the classical β-agonist isoprenaline (Konkar et al., 2000; Lowe et al., 2002). This "CGP 12177" conformation is characterised by its relative resistance to classical β-blockers (Konkar et al., 2000). The aim of this study was to investigate the potential presence of these two conformations in CHO-K1 cells expressing the human β_1-adrenoceptor and a secreted placental alkaline phosphatase (SPAP) reporter gene.

CHO-K1 cells expressing the human β_1-adrenoceptor were stably transfected with a secreted placental alkaline phosphate (SPAP) reporter gene under the transcriptional control of six (CRE) cyclic AMP response elements. Measurements of agonist stimulated SPAP secretion and [^{3}H]-CGP 12177 binding in whole cells (non-specific binding defined by the β_1 selective antagonist CGP 20712A, 100nM) were made as described previously (Baker et al., 2002).

[^{3}H]-CGP 12177 binding revealed a K_D value of 0.42 ± 0.01nM (n=3) for the radioligand. CGP 20712A had a log K_D value of –8.72 ± 0.04 (n=3) for inhibition of the specific binding of 0.4nM [^{3}H]-CGP 12177. The expression level of the receptor was 1146.7 ± 132.1 fmol/mg protein (n=3).

Isoprenaline stimulated a maximal increase in SPAP secretion of 2.76 ± 0.09 fold over basal with a log EC_{50} value of –8.54 ± 0.09 (n=15). This response was antagonised by CGP 20712A, to yield a log K_D value of –8.91 ± 0.08 (n=9).

Atenolol, another selective β_1-antagonist and propranolol, a non-selective β-antagonist also inhibited this isoprenaline-stimulated response to yield log K_D values of –6.79 ± 0.07 (n=6) and –8.13 ± 0.09 (n=5) respectively.

In this high expressing cell system, CGP 12177 (log EC_{50}–9.13 ± 0.04, n=16) was virtually a full agonist, stimulating a maximal SPAP response equivalent to 84.9 ± 2.7% of that produced by isoprenaline. CGP 20712A inhibited this CGP 12177 stimulated response to yield a log K_D value of –7.93 ± 0.08 (n=10). Atenolol and propranolol also inhibited this CGP 12177-induced response to yield log K_D values of –5.21 ± 0.18 (n=6) and –6.91 ± 0.14 (n=5) respectively.

No response was seen to any of the ligands used in CHO-K1 cells transfected with only the CRE-SPAP reported gene.

In summary, CGP 12177 behaves as a potent agonist of SPAP reporter gene transcription response in cells expressing high levels of the human β_1-adrenoceptor. However, the concentration of all three antagonists required to block the CGP 12177-induced response was approximately ten fold higher than that needed for the isoprenaline-induced responses. These data show that agonist specific differences in antagonist affinity can be observed using a reporter gene system and are consistent with the suggested two agonist conformations of the β_1-adrenoceptor.

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Baker J.G., et al., (2002) *Br J Pharmacol.* **135**, 182P.
Konkar A.A.,et al., (2000) *J. Pharmacol. Exp. Ther.* **294**, 923-932.
Lowe M.D., et al., (2002) *Br J Pharmacol.* **135**, 451-461.

96P THE OREXIN 1 RECEPTOR INTERACTS WITH BETA-ARRESTIN 2 AT ITS EXTREME C-TERMINUS

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The orexin 1 receptor belongs to the superfamily of G protein-coupled receptors (GPCRs). Another member of this small group of GPCRs is the orexin 2 receptor. The endogenous ligands, orexin A and B, are hypothalamic neuropeptides involved in the control of feeding and energy metabolism (Sakurai *et al.*, 1998), modulation of neuroendocrine functions (Van den Pol *et al.*, 1998, Smart, 1999) and regulation of the sleep-wake cycle (Smart, 1999). Haynes *et al.* (2000) also showed that the orexin 1 receptor appears to mediate the orexigenic response to orexin A, and stimulation of the orexin 1 receptor is necessary for normal feeding. β-arrestins are cytosolic proteins that play an important role in regulating the responsiveness of GPCRs. They uncouple GPCRs from their effector by sterically blocking G protein binding producing a nonsignalling, desensitised receptor. They are also able to associate with clathrin and the clathrin adaptor protein AP-2, implicating them in playing an integral part in the internalisation of GPCRs (Goodman *et al.*, 1996, Laporte *et al.*, 1999). The aims of this study were to identify the region of the orexin 1 receptor that interacts with β-arrestin 2, to determine if binding of β-arrestin 2 is a prerequisite for internalisation and also to ascertain if this interaction is phosphorylation dependent.
HEK293T cells were transiently transfected with β-arrestin 2 GFP and epitope tagged mutations of the orexin 1 receptor. These mutations were generated using overlap PCR (Fong, 1999). In order to label the receptor, the cells were incubated with the anti-epitope antibody and stimulated with or without agonist. After permeabilisation the cells were incubated with a fluorescently labelled secondary antibody and the receptor and β-arrestin localisation visualised using a confocal microscope. Increasing the osmolarity of the medium inhibits internalisation of the orexin 1 receptor. In contrast mutation of the Ser/Thr residues of cluster C1 (amino acids 418-422), but not C2 (amino acids 393-396) prevents high affinity binding of β-arrestin 2 to the receptor but not receptor internalisation.

This data indicates that the orexin 1 receptor internalises in clathrin coated pits via binding of β-arrestin 2 to the extreme C terminal region . This tight interaction between the receptor and β-arrestin 2 is mediated by a cluster of Ser/Thr residues at position 418-422.

Fong T. M., (1999). *In Structure-Function Analysis of G Protein-Coupled Receptors* (ed. J. Wess) p. 12. Wiley-Liss, New York.
Goodman, O.B., Krupnick, J.G., Santini, F. *et al.* (1996). *Nature* **383**, 447-450.
Haynes, A.C., Jackson, B., Chapman, H. *et al.* (2000). *Regul. Pept.* **96**, 45-51.
Laporte, S.A., Oakley, R.H., Zhang, J. et al. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 3712-3717.
Sakurai, T., Amemiya, A., Ishii, A. *et al.* (1998). *Cell* **92**, 573-585.
Smart, D. (1999). *Br. J. Anaesththes.* **83**, 695-697.
Van den Pol, A.N., Gao, X.B., Obrietan, K. *et al.* (1998). *J. Neurosci.* **18**, 7962-7971.

97P THE EFFECT ON SIGNALLING OF MUTATIONS TO PROLINE RESIDUES WITHIN THE TRANSMEMBRANE REGIONS OF HUMAN CALCITONIN RECEPTOR-LIKE RECEPTOR (CL)

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CGRP receptors require the heterodimerisation of a type II G-protein coupled receptor (GPCR) known as the calcitonin receptor-like receptor (CL) and a single-pass transmembrane (TM) protein known as receptor activity modifying protein 1 (RAMP1) (McLatchie *et al.*, 1998). Proline (P) residues within the TM domains of GPCRs often form functionally important kinks in the helices (Sansom & Weinstein, 2000). The function of these residues within CL has not been studied.

Proline residues within TM domains 4, 5 and 6 of CL (P242, P245, P276 and P322) were mutated to alanine (A) using the Stratagene Quick-change Mutagenesis method. Genes encoding the mutant and wild-type (WT) CL contained within the pcDNA3- mammalian expression vector were transiently transfected (with RAMP1) into Cos-7 cells. Cells were transfected at 60-80% confluency on 48-well plates according to the CalPhos transfection system (Clontech). The (1pM to 1μM) human αCGRP-induced cAMP response of transfected cells was measured using a ^{3}H-cAMP radio-receptor assay as described previously (Poyner *et al.*, 1998). pEC_{50} (-log EC_{50}) values were calculated using Graphpad Prism version 3.00. Mutant and wild-type (WT) pEC_{50} values were compared using the two-tailed, unpaired Student's t-test.

Table 1. pEC_{50} values for CGRP in mutant and WT receptors

	pEC_{50} ± s.e.mean (n)	
	Alanine substituent	WT
P242A	8.79 ± 0.54 (3)	9.15 ± 0.37 (3)
P245A	9.01 ± 0.20 (4)	9.05 ± 0.16 (4)
P276A	9.56 ± 0.37 (3)	9.80 ± 0.18 (3)
P322A	6.86 ± 0.20 (3)	9.18 ± 0.46 (3) **

** Significantly different from WT, $P < 0.01$

The results showed that the P322A mutation significantly decreased the pEC_{50} for CGRP. This was not observed for the other mutants.

This study suggests that of the transmembrane pralines only P322 is important for the functioning of CL/RAMP1. It may be involved in the ligand-receptor interaction, G-protein coupling or receptor expression.

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McLatchie, L M., Fraser, N, J., Main, M, J. *et al.,* (1998), *Nature*, **393**, 333-339.

Poyner, D.R., Soomets, U., Howitt, S.G. *et al.*, (1998), *Br. J. Pharmacol.*, **124**, 1659-1666.

Sansom, M.S.P. & Weinstein, H, (2000), *TiPS*, **21**, 445-451.

98P INCIDENCE OF SUBCONDUCTANCE CURRENTS AT SINGLE NICOTINIC RECEPTORS IS INVERSELY PROPORTIONAL TO FLEXIBLE AGONIST CONCENTRATION, BUT INDEPENDENT OF RIGID LIGAND CONCENTRATION

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Most kinetic schemes for the activation of ligand gated ions channels such as the nicotinic receptor (nAChR) focus on the two high affinity binding sites long recognised to be present. Occupation of either or both of these sites is often adduced as explanation for the populations of channel currents differentiated on the basis of duration. Subconductances, or currents of less than the normal magnitude, have been acknowledged, but their position in kinetic schemes has been ignored or avoided. The possibility that there are also binding sites of lower affinity, the occupation of which contributes to the overall function of the receptor was first mooted in 1982 (Dunn and Raftery, 2000). We have noted that while subconductances were relatively common when carbachol was used as a ligand, they totally dominated when the semirigid ligand, arecolone methiodide, was used (Kawai *et al.*, 2000). We report here that this appears to be a general rule, as flexible ligands, used at concentrations substantially below those required for equilibrium saturation of low affinity binding sites, evoke subconductances inversely proportional to the ligand concentration, while the incidence of subconductance currents is unaffected by the concentration of more rigid ligands

Native nAChR enriched membrane fragments were obtained from *Torpedo californica* electroplax and incorporated into giant liposomes (Riquelme *et al.* 1990). Single channel recordings were made from inside-out patch configurations using an Axopatch 1D amplifier, and pClamp® software. Ligands were applied to the receptors from the lumen of the recording electrodes. Under conditions of low ligand concentration currents were recorded throughout the period of observation, and not in bursts. Current amplitudes were collected and expressed initially as population frequency histograms and the fraction of the whole represented by full conductance events determined. Experiments were carried out in replicates of at least three.

Flexible ligands increased the proportion of full conductances over three decades of concentration. Carbachol full conductance current fraction rose from 0.1 to 0.55 between 0.1 and 10 μM (n>2000), while acetylcholine showed a similar increase between 0.3 and 50 μM. Although tetramethyl ammonium is not a flexible ligand, it is small enough not to impede binding site flexion and demonstrated a low full current fraction over the concentration range from 5 to 7 μM and at 10 μM, the duration of the currents was greatly extended, although only a small proportion (10%) were in the full conductance category. Of the semi-rigid ligands investigated, arecolone methiodide, dimethylphenylpiperazinium, phenyltrimethylammonium, nicotine, and lobeline, the observed currents were almost exclusively of the subconductance type over concentrations ranging from 10 to 50 μM. Full conductance currents may require the progressive occupation of multiple binding sites and a conformational change at a binding site.

Dunn, S.M.J. and Raftery, M.A. (2000) *Biochem. Biophys. Res Comm.* **279**, 358-362.

Kawai, H. *et al.* , (2000) *Biochemistry.* **39**, 3867 – 3876.

Riquelme. G. *et al.*, (1990) *Biochemistry*, **29**, 11215 – 11222.

99P TRANSMITTER MOBILIZATION AT THE MAMMALIAN NEUROMUSCULAR JUNCTION IS MEDIATED BY CGRP ACTING ON SALMON CALCITONIN SENSITIVE RECEPTORS COUPLED TO ADENYLATE CYCLASE BY G_S

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Elmqvist and Quastel (1965) first used the term "mobilization" in the restricted sense of recruiting reserve stores of quanta into the pool that supplied the release mechanism itself during a period of tetanic stimulation of motor nerves. Wilson (1979) found evidence that during the later stages of tetanus, the probability of release was unaffected, and that pool size was the determinant of release rates. The detailed mechanism behind the phenomenon has received scant attention in the literature although recently we reported that activation of a cyclic AMP dependent kinase was a necessary step (Dryden *et al.* 2000). The aim of this study was to determine if a co-transmitter acting on presynaptic receptors was involved.

Mouse phrenic nerve cut hemidiaphragm preparations were superfused with physiological saline and stimulated with trains of stimuli at 30 or 50 Hz for 500 ms. Amplitudes of epps were plotted for each train, and the curves integrated to provide parameters suitable for statistical analysis by Student's t-test. Mepps were counted over successive 1 min intervals for periods of up to 15 min after the addition of drugs. G-protein antagonists were encapsulated in liposomes and applied to the preparations as described by Dryden and Chu (1995).

Suramin (100 μM), $ACTH_{(7-38)}$ (5 μM) and $CGRP_{(8-37)}$ (5 μM) applied to the bathing fluid had no effect on the profile of epp rundown and stabilization at either frequency of stimulation. The frequency of mepp's rose steadily between 5 and 15 min after addition of CGRP to reach levels of 170% (4 nM) or 270% (50 nM) of control values (n=7). The effect of 50 nM CGRP was abolished by the presence of 1 μM salmon calcitonin. When 1 μM salmon calcitonin was applied to preparations stimulated via the phrenic nerve, the area under the curve was significantly reduced only at 50 Hz. However, when the concentration of salmon calcitonin was increased to 10 μM, the areas under the curves were significantly ($P<0.05$) reduced at both 30 and 50 Hz. (1041 ± 24.2 vs 835.9 ± 98.3 units; and 1708 ± 113 vs 1072 ± 180 units, n = 11, respectively). Application of liposomally encapsulated suramin (100 μM), an inhibitor of G-proteins, to the nerve terminal cytoplasm resulted in a significant reduction in the areas under the curve at 30 and 50 Hz stimulation. (998.2 ± 34.99 vs 501 ± 115 units, n =11; and 1380 ± 56.8 vs 299.6 ± 61.57 units, n = 11, respectively). When 4,4',4",4'"-[carbonylbis[imino-5,1,3-benzenetriyl bis-(carbonylimino)]] tetrakis-(benzene-1,3 disulphonate (Nf 449), a selective inhibitor of $G_{S\alpha}$ was used instead of suramin, the areas under the curves were again significantly reduced (1006 ± 42.76 vs 683.5 ± 142, n=9; and 1387 ± 69.1 vs 573.1 ± 176.7, n = 11 respectively).

The data suggest that the co-transmitter, CGRP, and not ACTH or ATP, acts as a feedback stimulant of mobilization at the motor nerve terminal, acting on a salmon calcitonin sensitive CGRP receptor which is coupled to adenylate cyclase by $G_{S\alpha}$, and not on $CGRP_1$ or $CGRP_2$.

Dryden, W.F. & Chu, J. (1995) *Br. J. Pharmacol.*, **116,** 366P.
Dryden, W.F., Y. Gao & H.R. Jamshidi, (2000) Soc. Neurosci. Abst., **30,** 333.13.
Elmqvist, D. & Quastel, D.M. (1965) *J. Physiol.*, **178,** 505-529.
Wilson, D.F. (1979) *Am. J. Physiol.*, **237,** C31 – C37.

100P A ROLE FOR SYNAPSIN-I IN THE MOBILISATION OF ACETYLCHOLINE FOR RELEASE FROM MOTOR NERVE TERMINALS OF THE MOUSE

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In addition to its postjunctional action, vecuronium depresses acetylcholine (ACh) release from motor nerve terminals during high frequency nerve stimulation and this has been attributed to its ability to inhibit prejunctional autoreceptors through which ACh usually facilitates its own mobilisation thus allowing release to keep pace with demand (see Prior *et al.*, 1995 for review). More recent studies (Singh & Prior, 1998) have suggested that this effect of vecuronium might involve a change in the calmodulin-dependent interaction of synaptic vesicles with the nerve terminal protein synapsin-I. Therefore to further investigate the involvement of synapsin-I in ACh mobilisation we have studied how its removal affects the vecuronium-induced neuromuscular depression at a high frequency of motor nerve stimulation (50 Hz).

Studies were performed on the B6,129-*Syn1*^{tm1Sud} strain of mouse that is genetically deficient in synapsin-I. Animals were supplied by Jax Mice (Bar Harbor, Maine) and non-mutated wild-type mice (B6,129F2/J) from the same source were used as control for the comparison of all data. ACh mobilisation was measured as the stimulation-induced depression in the amplitude of end-plate potentials (e./p./ps) during 2 s trains of 50 Hz stimulation recorded from hemidiaphragm muscle/phrenic nerve preparations using a conventional single microelectrode voltage recording technique (18 – 22 °C). All recordings were made in the presence of 1 μM vecuronium.

The absence of synapsin-I had no effect on the shape of the e./p./ps as determined from an analysis of the amplitude and rise time of the first e.p.p. in each train of stimuli (Table 1).

Table 1: Peak amplitude and rise time of first e./p./p/. in the train in normal (+/+) (n=10) and synapsin-I deficient (-/-) (n=8) mice.

	(+/+)	(-/-)
E./p./p amplitude (mV)	3.0 ± 0.2	3.0 ± 0.2
E./p./p. rise time (ms)	0.52 ± 0.02	0.52 0.02

However, although there was no difference in the first e.p.ps in the two strains, there was a significantly reduced stimulation-induced depression of e.p.p. amplitude throughout the course of the train in the synapsin-I deficient mice when compared to the control animals: synapsin-I deficient, 62.9 ± 1.0 % depression (n=10) versus control, 69.5 ± 0.8 % depression (n=8, $P<0.05$, two tailed unpaired Student's *t* test). Thus, in the absence of synapsin-I the motor nerve terminals were better able to sustain vesicular release during sustained periods of activity. This observation is in keeping with the proposed role of synapsin I as a vesicular anchor and suggests that at mice motor nerve terminals a reduction in the binding of synaptic vesicles to synapsin I could be one mechanism by which more synaptic vesicles are made available for release during sustained periods of vesicular exocytosis.

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Prior, C. *et al.* (1995). *Gen. Pharmacol.*, **26,** 659-666.
Singh, S. & Prior, C. (1998). *J. Physiol.*, **511,** 451-460.

101P ATTENUATION OF VECURONIUM-INDUCED TETANIC FADE IN THE RAT HEMIDIAPHRAGM BY THE P2X RECEPTOR ANTAGONIST, PYRIDOXAL PHOSPHATE-6-AZOPHENYL-2',4'-DISULPHONIC ACID (PPADS)

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Exogenous ATP depresses transmission at the skeletal neuromuscular junction (Ribeiro & Walker, 1975). This was initially thought to be due to degradation to adenosine, which then acts at presynaptic adenosine A_1 receptors to depress acetylcholine (ACh) release (Redman & Silinsky, 1994). However, P2X receptors are present at the neuromuscular junction, suggesting a direct effect of endogenous ATP on neuromuscular function (Giniatullin & Sokolova, 1998). To examine this we have studied the effects of the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) in a functional assay of neuromuscular depression – the ability of vecuronium to produce a waning of tetanic contractions during high frequency nerve stimulation.

Tension responses were recorded from the rat phrenic nerve/hemidiaphragm muscle using a standard *in vitro* setup (32°C) at low (0.1 Hz) and high (50 Hz/2 s) frequencies of nerve stimulation. Preparations were exposed to sufficient vecuronium to produce around 50 % fade of the tetani (1.0 – 1.3 μM). They were then exposed to 30 μM PPADS for 25 min and the effect that this had on the vecuronium-induced tetanic fade was assessed. Experiments were also performed in the presence of 1 μM of the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) to eliminate the possibility that any effect of PPADS observed was due to its ability to prevent ATP being converted to adenosine.

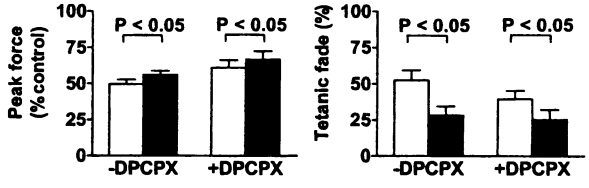
Vecuronium produced a reduction ($P < 0.05$, paired Student's t test) in tension elicited at 0.1 Hz in the absence and presence of DPCPX. This was not affected by 30 μM PPADS (Table 1).

Table 1: Effect of 30 μM PPADS on the depression of twitch force (0.1 Hz) by vecuronium. Data are %control.

	Before PPADS	After PPADS
-DPCPX (n=8)	86.5 ± 3.4 (n=8)	89.5 ± 5.8 (n=8)
+DPCPX (n=8)	90.4 ± 3.7 (n=8)	88.2 ± 5.1 (n=8)

For tetanic contractions, in addition to vecuronium producing around 40 - 50% fade, (Fig. 1, right) it depressed the peak of the tetani by around 40 % (Fig. 1, left). PPADS increased the peak height of the tetanus and attenuated tetanic fade irrespective of the presence or absence of DPCPX (Fig. 1).

Figure 1: Depression of tetani by vecuronium in the absence (open) and presence (filled) of 30 μM PPADS.



Further studies are required to determine the exact mechanism underlying this facilitatory effect of PPADS. However, the results are consistent with the idea that endogenous ATP, presumably derived from vesicular exocytosis and acting via P2X receptors, has a direct inhibitory effect on neuromuscular function in the rat, possibly by decreasing ACh release.

Giniatullin, R. A. & Sokolova, E. M. (1998). *Br. J. Pharmacol.*, **124,** 839-844.
Redman, R. S. & Silinsky, E. M. (1994). *J. Physiol.*, **477,** 117-127.
Ribeiro, J. A. & Walker J. (1975). *Br. J. Pharmacol.*, **52,** 213-218.

102P CHARACTERISATION OF P2X RECEPTORS IN RAT DORSAL ROOT GANGLION SENSORY NEURONS

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Adenosine 5'-triphosphate (ATP) stimulates sensory neurons via P2X receptors. Seven P2X subtypes have been cloned ($P2X_{1-7}$) and the predominant one found in sensory neurons is the $P2X_3$ receptor. We have shown that neurones in intact rat dorsal root ganglia (DRG) do not respond to the $P2X_3$ receptor agonists ATP or α,β-meATP (Assis *et al.*, 2002), but after acute enzymatic dissociation these agonists evoke an inward current (Robertson *et al.*, 1996; Rae *et al.*, 1998) and a rise in intracellular [Ca^{2+}] (Assis *et al.*, 2002). Thus, dissociation appears to induce upregulation of $P2X_3$ receptors. The aim of this study was to further characterise the rise in intracellular [Ca^{2+}] evoked by $P2X_3$ receptor agonists in acutely dissociated rat DRG neurons.

DRG were removed from all spinal levels of neonatal rats, and incubated with dispase and collagenase (both 2.5 mg.ml^{-1}) for 45-60 min at 37°C. Cells were released by gentle trituration, plated onto precoated coverslips and kept under tissue culture conditions for up to 36 h. The cells were then perfused at 2-4 ml.min^{-1} with a HEPES-buffered solution and the intracellular [Ca^{2+}] monitored in single cells (20-25 μm dia) using the Ca^{2+} sensitive dye fura-2. Drugs were applied in the superfusate and cells were only accepted for analysis if they responded to KCl (50 mM). The data are expressed as mean ± s.e.m mean and were analysed statistically by 1-way ANOVA and Tukey's comparison.

Initial control experiments determined the reproducibility of the response to KCl. KCl (50 mM) evoked a rapid rise in intracellular [Ca^{2+}] in all cells used and when it was applied four times at 15 min intervals to 8 cells, the peak response was 83 ± 6%, 81 ± 4% and 77 ± 5% respectively, of the first response. These values were not significantly different from control.

ATP (100 μM) evoked a rise in intracellular [Ca^{2+}] in 19/28 cells. Only 12/19 cells responded to a second application of ATP. When ATP was applied three times at 15 min intervals the peak response was 69 ± 9% and 64 ± 8% of the first response (n=10). Again, these values were not significantly different from control. In contrast, α,β-meATP (3 μM) evoked a rise in intracellular [Ca^{2+}] in only 2/35 cells tested.

These results show that acutely dissociated rat DRG neurones respond to repeated application of agonists with reproducible changes in intracellular [Ca^{2+}]. Although all cells used responded to KCl, only 68% of cells were responsive to ATP. Surprisingly, α,β-meATP (3 μM) evoked a rise in intracellular [Ca^{2+}] in only 6 % of cells. The reason for this difference between ATP and α,β-meATP is currently under study.

Assis, T.S., Rowan, E.G. & Kennedy, C. (2002). *Br. J. Pharmacol.*, **135,** 62P.
Rae, M.G., Rowan, E.G. & Kennedy, C. (1998). *Br. J. Pharmacol.*, **124,** 176-180.
Robertson, S.J., Rae, M.G., Rowan, E.G. *et al.* (1996). *Br. J. Pharmacol.*, **118,** 951-956.

103P P2X RECEPTOR-LIKE IMMUNOREACTIVITY IN THE GUINEA-PIG VAS DEFERENS

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Adenosine 5'-triphosphate (ATP) is released as a cotransmitter with noradrenaline from sympathetic nerves of the guinea-pig vas deferens and evokes smooth muscle contraction via ionotropic $P2X_1$ receptors (Sneddon *et al.*, 1996). We and others have characterised pharmacologically a second site of action of ATP in this tissue that is distinct from the $P2X_1$ receptor (Bailey & Hourani, 1994; Reilly & Hirst, 1996; Kennedy & Westfall, 2002). The aim of this study was to investigate the expression of $P2X_1$, $P2X_2$ and $P2X_4$ receptors in the guinea-pig vas deferens using immunohistochemistry.

Four male albino guinea-pigs (400-500 g) were killed by cervical dislocation. The vasa deferentia were fixed in 10% neutral buffered formalin, wax embedded and sectioned at 6 μm. Three rat anti-P2X subunit antisera were used, each recognising sequences near the intracellular carboxy terminus (anti-$P2X_1$: DPVATSSTLGLQENMRTS, residues 382-399; anti-$P2X_2$: SQQDSTSTDPKGLAQL, residues 457-472; anti-$P2X_4$: KKYKYVEDYEQGLSGEMNQ, residues 370-388). In sections of guinea-pig vas deferens, P2X subunit-like immunoreactivity (lir) was visualised using the avidin-biotin technique. Omission of the primary antiserum or pre-absorption with the cognate peptides served as negative controls. Though raised against rat P2X receptor antigens, these antisera are known to recognise the guinea-pig homologues (Zhong *et al.*, 2000). To ensure that the anti-$P2X_2$ and anti-$P2X_4$ antisera possessed avidity for the rat receptor subunits we performed standard Western blotting experiments using HEK293 cells stably expressing rat $P2X_{1,2,3}$ or $_4$ subunits (HEK293-$P2X_{1-4}$).

In sections of guinea-pig vas deferens, $P2X_1$-lir was evident in the plasma membrane of smooth muscle cells; immunostaining was particularly strong in the outermost layer. $P2X_2$-lir was detected on the apical membrane of epithelial cells. No specific $P2X_2$-lir was observed in the smooth muscle. $P2X_4$-lir was present in diffuse patches throughout the smooth muscle layers. Intense, punctate immunostaining was seen within these patches. No immunostaining was observed in negative controls. In Western blots using the anti-$P2X_2$ antiserum, bands were observed only in HEK293-$P2X_2$ membrane preparations. Similarly, in HEK293-$P2X_4$ preparations only the anti-P2X4 antiserum possessed high avidity.

In summary, the pattern of $P2X_1$-lir is likely to correspond to the functional $P2X_1$ receptors seen in several species. The localisation of $P2X_2$-lir to the epithelial cell membrane suggests a role for this receptor subunit in absorption or secretion. Moreover, this study shows that the $P2X_4$ receptor is expressed in vas deferens smooth muscle and so is a candidate for the second site of ATP action in this tissue.

Bailey, S.J. & Hourani, S.M.O. (1994). *Br. J. Pharmacol.*, **112**, 219-225.
Kennedy, C. & Westfall, T.D. (2002). *Br. J. Pharmacol.*, **135**, 51P.
Reilly, M.J. & Hirst, G.D.S. (1996). *J. Autonom. Nerv. Sys.*, **57**, 93-100.
Sneddon, P., *et al.* (1996). *Seminars in Neurosci.*, **8**, 201-205.
Zhong, Y., *et al.* (2000). *J. Physiol.*, **523**, 391-402.

104P BIOCHEMICAL CHARACTERISATION OF A SOLUBLE ATPASE RELEASED FROM SYMPATHETIC NERVES

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Adenosine 5'-triphosphate (ATP) is a cotransmitter in many peripheral neurons. We have described previously the kinetic and pharmacological characterisation of a soluble ATPase released from sympathetic nerves of the rabbit isolated vas deferens (Westfall *et al.*, 2000). The aim of this study was to characterise this enzyme biochemically.

Samples were collected from electrically-stimulated (8 Hz, 25 sec) rabbit isolated vas deferens superfused with HEPES buffer (pH 7.4) maintained at 37°C and bubbled with O_2. Superfusates were assayed for ATPase activity using a luciferin-luciferase assay system or by HPLC, as previously described (Westfall *et al.*, 2000). Samples of active superfusate were concentrated using microcentrifuge filters and subjected to standard SDS-PAGE gel electrophoresis or non-denaturing gel electrophoresis. Some samples of active superfusate were incubated with 8-azido[γ-^{32}P]ATP (80 μM; 5 μCi; 10 min), photoaffinity labeled by UV irradiation (2-10 min) and separated on SDS-PAGE gels prior to exposure to X-ray film overnight. In these experiments, a recombinant GST-p38 MAP kinase was used as a positive control. ATP-binding proteins were isolated from active superfusates by affinity binding with agarose beads crosslinked to ATP, followed by elution using high salt concentrations and separation on SDS-PAGE gels. Selected silver-stained bands obtained by SDS-PAGE electrophoresis were destained and sequenced (automated Edman degradation; M. Berne, TUCF, Boston).

Silver staining of denaturing and non-denaturing gels revealed five distinct bands of between ~45 and ~150 kDa (n = 9). A strongly stained band was observed at ~70 kDa. Photoaffinity labelling experiments (n = 3) also revealed a strongly labelled band with a molecular weight of ~70 kDa. In two control experiments, radiolabeled bands representing the GST-p38 MAP kinase were detected at the expected molecular weights. Affinity binding experiments (n = 4) showed that the 70 kDa band bound specifically to crosslinked ATP. Four bands with molecular weights approximating those of cloned ecto-ATPases (90, 70, 60 and 45 kDa; see Zimmerman, 2000) were prepared for sequencing. 100% homology was observed with rabbit transferrin precursor (35% coverage; MW ~77 kDa), rabbit serum albumin precursor (66% coverage; MW ~69 kDa) and rabbit α-1-antiproteinase F precursor (8% coverage; MW ~46). Insufficient protein was present in the 90 kDa band to allow sequencing.

These data suggest that the detection of a 70 kDa band in photoaffinity labelling experiments may be due to non-specific binding of a charged ATP species to serum albumin. Rabbit serum albumin appears to bind ATP, but has no ATPase activity (unpublished observations). It is probable, then, that the protein responsible for the ATPase activity is the heaviest (unsequenced) band or is present in quantities lower than the limit of silver staining detection. Bulk purification using affinity binding is likely to allow its isolation and sequencing.

Westfall, T.D., *et al.*, 2000. *Br. J. Pharmacol.* **131**, 909-914.
Zimmerman, H., 2000. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 299-309.

105P USE OF FLIPR™ IN THE CHARACTERISATION OF VOLTAGE-GATED SODIUM CHANNELS IN THE SH-SY5Y HUMAN NEUROBLASTOMA CELL LINE

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Voltage-gated sodium channels (VGSC's) are critical in the regulation of neuronal excitability and are potential targets for drugs to treat pain, cardiac arrhythmia and epilepsy (Clare *et al.*, 2000). Current electrophysiological methods of measuring sodium channel activity do not support the demands of high-throughput screening (HTS). The present study aimed to develop a fluorescence-based HTS assay using the Fluometric Imaging Plate Reader (FLIPR™) platform to characterise VGSC's in SH-SY5Y cells which possess a TTX sensitive inward sodium current (Toselli *et al.*, 1996).

Undifferentiated SH-SY5Y cells, passage 15-20, were routinely cultured and plated in Dulbecco's Modified Eagle's Medium with 20% Fetal Calf Serum. Fluorescent membrane potential based assays were performed by pre-treating the cells (200µl final volume, 30,000 cell/well) with Na^+ channel antagonists and FLIPR™ membrane potential dye made up in Hanks' Balanced Salt Solution buffer, final pH =7.4. VGSC activation by 30µM veratridine induced a fluorescence change, which was measured over a 90 second period. Data were quantified as a percentage of the 30µM veratridine response. Electrophysiological recordings were made to confirm TTX sensitivity using subconfluent undifferentiated cells on cover slips. Whole-cell voltage-clamp recordings were made at 20 ± 2°C with microelectrodes containing CsF *inter alia*. Inward sodium currents were activated by voltage steps from –100 to –20 mV (20ms at 0.2Hz). All data are shown as mean pIC_{50} ± s.e.m.

Table 1. Effect of sodium channel blockers on veratridine evoked Na^+ channel activation in SH-SY5Y cells (n=11-21).

Compound	Mean	s.e.m
Tetrodotoxin	7.60	0.05
Amitriptyline	5.65	0.04
Sipatrigine	5.39	0.03
Flecainide	4.50	0.02
Lidocaine	3.86	0.05
Procaine	3.04	0.06

The rank order of pIC_{50} for the Na^+ channel blockers tested using FLIPR™ are shown in Table 1. The data are in accord with published data using rat cortical synaptosomes (Deffois & Carter., 1996), and our electrophysiological recordings confirm TTX sensitivity in these cells (pIC_{50} 8.6 ± 0.1). This study demonstrates that Na^+ channel blockers can be characterised using commercially available FLIPR™ membrane potential dye in an HTS paradigm that utilises non-transfected human derived cells. FLIPR™ pIC_{50}'s and TTX sensitivities were consistent with data from our electrophysiology studies and previously published data.

Clare, J.J., *et al.*, (2000) *Therapeutic Focus.* 5, 506-520
Deffois, A. & Carter, C., (1996), *Neurosci. Lett.* 220, 117-120
Toselli,, M., *et al.*, (1996) *J. Neurophysiol.* 76, 3920-7

106P EFFECT OF SOMATOSTATIN ON CATECHOLAMINE RELEASE FROM BOVINE ADRENAL MEDULLARY CELLS

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Somatostatin (SS) acts as a neurotransmitter and neuromodulador in the central nervous system and in the periphery, producing multiple effects, through interactions with membrane receptors. It has been shown to decrease the release of noradrenaline from sympathetic nerve terminals (Goethert, 1980; Calhau *et al.*, 2000). The aim of this work was to investigate, using bovine adrenal chromaffin cells, the effect of somatostatin on adrenal catecholamine (CA) release, under basal conditions and during KCl or cholinergic stimulation.

Bovine adrenal chromaffin cells were isolated from fresh adrenal glands by collagenase digestion using a standard procedure (Livett, 1984) with minor modifications. Chromaffin cells, cultured in Dulbecco's Modified Eagle's medium/F12 Ham supplemented with 10% fetal calf serum, were plated in collagen-coated 24-well plastic culture dishes at a density of 4-5x10^5-10^6 cells/well. Culture medium was replaced every 24 h. For the experiments, cells were used after 4 days in culture. For studies on endogenous CA release, cells were preincubated for 10 min followed by a 15 min incubation period under basal conditions or in the presence of KCl (50 mM) or acetylcholine (ACh) (10 mM). Simultaneously, the same conditions were tested in the presence of SS (0.01-1 µM) or the stable analogue, octreotide (1, 10 µM). Values (mean ±SEM) were compared by Student's t-test.

Chromaffin cells synthesised and accumulated large amounts of adrenaline (AD), noradrenaline (NA) and dopamine (DA). The release of the three CAs by the cells was markedly increased by KCl (to 363±60%, 326±57% and 267±35% of the spontaneous release of AD, NA and DA, respectively; n=18). ACh was also found to enhance, albeit to a lesser extent than KCl, the release of AD, NA and DA (to 208±22%, 213±24% and 216±33% of their respective spontaneous release; n=15).

At all concentrations tested neither SS nor octreotide had any effect on basal CA secretion. However, CA release induced by ACh was significantly enhanced by SS in a concentration-dependent manner. The maximal effect of SS on AD, NA and DA release induced by Ach was an increase to 219±24% (n=6), 159±9% (n=6) and 142±10% of control (n=6), respectively. This produced an increase in the AD/NA ratio from 0.97±0.03% to 1.40±0.15% (n=6). Moreover, SS was able to enhance, in a concentration-dependent manner, the KCl-evoked release of NA (to a maximum of 159±24% of control; n=7).

Except for an inhibitory effect on the KCl-induced release of AD (to 36±6% of control; n=6) at 10 µM, octreotide had no significant effect on KCl- or ACh-induced CA release

In conclusion, SS exerts an effect on adrenal medullary cells opposite to that observed in sympathetic nerve terminals. Comparison of SS effects with those of octreotide suggest that the increase in ACh-elicited CA release by SS involves SS receptors 1 and/or 4.

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Calhau, C. *et al.* (2000) *Pharmacol. Res.* 41, 497-501.
Goethert, M. (1980) *Nature* 288, 86-89.
Livett, B.G. (1984) *Physiol. Rev.* 64, 1103-61.

107P STUDIES ON THE CHOLINERGIC INNERVATION OF THE MOUSE ISOLATED VAS DEFERENS

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The rodent vas deferens receives a dense sympathetic innervation and has been used extensively to study transmitter release mechanisms. Recently, Jackson and Cunnane (2002) described a population of nerves in the mouse vas deferens, which were insensitive to high concentrations of the adrenergic neurone blocker, bretylium.

When set up for tension recording, the vas deferens responds to long trains of 200 stimuli (10 Hz) with a biphasic contraction (amplitude 3.3 ± 0.7 mN (fast), 2.3 ± 0.4 mN (slow), n = 6): the first has been attributed to ATP and the second, to noradrenaline. In this study, we have examined the pharmacology of this nerve-evoked contraction in more detail.

Aganglionic mouse (Balb/C, 8-12 weeks) vasa deferentia were suspended in 25 mL organ baths, attached to isometric recording transducers and allowed to equilibrate for 1 h under an initial resting tension of 9.8 mN. Platinum ring electrodes (positioned around the proximal end of the vas deferens) were used to apply trains of stimuli (pulse width 0.5 ms, 80 V, 10 Hz) to evoke neurogenic contractions.

Neostigmine increased the amplitude of the slow component (200 pulses, 10 Hz) from 1.3 ± 0.2 mN to 4.2 ± 0.3 (1 μM, $P < 0.05$ (Student's t-test); n = 6) or to 5.6 ± 0.6 mN (10 μM, $P < 0.05$, n = 6) and this effect was reversed by atropine (1 μM; 1.3 ± 0.4 $P < 0.05$, n = 4). Bretylium (20 μM) blocked the characteristic biphasic neurogenic contraction (0.04 ± 0.05 mN (fast), 0.08 ± 0.06 mN (slow), n = 6) but a new residual, low-amplitude component was revealed in 5 out of 12 preparations. Interestingly, this residual component increased in amplitude over the time period of exposure to bretylium, typically reaching a maximum amplitude (3.6 ± 0.6 mN, n = 5) after approximately 5 hours. In four of the five preparations, the residual component was of greater amplitude than the slow component in control preparations. Amphetamine (20 μM) reversed the inhibitory effect of bretylium.

Cyclopentolate (0.1 μM) reduced the amplitude of the residual component from a control amplitude of 3.6 ± 0.6 mN (n = 5) to 0.1 ± 0.04 mN (n = 5). Neostigmine (1 μM) reversed the cyclopentolate-induced blockade of the residual component in 2 out of 3 preparations.

Next we determined whether nicotinic acetylcholine receptors (nAChRs) were present on the nerve terminals of the cholinergic nerves. Nicotine (30 μM) was added to preparations in which the residual component remained in the presence of cyclopentolate and neostigmine (n = 2), 10 s prior to the expected onset of the evoked contraction. Nicotine potentiated the residual component of contraction (136%, 152%). Thus the mouse vas deferens is innervated by functional cholinergic nerves, limited by cholinesterase action, and release can be modulated by activation of nAChRs.

Jackson, V.M. & Cunnane, T.C. (2002), *Br. J. Pharmacol.* **135**, 1845 – 1850

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108P EFFECTS OF LYSOPHOSPHATIDIC ACID ON PERIPHERAL SOMATOSENSORY PROCESSING

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Lysophosphatidic acid (LPA) is a bioactive phospholipid controlling numerous cellular responses through the activation of specific G-protein coupled receptors. The G_i-coupled LPA receptor vzg-1 (edg 2) is present on polymodal sensory neurons in adult mice (Renbäck *et al.*, 2000). This study aimed to determine the effects of peripheral injection of LPA on somatosensory processing in anaesthetised rats.

Extracellular recordings of convergent dorsal horn neurons were made in anaesthetised (1% halothane in 66% N_2O / 33% O_2) male Sprague Dawley rats (230-300g) (Chapman *et al.*, 1994). Spinal Neuronal responses to peripheral injection of LPA (0.05-5.0μg / 5μl) into the receptive field were characterised (n=10 rats). Responses were quantified as neuronal firing rate (Hz) and duration of firing (s). Spinal neuronal responses to mechanical von Frey stimulation (6, 8, 12, 21, 45 and 80g) of the receptive field were quantified as neuronal firing rate (Hz) during a 10sec stimulus duration. Control responses were determined, effects of peripheral injection of LPA (5.0μg / 5μl) into the receptive field, on von Frey evoked responses of spinal neurons was followed for 60 minutes (n=8 rats). Data are presented as mean maximal effects and standard error of the mean; statistical analysis was performed using repeated measures ANOVA and Dunnett's *post hoc* test.

Peripheral injection of LPA increased the total firing of the neuronal population studied in a concentration dependent manner (Fig.1A).

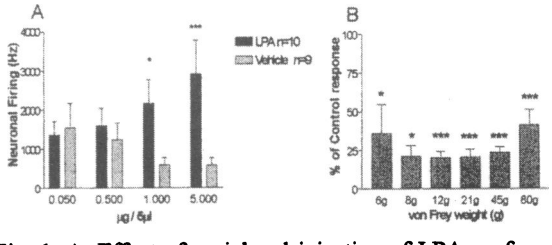

Fig. 1. A. Effect of peripheral injection of LPA on frequency of firing of spinal neurons. B. Effect of peripheral injection of 5μg / 5μl LPA on mechanical punctuate-evoked responses of spinal neurons at 60 mins post injection. $^{*}p<0.05$, $^{***}p<0.001$

Peripheral injection of LPA (5μg / 5μl) significantly reduced the mechanical punctuate-evoked responses of spinal neurons (Fig. 1B).

Peripheral injection of LPA produced a dose-related increase in the frequency of firing of spinal neurons, indicative of activation of primary afferent fibres. Mechanical evoked responses were attenuated by LPA, this effect may represent desensitisation of the LPA receptor(s) which influences mechanical transduction mechanisms.

Chapman V, Haley JE & Dickenson AH (1994). *Anesthesiol.* 81,1429-1435
Renbäck K, Inoue M, Yoshida A *et al.* (2000). *Mol. Brain Res.* 75, 350-354

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109P EFFECTS OF CANNABINOID (CB) AGONISTS ON RAT HIPPOCAMPAL NEURONAL FIRING RATE ACTIVITY USING MULTI-CHANNEL EXTRACELLULAR RECORDING

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In view of recent evidence supporting the existence of a novel CB receptor in addition to CB_1 receptors expressed in the hippocampus (Hajos *et al* 2001), an evaluation of cannabinoid-evoked responses was undertaken. The effects of the non-selective $CB_{1/2}$ receptor agonists Δ-9-THC, CP 55,940, WIN 55,212-2, and the endogenous CB ligand noladin ether (Fezza *et al* 2002), were examined on the spontaneous discharge activity of rat hippocampal neurones.

Multiple single-unit extracellular activity was recorded from spontaneously discharging hippocampal CA1 neurones (mean firing rat 18.0 ± 1.9 Hz s.e.m; n=143) in fourteen adult male Sprague-Dawley rats under isoflurane-N_2O:O_2 anaesthesia. Stereotactically manipulated eight-channel microwire electrode arrays (NB Labs, Texas USA) were used connected to a Plexon Multineurone Acquisition Processor system (Jagger *et al* 1999); multichannel data was analysed using NeuroEXplorer (Plexon Inc., Texas USA). Cannabinoid agonists (0.05-1.0 $mg.kg^{-1}$), the CB_1 antagonist SR141716A (0.1$mg.kg^{-1}$), or vehicle were administered i.v. Data are presented as mean ± s.e.m. and compared by Student's t-test (Prism version 3; GraphPad, USA).

The predominate effect of cannabinoid agonists was an inhibition of CA1 neuronal firing. Δ-9-THC induced a dose-dependent inhibition of firing rate (46 ± 8% response at 0.3 mg kg^{-1}, $p<0.05$) in 19/35 (54%) neurones, which was reversed by SR141716A; 13/35 (37%) neurones were activated and 3 cells (9%) were not affected. WIN55,212-2 produced a dose-dependent inhibition of firing rate (48 ± 4% response at 0.1mg kg^{-1}, $p<0.001$) in 33/42 (79%) neurones; 2 (5%) neurones were activated and 7/42 (17%) were not effected. CP55,940 at 0.1 $mg.kg^{-1}$ inhibited firing rate (50 ± 4% response, $p<0.01$) in 40/43 (93%) neurones and activated 3 (7%) neurones, with only a partial reversal (67%) of inhibition in the forty neurones by SR141716A; CP55,940 at doses >0.1$mg.kg^{-1}$ induced respiratory depression. Noladin at 0.2 $mg.kg^{-1}$ inhibited (52 ± 8% response, $p<0.05$) firing rate in 15/23 (65%) neurones; 6 cells (26%) were activated and 2 (9%) not affected.

Multichannel recording revealed simultaneously sub-populations of neurones exhibiting inhibition or activation by cannabinoid agonists. CB_1 agonists produced a dose-dependent inhibition of firing in the majority of neurones recorded with an EC_{50} rank order of WIN55,212-2 = CP55,940 > Δ-9-THC, responses were blocked by the CB1 antagonist SR141716A. These data support the view of differential sensitivity of hippocampal neurones to cannabinoid agonists. The study also illustrates the effectiveness of multichannel recording in obtaining a high neuronal sample per animal.

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Jagger E *et al* 1999 *Brit. J. Pharmacol.* **128** 206P
Fezza F *et al.* 2002 *F E B S Letters* **513** 294-298
Hajos *et al* 2001 *Neuroscience* **106** (1) 1-2

110P EFFECT OF NEUROPEPTIDE Y RECEPTOR ANTAGONISTS BIBO3304 AND CGP71683A ON NEUROPEPTIDE Y-EVOKED RESPONSES IN THE RAT LATERAL HYPOTHALAMUS

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Neuropeptide Y (NPY) is the most potent physiological stimulator of food intake yet identified (Duhault *et al.,* 2000). In the hypothalamus, NPY neurones are found primarily in the arcuate nucleus (ARC), where they project to other areas involved in the control of energy balance, including the lateral hypothalamus (LH; Williams *et al.,* 2000). NPY is thought to mediate its effects on food intake through Y1 and Y5 receptor subtypes (Duhault *et al.,* 2000). The aims of this study were to determine electrophysiological responses of single lateral hypothalamic neurons to NPY, and further to use the Y1 antagonist (BIBO3304) and the Y5 antagonist (CGP71683A; Duhault *et al.,* 2000) to determine the contribution of these receptors in the response to NPY.

Male Sprague-Dawley rats (250-350g) were anaesthetised using isofluorane, decapitated and the brain removed, trimmed and sliced to a thickness of 500μm using a vibrotome to obtain hypothalamic slices. Slices were then transferred to a recording chamber and incubated for 1h while being perfused (1$ml.min^{-1}$) with gassed (95%O_2:5%CO_2; 35°C) aCSF. Spontaneous single-unit extracellular activity was recorded from LH neurons using a *NeuroLog* recording system (Digitimer, U.K.), collected using *Spike 2* (CED, UK), and analysed off-line using *NeuroExplorer* (Nex, USA). All data are presented as mean±s.e.m. Statistical significance levels were determined using an unpaired t-test or one way ANOVA.

LH cells exhibited spontaneous discharge activity (4.5±0.8Hz, n=24). NPY (100-500nM) applied for 1 minute, caused significant dose-dependent decreases in neuronal firing rate in 11/12 cells tested (100nM: 44.5±11.3%, n=10, $p<0.01$; 200nM: 81.2±10.6%, n=7, $p<0.001$; 500nM: 90.3±4.3%, n=3, $p<0.001$). The response to NPY did not show recovery within 45 minutes in any of the 11 responsive neurons tested, thus the antagonist experiments were performed on different populations of neurons. N-methyl-D-aspartate acid (50μM, 1 minute) was applied at the end of each experiment to excite the cell as a measure of viability.

The 200nM NPY-evoked decrease in neuronal firing (81.2±10.6%) was significantly attenuated ($p<0.01$) when NPY (200nM) was co-perfused with either the Y1 antagonist BIBO3304 (12.2±19.4%; 1μM; n=6) or Y5 antagonist CGP71683A (15.5±7.1%; 1μM; n=6;). Antagonists alone caused no significant decrease ($p>0.05$) on firing rate (BIBO3304: 4.8±9.3%; CGP71683A: 16.6±6.8%; n=6 per antagonist).

These results suggest that both NPY Y1 and NPY Y5 receptors have a role to play in the lateral hypothalamic response to NPY.

Supported by Knoll Ltd & University of Nottingham

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Duhault J. *et al.,* (2000). *Can. J Physiol. Pharm..* 78, 173-185
Williams G. *et al.,* (2000). *Proc. Nutr. Soc.* 59, 385-396

111P THE EFFECT OF 5,7-DIHYDROXYTRYPTAMINE ON CENTRAL CANNABINOID RECEPTOR FUNCTION.

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5-HT is a neurotransmitter known to be intimately involved in affective disorders and several *in vitro* and *in vivo* studies have shown that serotonergic and cannabinergic neuronal systems interact (Nakazi *et al* 2000, Malone and Taylor 1998). We have previously shown that *in vivo* depletion of 5-HT levels using pCPA (p-chlorophenylalanine) abolished cannabinoid receptor agonist-stimulated [^{35}S]-GTPγS binding *in vitro* in the cerebral cortex and cerebellum and attenuated it in the striatum and hippocampus. (Overbury *et al* 2002). Our interpretation of these data is that an intact 5-HT system is required for the full expression of CB_1 coupling to G proteins. The present study tested the selectivity of the pCPA-mediated inhibitions by determining the effect of 5,7-dihydroxytryptamine (5,7-DHT), a serotonergic neurotoxin, on CB_1-stimulated [^{35}S]-GTPγS binding in rat brain sections.

Male Lister-Hooded rats were treated with desipramine (10mg/kg, i.p.) to prevent 5,7-DHT uptake into noradrenergic terminals and 30 mins later they were anaesthetised using isofluorane and placed in a stereotaxic frame. Bilateral i.c.v. (A.P. -0.8mm, M.L. ± 1.5mm, D.V. -3.8mm measured from bregma) injections (5μl per ventricle) of either vehicle (artificial C.S.F.) or 5,7-DHT (diluted to 15mg/ml in artificial C.S.F., resulting in a dose of 150μg per rat) were given to each rat. The rats were allowed to recover and were singly housed for 2 weeks before being killed by decapitation on day 14. The frontal cortex of each brain was removed and stored at –80°C for analysis of 5-HT by HPLC with electrochemical detection. The brains were then frozen on dry ice; 20μm sections were cut using a cryostat and mounted on gelatin-coated glass slides for autoradiographic measurement of [^{35}S]-GTPγS binding as described by Sim et al 1995. Sections were incubated with [^{35}S]-GTPγS in the presence and absence of the cannabinoid receptor agonist HU-210 (1μM). The slides were exposed to photographic film, which was developed after 3 days. [^{35}S]-GTPγS binding was quantified in four brain areas using "NIH image" software. Data were converted into % of basal and statistically analysed using an unpaired *t*-test.

HPLC analysis showed a 73% reduction in 5-HT levels (vehicle-treated: 6.7 pmol/mg (± 1.2), 5,7-DHT-treated: 1.8 pmol/mg (±0.3)) in the frontal cortex of brains treated with 5,7-DHT (p=0.03, n=3, unpaired *t*-test)

The CB receptor agonist HU-210 (1μM) increased [^{35}S]-GTPγS binding in brains of vehicle-treated rats to the following levels cortex: 210% (±35%) striatum: 430% (±155%), hippocampus: 190% (±17%), cerebellum: 182% (±31%) (% basal, n=3). Pre-treatment with 5,7-DHT had no effect on basal binding but decreased binding in the presence of HU-210 (cortex: 90% (±9%); p=0.029, striatum: 100% (±9%); p=0.099, hippocampus: 98% (±12%); p=0.011, cerebellum: 86% (±9%); p=0.040. n=3).

These results mirror those seen with pCPA, thereby providing further evidence that depletion of 5-HT causes the decrease in agonist stimulated [^{35}S]-GTPγS binding seen in these brains. This further corroborates the suggestion that an intact 5-HT system is required for the full expression of CB_1 coupling to G proteins, although the mechanism underlying this effect is yet to be determined.

Malone D.T & Taylor D.A. (1998). *Br. J. Pharmacol.* **124**, 1419-24

Nakazi M., *et al* (2000). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **361**, 19-24

Overbury A.L., Kendall D.A., Marsden C.A. (2002) *Br. J.Pharmacol (in press).*

Sim L.J., Selley D.E. & Childers S.R. (1995). *Proc. Natl. Acad. Sci. USA* ,**92**, 7242-7246

112P A FUNCTIONAL STUDY OF REGIONAL DIFFERENCES IN CANNABINOID AND VANILLOID RECEPTORS IN THE ANAESTHETISED RAT

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Anandamide is one of several endogenous cannabinoid (CB) receptor ligands, and is also a full agonist at the vanilloid receptor (VR1) (Smart *et al.*, 2000). Whilst CB_1 and VR1 receptors are co-expressed on sensory nerves, it is possible that differences in the receptors exist between nerves innervating arteries and veins. Previous work in our laboratory suggests arterial vasosensory nerves have a greater proportion of VR1 relative to CB_1 (Smith & McQueen, 2001). The aim of the present study was to compare the reflex cardiovascular and respiratory (CVR) responses evoked by intra-arterial (i.a.) and intra-venous (i.v.) anandamide, and to determine the relative contribution of CB_1 and VR1 receptors mediating the responses, using selective receptor antagonists.

Experiments were licensed under U.K. Home Office regulations. Male Wistar rats (354 ± 15 g, n = 18) were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p., maintained with a 25 mg $kg^{-1}hr^{-1}$ i.v. infusion *via* a jugular vein cannula). A tracheal cannula was inserted and connected to a pneumotachograph head linked to an electrospirometer and a computerised recording system for measuring and recording tracheal airflow, and respiratory minute volume (RMV). Animals breathed room air spontaneously. The right carotid artery was cannulated for measurement of mean arterial pressure (MAP) via a blood pressure transducer. The left femoral artery and vein were cannulated for drug administration. CVR reflexes evoked by bolus injections (0.1 ml, washed in with 0.2 ml saline, over 2 sec) of anandamide (288 - 1725 nmoles; ≥ 20 min between doses), before and after antagonist for either the CB_1 (SR141716, 1 mg kg^{-1}; 5 min pre-treatment) or VR1 (capsazepine, 1mg kg^{-1}; 5 - 10 s pre-treatment) receptor, were investigated.

Data (mean ± s.e.mean) for ~ equieffective doses of anandamide i.a. and i.v. are presented in Table 1. Anandamide caused a fall in MAP and an increase in RMV. Sensitivity to antagonists at CB_1 or VR1 depended on the route of drug administration.

Table 1a. Anandamide-induced changes in blood pressure (ΔMAP, mmHg)

	i.a. (575 nmoles)	i.v. (863 nmoles)
before SR141716	-23 ± 13	-23 ± 5
after SR141716	-18 ± 3	2 ± 1 **
before capsazepine	-19 ± 4	-14 ± 4 †
after capsazepine	8 ± 4 **	-3 ± 3 †

Table 1b. Anandamide-induced changes in ventilation (ΔRMV, ml min^{-1})

	i.a. (288 nmoles)	i.v. (863 nmoles)
before SR141716	31.6 ± 3.0	36.7 ± 9.8
after SR141716	24.4 ± 9.2	-1.1 ± 2.2 *
before capsazepine	54.3 ± 15.5	26.7 ± 0.9 †
after capsazepine	6.5 ± 3.1 *	-0.7 ± 4.1 †

*$P \leq 0.05$; **$P \leq 0.01$, before vs. after; paired Student's *t*-test (where n = 4-6). † n = 3.

These data suggest that while both CB_1 and VR1 receptors contribute to the CVR response to i.v. administered anandamide, the i.a. route is predominantly VR1-mediated. Further experiments are required to determine whether this is due to differences in the proportion of CB_1:VR1 located on arterial and venous sensory nerves.

Smart, D., Gunthorpe, M.J., Jerman, J.C., *et al.* (2000). *Br. J. Pharmacol.*, **129**, 227-230.

SMITH, P.J.W. & McQUEEN, D.S. (2001). *Br. J. Pharmacol.*, **134**, 655-663.

113P THE CB_2 SELECTIVE CANNABINOID AGONIST JWH-015 ATTENUATES SENSORY NEUROTRANSMISSION IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

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In the rat mesenteric arterial bed calcitonin gene-related peptide (CGRP) is released upon activation of sensory nerves producing vasodilatation (Kawasaki *et al.*, 1988). We have previously reported that cannabinoid agonists WIN55,212 and CP55,940 inhibit sensory neurotransmission via prejunctional CB_1-like cannabinoid receptors in the rat mesenteric bed (Duncan *et al.*, 2001a,b). Putative CB_2-like receptors have been implicated in the antinociceptive actions of palmitoylethanolamide (Pertwee 2001), and the antinociceptive and hypotensive effects of HU-308 (Hanuš *et al.*, 2001). Since CB_1 and CB_2 receptor protein has been detected in dorsal root ganglion cells (Ross *et al.*, 2001), we investigated whether the selective CB_2 receptor agonist JWH-015 can attenuate sensory neurotransmission in the rat mesenteric arterial bed.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused with oxygenated Krebs' solution containing guanethidine (5μM) to block sympathetic neurotransmission (Ralevic & Kendall, 2001). After 30 min equilibration, preparations were preconstricted with methoxamine (10-100μM) and three consecutive frequency response curves to electrical field stimulation (EFS, 1-12Hz, 60V, 0.1ms, 30s) (EFS control, EFSI and EFSII) were constructed in each preparation. JWH-015 or vehicle (0.01% ethanol) was added after EFS control, 15 min before EFSI. Antagonists were added at the start of the equilibration period. In separate preparations, dose response curves were constructed to CGRP (0.05 pmol – 0.5 nmol) in the presence of JWH-015 (1μM) and ethanol (1.7 μM/0.01%). Data are expressed as mean±s.e.m. and analysed by ANOVA with Tukey's post hoc test or by Student's unpaired t test.

EFS produced frequency-dependent relaxation (1-12Hz) of the rat mesenteric bed. JWH-015 (0.1 - 1μM) attenuated sensory neurogenic relaxation evoked during EFSI and EFSII compared with EFS control in a concentration-dependent manner. In the presence of 1μM JWH-015 the response at a submaximal frequency of 8Hz was reduced from 51.65±2.72%, EFS control, to 33.52±2.84%, EFSII (n=6, $P<0.001$).

The CB_2 receptor antagonist SR144528 had no effect on JWH-015-mediated inhibition (8Hz, EFS control, 59.41±6.62% to EFSII, 33.43±5.11%, n=9, $P<0.05$). The selective CB_1 receptor antagonist SR141716A also failed to block inhibition of the relaxation response by JWH-015 (8Hz, EFS control, 74.77±5.11% to EFSII, 40.74±8.9%, n=5, $P<0.05$). There was no significant difference between EFS control, EFSI and EFSII generated in the presence of 0.01% ethanol. JWH-015 failed to affect the response to CGRP (pEC_{50} =10.13±0.2 and 10.5±0.1 in the presence and absence respectively of 1μM JWH-015; $P<0.05$, unpaired t test).

These data show that JWH-015 attenuates sensory neurogenic relaxation in the rat isolated mesenteric arterial bed. The inhibitory actions of JWH-015 cannot be blocked by CB_1 and CB_2 antagonists possibly indicating that its actions are mediated by a novel receptor subtype. JWH-015 was found to have no inhibitory actions on vasorelaxation produced by exogenous CGRP indicating that its site of action is prejunctional.

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Duncan, M., Kendall, D.A. & Ralevic, V., (2001a) *Br. J. Pharmacol.* **135**, 65P
Duncan, M., Kendall, D.A. & Ralevic, V., (2001b) *Br. J. Pharmacol.* **135**, 285P
Pertwee, R.G., (2001) *Prog. in Neurobiol.*, **63**, 569-611.
Kawasaki, H. *et al.* (1988) *Nature*, **335**, 165-167.
Hanuš et al., (1988) *Gen. Pharmacol.*, **19**, 1-43.
Ross, R.A. *et al.* (2001) *Neuropharmacology* **40**, 221-32.

114P OLEAMIDE STIMULATES [^{35}S]GTPγS BINDING IN RAT BRAIN PREPARATIONS VIA A CANNABINOID RECEPTOR

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Oleamide (cis-9,10-octadecamide, ODA) is the primary amide of oleic acid and a member of a growing family of fatty acid amide transmitter substances which include the endogenous cannabinoid anandamide. Systemic administration of ODA induces sleep in rats (Murillo-Rodriguez *et al*, 2001) and has been shown to produce many of the characteristics associated with the cannabinoids including the behavioural tetrad (Mechoulam *et al*, 1997). It has, however, been proposed that the effects of ODA do not result from direct activation of cannabinoid receptors but are instead mediated by an 'entourage' effect due to competition for the endocannabinoid metabolising enzyme fatty acid amide hydrolase (Mechoulam *et al*, 1997). However, a recent study by Cheer *et al* (1999) demonstrated that micromolar concentrations of oleamide inhibited binding of the CB_1 receptor agonist [^{3}H]CP55,940 to rat cerebellar membranes. The current study used [^{35}S]GTPγS binding and autoradiographic techniques to determine whether ODA has direct agonist activity at brain CB_1 receptors.

Male Lister Hooded rats (350g, Nottingham University BMSU) were decapitated and the brains removed. Brains were either frozen on dry ice for later autoradiographic analysis or immediately processed for membrane binding. Washed whole brain homogenates (minus brain stem) were immediately prepared on ice in Tris buffer (50mM Tris, 10mM EDTA). These were homogenised using a glass/Teflon homogeniser and centrifuged at 20000 × *g* for 10 mins at 4°C. The pellet was resuspended in Tris buffer and homogenisation and centrifugation were repeated a further two times. The final pellet was resuspended in storage buffer (50 mM Tris, 1mM EDTA) and stored at –80°C until required. For autoradiography 20μm coronal brain sections (including representative examples of the cortex, striatum, hippocampus and cerebellum) were cut using a cryostat, mounted on gelatin coated slides and stored at –80°C. [^{35}S]GTPγS autoradiography was carried out according to Sim *et al* (1995), and [^{35}S]GTPγS binding according to Traynor and Nahorski (1995). Curve fitting was carried out using Graphpad Prism3 software.

At a maximally effective concentration (1μM), the potent CB receptor agonist HU210 stimulated [^{35}S]GTPγS binding to 194±6% of basal (n=10) with an EC_{50} of 1.46nM. A Maximally effective concentration of ODA (100μM) stimulated binding to 188±14% of basal (n=9) with an EC_{50} of 1.24μM. Both HU210- and ODA-stimulated increases in [^{35}S]GTPγS binding were reversed by the CB_1 antagonist SR141716A. The IC_{50} value for SR141716A in the presence of ODA was 1nM. Another CB_1 antagonist, LY320135 (1μM), reduced ODA stimulated binding to 116±3% of basal (n=3). Similar increases in [^{35}S]GTPγS binding were seen in brain regions analysed using autoradiography. In the hippocampus HU210 (1μM) increased binding to 190±16% ($P<0.05$) of basal (n=3). ODA (10μM) increased levels of binding to 173±19% ($P<0.05$) of basal (n=3).

These findings provide evidence that oleamide both binds to, and activates central CB_1 cannabinoid receptors.

Traynor et al, (1995), *Molecular Pharmacology*, 47(4), p848-854
Sim *et al*, (1995), *Proc. Natl. Acad. Sci.*, 92, p7242-7246
Mechoulam *et al*, (1997), *Nature*, 389, 6646, p25-26
Cheer *et al*, (1999), *Neuropharmacology*, 38, 4, p533-541
Murillo-Rodriguez *et al*, (2001), *Neuro. Letts.*, 313, p61-64

115P THE EFFECT OF *N*-SUBSTITUTION OF (*RS*)-4-CARBOXYPHENYLGLYCINE ON ANTAGONIST ACTIVITY AT GROUP I METABOTROPIC GLUTAMATE RECEPTORS EXPRESSED ON NEONATAL RAT MOTONEURONES

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(*S*)-4-Carboxyphenylglycine (4-CPG) antagonises group I metabotropic glutamate (mGlu) receptors expressed on neonatal rat spinal motoneurones with an apparent K_D of 208 μM (Pook et al., 1993). Here we examine the effects of three structurally related compounds that differ only in the nature of the *N*-substituent, UBP1105, UBP1106 and UBP1115 (Figure 1) as group I mGlu receptor antagonists.

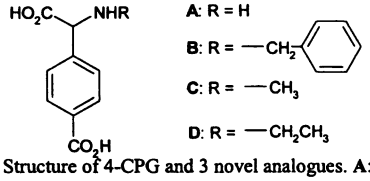

Figure 1. Structure of 4-CPG and 3 novel analogues. **A**: 4-CPG, **B**: UBP1115, **C**: UBP1105, **D**: UBP1106.

All experiments were performed on isolated hemisected spinal cords from neonatal rats (2-5 days old) bathed in a physiological saline (Evans et al., 1982) with tetrodotoxin (TTX: 10 μM for 2 min, then 0.1 μM continuously) added to the perfusate. Saline flow was 1 $ml.min^{-1}$ throughout the experiment. Recordings were made from a ventral root. All agonists were applied for 1 min with a 20 min interval between applications to avoid desensitisation. The antagonists were applied for 15 min prior to agonist application.

UBP1105 and UBP1106 (both 1 mM) were examined as antagonists of group I mGlu receptors by measuring the % antagonism of (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid ((1*S*,3*R*)-ACPD)-induced depolarisations. This produced a 49 ± 5% and 34 ± 10 % reduction of (1*S*,3*R*)-ACPD-induced depolarisations, respectively (mean ± SEM, n=3). In contrast, 200 μM UBP1115 produced a 72 ± 5 % reduction of depolarisations induced by the group I mGlu receptor agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) (mean ± SEM, n=3). UBP1105 and UBP1106 were not as potent as 4-CPG as antagonists of group I mGlu receptors and were not characterised further.

A non-cumulative, non-sequential concentration-response curve (CRC) was constructed to DHPG. After the first CRC was constructed 400 μM UBP1115 was added to the perfusate and a second CRC constructed. In the presence of 400 μM UBP1115 the CRC to DHPG was shifted rightwards with an apparent K_D of 96.6 ± 29.4 μM (mean ± SEM, n=4).

UBP1115 (200 μM) was then tested for antagonist activity against 10 μM NMDA, 2 μM kainate and 0.5 μM AMPA-induced depolarisations and did not alter any of these depolarisations (n=3).

These experiments show that *N*-substitution can enhance the potency of group I mGlu receptor antagonists making it a novel site for improving the potency and possibly the selectivity of group I mGlu receptor antagonists. In addition, substitution at this site does not alter the selectivity of the antagonists with respect to ionotropic glutamate receptors.

The authors acknowledge financial support from the MRC.

Evans, R.H., Francis, A.A., Jones, A.W. et al., (1982). *Br. J. Pharmacol.* **75**:65-75.
Pook, P.C.-K., Birse, E.F., Jane, D.E. et al., (1993). *Br. J. Pharmacol Proceedings Supplement* **108**:87P.

116P EFFECT OF 5-SUBSTITUTION OF N^3-SUBSTITUTED WILLARDIINE DERIVATIVES ON ANTAGONIST ACTIVITY AT AMPA RECEPTORS EXPRESSED ON NEONATAL RAT MOTONEURONES

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Increasing the inter-acidic group chain length of willardiine by adding N^3-substituents to the uracil ring has been shown to convert its agonist action at AMPA receptors into antagonism (More *et al.*, 2001; More *et al.*, 2002). In the current study the effect of adding substituents to the 5-position of the uracil ring of such antagonists was examined. The ability of (*S*)-3-(2-carboxyethyl)-5-nitrowillardiine ((*S*)-5-N-CEW), (*S*)-3-(2-carboxyethyl)-5-iodowillardiine ((*S*)-5-I-CEW) and (*S*)-5-iodo-3-(2-tetrazol-5-ylethyl)willardiine ((*S*)-5-I-TEW) to act as AMPA receptor antagonists has been assessed using the neonatal rat hemisected spinal cord preparation.

To assess AMPA receptor antagonist activity the ability of the compounds to block the fast component of the dorsal root evoked ventral root potential (fDR-VRP) in the neonatal rat hemisected spinal cord preparation was measured (More *et al.*, 2001). Recordings were made from ventral roots from 2-5 day old rats following supramaximal stimulation of the corresponding dorsal root (Evans *et al.*, 1982). Non-cumulative concentration response curves were constructed for the antagonists (5 min applications) in the presence of 2 mM $MgSO_4$ / 50 μM (*R*)-AP5 (30 min pre-incubation) to block NMDA receptors. Results are expressed as mean ± s.e.m., n=3.

The IC_{50} values for the ability of the compounds to depress the fDR-VRP are summarised in Table 1. When these results are compared to that of (*S*)-3-(2-carboxyethyl)willardiine, which gave an IC_{50} value of 23.8 ± 3.9 μM (More *et al.*, 2002), it is

Table 1: IC_{50} values for the depression of the fDR-VRP

Compound	IC_{50} (μM)
(*S*)-5-N-CEW	79.9 ± 15.9
(*S*)-5-I-CEW	13.7 ± 1.7
(*S*)-5-I-TEW	3.7 ± 0.8

suggested that adding a nitro group to the 5-position of the uracil ring has decreased the potency of AMPA receptor antagonism. Conversely, adding an iodo group to this position appears to slightly enhance the potency. Similarly, (*S*)-5-I-TEW is more potent than (*S*)-3-(2-tetrazol-5-ylethyl)willardiine, which gave an IC_{50} value of 6.87 ± 1.39 μM (More *et al.*, 2002). The data also corroborate earlier findings which suggested tetrazole substituted derivatives are more potent than the corresponding carboxylic acid derivatives as AMPA receptor antagonists (More *et al.*, 2002).

It is suggested from this preliminary data that when substituents are added to the 5-position of N^3-substituted willardiine derivatives the order of potency of AMPA receptor antagonism is $I \geq H > NO_2$. (*S*)-5-I-TEW is currently the most potent AMPA receptor antagonist derived from the willardiine structure. It is hoped that further structural changes using this information may lead to the development of compounds with high potency and selectivity as AMPA receptor antagonists.

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Evans, R.H., Francis, A.A., Smith, D.A.S. *et al.* (1982) *Br. J. Pharmacol.* 75, 65-75.
More, J., Troop, H. & Jane, D. (2001) *Br. J. Pharmacol.* 133, 152P.
More, J., Troop, H. & Jane, D. (2002) *Br. J. Pharmacol.* (in press).

117P MICRODIALYSIS STUDIES IN THE FRONTAL CORTEX OF THE FREELY MOVING RAT TO INVESTIGATE THE INTERACTION BETWEEN THE NOVEL AMPAKINE™ CX 516 AND SEROTONERGIC NEUROTRANSMISSION

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CX 516 is a brain penetrating, positive allosteric modulator of AMPA receptors (Yamada, 2000). AMPA receptors are distributed throughout the CNS and may be localised on 5-HT nerve terminals to tonically regulate 5-HT neurotransmission (Tao et al., 1997). Here we report the effects of CX 516 alone and in combination with AMPA on 5-HT release and metabolism in the rat frontal cortex and the possible involvement of 5-HT_{1A} receptors in the mechanisms.

Male rats (Wistar, 250 - 300 g, Harlan) were anaesthetised using a mixture (1:1) of hypnorm and hypnovel before guide cannulae were stereotaxically inserted; rats were allowed at least a week for recovery. A microdialysis probe (4 mm membrane) was inserted into the frontal cortex (mm, A +3.5, L -1.5, V -5.5, relative to Bregma, Paxinos & Watson, 1986) and perfused with artificial cerebrospinal fluid (aCSF) at 2 µl/min. Dialysate 5-HT and 5-HIAA levels were analysed by HPLC coupled with electrochemical detection (ECD).

Local administration of AMPA (100 and 300 µM, via the microdialysis probe) increased the 5-HT levels to 103 ± 13 (n=5) and 175 ± 20 % (n=4, $P<0.05$, Student's t test vs basal) of basal (defined as mean of 3 samples prior to drug administration). The 5-HIAA levels were reduced to 80 ± 4 (n=5, $P<0.05$) and 68 ± 2 % (n=4, $P<0.01$, Student's t test vs basal) of basal. CX 516 (100 mg/kg, i.p.) alone reduced 5-HT levels to 60 ± 3 % (n=4, $P<0.01$, Student's t test vs basal) of basal but failed to modify 5-HIAA levels.

The reduction in 5-HT levels induced by CX 516 was reversed by the AMPA antagonist CNQX (2.5 mg kg^{-1} i.p.) which by itself had no effect on 5-HT levels. When co-administered with AMPA (100 µM), CX 516 (100 mg kg^{-1}, i.p.) significantly potentiated the 5-HT levels to 191 ± 19 % (n=4, $P<0.05$, Student's t test vs basal) of basal. CNQX (50 µM), alone had no effect on either 5-HT or 5-HIAA levels, but completely prevented the effect of co-administration of CX 516 and AMPA on 5-HT levels. Local administration of CX 516 (1.0 mM) alone or in combination with AMPA (100 µM) failed to modify 5-HT levels in rat frontal cortex. WAY100635 (0.5 mg kg^{-1}, i.p.) alone had no effect on either 5-HT or 5-HIAA levels. However, when given 60 min prior to CX 516 (100 mg kg^{-1}, i.p.) plus AMPA (100 µM), WAY100635 (0.5 mg kg^{-1}, i.p.) significantly attenuated the potentiation of 5-HT release but failed to modify the reduction in 5-HIAA levels.

The present studies demonstrate that AMPA receptors can modulate 5-HT release and metabolism in rat frontal cortex, and in particular CX 516 may potentiate serotonergic function via an interaction with glutamatergic neurotransmission in the CNS. Somatodendritic 5-HT_{1A} receptors localised in raphe nuclei may be involved in the response of the 5-HT system to CX 516 plus AMPA since the effect is attenuated by systemic WAY100635. Local administration of CX 516 plus AMPA to the frontal cortex failed to modify 5-HT release.

Cotton, J. & Partin, K. (2000) *Neuropharmacology* 39, 21-31.
Yamada K.A. (2000) *Expert Opinion on Investigational Drugs*. 9, 765-778.
Paxinos, G. & Watson, C. (1986), *Academic Press*, London.
Tao, R., Ma, Z.Y. & Auerbach, S.B. (1997) *Br. J. Pharmacol.* 121, 1707-1715.

118P UP-REGULATION OF METABOTROPIC GLUTAMATE RECEPTOR 5 ($MGLUR_5$) MRNA IN SPINAL CORD IN A CLINICAL MODEL OF INFLAMMATION AND HYPERALGESIA

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A specific role for spinal group I mGluRs ($mGluR_1$ and $mGluR_5$) in acute nociceptive processing (Dolan and Nolan, 2000) and inflammation-evoked hyperalgesia has been described previously in behavioural studies (Fisher and Coderre, 1996). The aim of this study was to characterise the expression of $mGluR_1$ and $mGluR_5$ mRNA in spinal cord in a clinical model of acute inflammatory disease and hyperalgesia.

Thresholds to noxious mechanical stimulation of each leg were measured in Newtons (as described in Nolan *et al.*, 1987) in adult female sheep affected by unilateral hindlimb lameness, clinically diagnosed as 'footrot', a bacterial infection of the digital tissues (n = 6), and healthy-control sheep (n = 6). Animals were euthanased and spinal cord tissue (L6-S2) collected, sectioned mid-line and processed for $mGluR_1$ and $mGluR_5$ mRNA expression using real-time RT-PCR, and *in situ* hybridisation. Relative quantification of mRNA by real-time PCR was performed by normalizing mGluR levels to the "housekeeping genes" β-actin and glyceraldehyde-3-phosphate dehydrogenase. Optical density of silver grain staining (*in situ* hybridisation) was measured using a computerised image analysis system (Scion Image, v.beta 3b, Scion Corporation, USA). Data presented are mean ± S.E.M., and were analysed using analysis of variance with post-hoc Tukey's test.

Mechanical thresholds were significantly reduced on the limb affected by lameness compared to healthy-control animals (9.5 ± 2 N and 18.9 ± 1 N, respectively; $p < 0.001$), and compared to all other limbs (48.8 ± 6% decrease; $p < 0.01$). There was no difference between non-affected limb thresholds and control sheep limb thresholds. Real-time PCR revealed a significant up-regulation of $mGluR_5$ mRNA in ipsilateral spinal cord, relative to control spinal cord (> 10-fold increase, $p < 0.05$). *In situ* hybridization analyses identified $mGluR_1$ and $mGluR_5$ mRNA in spinal cord dorsal horn, with particularly high levels of $mGluR_5$ in laminae I-II neurons. A significant increase in $mGluR_5$ expression in laminae I-II in ipsilateral spinal cord was detected relative to control levels (33 ± 5% increase; $p < 0.05$). No change in expression of $mGluR_1$ mRNA was detected in spinal cord from lame animals compared to control animals.

The up-regulation of $mGluR_5$ in laminae I-II neurons (corresponding to terminal fields of primary afferent nociceptive fibres), ipsilateral to the site of inflammation, suggests that $mGluR_5$ may be involved in mediating the hyperalgesic response in this model of clinical inflammation.

Dolan, S., & Nolan, A.M., (2000) *Neuropharmacol.* 39, 1132-1138.
Fisher, K., & Coderre, T.J., (1996) *Pain* 68, 255-263.
Nolan, A., Livingston, A., Morris, R., *et al.*, (1987_ *J. Pharmacol. Meth.* 17, 39-49.

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119P HYDROGEN PEROXIDE AND FERROUS CHLORIDE POTENTIATE LIPID PEROXIDATION BY QUINOLINIC ACID IN RAT BRAIN HOMOGENATES

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Quinolinic acid induces lipid peroxidation in rat brain homogenates, an effect which may contribute to its neurotoxicity (Rios & Santamaria, 1991; Behan *et al.*, 1999). This induction of lipid peroxidation *in vitro* has been shown to be dependent on the presence of iron (Stipek *et al.*, 1997), but few data exist regarding the interaction of quinolinic acid with other known oxidising agents, and the precise mechanism by which quinolinic acid exerts its effects on lipid peroxidation remains unknown. The aim of this study was to examine the interaction of quinolinic acid with hydrogen peroxide and ferrous chloride on lipid peroxidation in rat brain homogenates.

Male Wistar rats (110-130g) were used in all experiments (n=4). Brains were removed and a 20% wv^{-1} homogenate was prepared in ice cold Tris-HCl buffer (20mM pH 7.4) with 5mM butylated hydroxytoluene. Homogenates (475µl) were incubated with quinolinic acid (100, 250 or 1000µM) alone and in combination with hydrogen peroxide 0.01% vv^{-1} and/or ferrous chloride (20 or 100µM) for 30 min at 37^0C. The concentrations of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal were measured using a Bioxytech LPO-586 colorimetric assay kit. Data are mean ± s.e.m. and statistical significance was determined by ANOVA followed by a Bonferroni post-test for multiple comparisons with $p<0.05$ as the limit of significance.

Quinolinic acid alone up to 1mM exerted no significant effect on lipid peroxidation when compared to Tris control. However, when hydrogen peroxide was present, quinolinic acid exerted a dose-dependent increase in lipid peroxidation, reaching significance ($p<0.01$) at 1mM [0.931±0.096 nmol mg^{-1} protein] when compared to quinolinic acid alone [0.514±0.106 nmol/mg protein]. This could be further potentiated by the addition of ferrous chloride 20µM, when levels of peroxidation products were increased [1.807±0.223 nmol mg^{-1} protein; ($p<0.001$)] compared to the combination of quinolinic acid 1mM with hydrogen peroxide. The addition of ferrous chloride at 100µM significantly increased lipid peroxidation by quinolinic acid 100µM [1.8±0.377 nmol mg^{-1} protein; ($p<0.01$)], 250µM [1.582±0.152 nmol mg^{-1} protein; ($p<0.01$)] and 1mM [2.463±0.242 nmol mg^{-1} protein; ($p<0.001$)] when compared to the respective combination of quinolinic acid with hydrogen peroxide.

These results would suggest that lipid peroxidation produced by quinolinic acid can be increased by hydrogen peroxide and Fe^{2+} ions

Behan, W.M.H., McDonald, M., Darlington, L.G., *et al.* (1999) *Br. J. Pharmacol.*, **128**, 1754-1760.
Rios, C. & Santamaria, A. (1991) *Neurochem. Res.*, **16**, 1139-1143.
Stipek, S., Stastny, F., Platenik, J., *et al.* (1997) *Neurochem. Int.*, **30**, 233-237.

120P DIFFERENTIAL EFFECTS OF PRENYLATION INHIBITORS ON LONG-TERM POTENTIATION IN AREA CA1 OF ADULT RAT HIPPOCAMPUS

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Isoprenylated proteins constitute approximately 0.5% of proteins in the cell and are characterized by the presence of either farnesyl or geranylgeranyl isoprenoids. Post-translational prenylation of these proteins is a prerequisite for their subsequent membrane localization, biological function and protein-protein interactions. Prenylation reactions are carried out by farnesyl transferase (FTase) or geranylgeranyl transferase (GGT) enzymes at the C-terminus "CAAX box" motif, where C is cysteine, A an aliphatic residue, and X any amino acid. Here we show contrasting effects on the degree of potentiation following high frequency stimulation (HFS) when hippocampal slice preparations are incubated with either an FTase inhibitor or GGT inhibitor.

Slices (450µm thick) of rat hippocampus were prepared from male Wistar rats (120-150 g) as described by O'Kane and Stone (2000). Test stimulation was given at 20s intervals via a concentric bipolar electrode placed in the stratum radiatum. The preparation was allowed to stabilize before recordings of orthodromic extracellular excitatory postsynaptic potentials (EPSP) were made from the stratum radiatum in area CA1. High frequency stimulation (100 Hz for 1 s) was delivered to slices after a baseline response was obtained and was sufficient to produce robust and reproducible long-term potentiation of evoked potentials. Responses were quantified as the slope of the negative-going arm of the evoked population EPSP response. Drugs were dissolved in DMSO and diluted in aCSF to make up to test concentrations. Slices were incubated with drugs for at least 2 hours before recordings were made, during which time drugs were added to the superfusing aCSF.

Incubation with, and perfusion of the FTase inhibitor, FTase I (200 nM) resulted in a significant decrease in potentiation of EPSP slope following HFS compared to control slices. 30 min following HFS, EPSP slope was potentiated 170.3 ± 8.8 % in control slices (n=4) compared with 117.4 ± 5.3 % in slices which had been previously incubated in, and perfused with FTase I (n=5, $p<0.001$, ANOVA).

In contrast, prior incubation with, and perfusion of the GGT inhibitor, GGTI-287 (30nM), resulted in a higher degree of potentiation of EPSP slope compared with controls (n=3) following HFS. In this case, 30min following HFS, EPSP slope was potentiated 147.3 ± 1.89 % in controls compared with 196.1 ± 15.9 % in drug-treated slices ($p<0.05$, ANOVA).

These results indicate that isoprenylated proteins play a role in the sequence of events following HFS to produce long-term potentiation in CA1 of adult rat hippocampus and suggest that different prenylation patterns may affect the degree of potentiation following HFS. We are currently investigating possible candidate proteins that may be involved.

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O'Kane, E. M. & Stone, T. W. *Eur J Pharmacol* **409**, 159-66. (2000).

121P CYTOSKELETAL CHANGES IN STRESS AND DEPRESSION: INVOLVEMENT OF THE MICROTUBULAR NETWORK IN NEURONAL PLASTICITY FAILURE

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Neuronal plasticity (i.e. structural adaptation of neurons to functional requirements) requires serotonin (5-HT) as regulator of synaptogenesis (Lauder, 1990) and synthesis of microtubular proteins (cytoskeletal component) such as tyrosinated isoform of α -tubulin (tyr-tub) (Contin & Arce, 2000). The aetiology of depression is correlated to changes in the CNS monoamine levels such as 5-HT and a major effect of antidepressant drugs consists in the increase of 5-HT at the synaptic level (Blier & de Montigny, 1994). Recent pre-clinical and clinical studies have shown that stress and depression result in neuronal atrophy and cell death (neuronal plasticity failure) (Duman *et al.*, 1999). This implies that not only neurotransmitter systems such as 5-HT but also central morphological events are involved in these pathologies. The hippocampus is the most important brain area in learning and memory processes. It has been reported that stressful conditions such as restraint stress (RS) increase 5-HT level (Kirby *et al.*, 1997) and generate neuronal plasticity failure in hippocampus (Duman *et al.*, 1999). This could result in the reduction of hippocampal volume and in the deficit in cognition and memory observed in depressed patients (Duman *et al.*, 1999).

The aim of the present work was to investigate the expression of tyr-tub in the hippocampus of rats submitted to RS. In addition, hippocampal 5-HT levels were monitored to analyse the influence of such a condition upon central 5-HT level. Naive Sprague Dawley adult rats (250-300g) were submitted to acute (6h for 1 day, n=4) and sub-chronic (6 h for 4 days every day, n=4) RS and were sacrificed after the immobilisation period. The brain was removed, the hippocampus dissected, homogenised and processed for: a) Differential Pulse Voltammetry (DPV) analyses of 5-HT levels (Crespi, 1990) b) Western Blot analyses of the tyr-tub expression.

Our results showed that following acute and sub-chronic stress hippocampal 5-HT levels were significantly increased ($p<0.05$) to 142±15% and 135±11% of control respectively. In contrast to the 5-HT levels the tyr-tub expression was decreased to 90±11% following acute stress or significantly decreased to 70±7% following chronic stress ($p<0.01$). While the electrochemical findings are in agreement with a previous report (Kirby *et al.*, 1997), the original finding of this work is the observation that RS induces molecular changes at the level of the microtubules which may result in the neuronal plasticity failure described above.

In conclusion, these molecular cytoskeletal changes may be novel target for the development of alternative antidepressant drugs.

Blier P. & de Montigny C. (1994), TiPS **15**, 220-226.
Contin M.A. & Arce C.A. (2000), Neurochem. Res., **25**, 27-36.
Crespi F. (1990), Neurosci. Methods, **34**, 53-65.
Duman R.S., Malberg J., Thome J. (1999), Biol. Psychiatry, 46,1181-1191.
Lauder J.M. (1990), Ann. N.Y. Accad. Sci., **600**, 297-310.
Kirby L.G., Chou-Green J.M., Davis K. *et al.* (1997), Brain Res., **760**, 218-230.

122P DIFFERENTIAL EXPRESSION OF BDNF EXONS IN RAT BRAIN AFTER SYSTEMIC ADMINISTRATION OF PAROXETINE AND TRANYLCYPROMINE

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The gene for brain-derived neurotrophic factor (BDNF) gives rise to four major transcript forms. All of which contain a common 3' exon, which codes for the BDNF protein as well as an additional unique 5' exon (Timmusk et al, 1993). Recently, BDNF has been implicated in the treatment of depression. Thus, by using probes directed to all BDNF transcripts, administration of electroconvulsive shocks (ECS) has been shown to produce robust and long-lasting changes in rat brain BDNF mRNA expression (Zetterström et al, 1998). The effect of antidepressant drugs on BDNF mRNA levels is however, less pronounced and complicated by a bi-phasic pattern (Coppel and Zetterström, 2000), involving down-regulation at 4h and up-regulation at 24h following repeated administration in the dentate gyrus (DG) of the hippocampus. Here, we report the effects of two different antidepressant drugs, paroxetine (parox) and tranylcypromine (TCP) on BDNF exon-specific mRNA distribution in the hippocampus. The exon-specific probes used comprise of that for exon IV which shares properties with immediate early genes (ieg) and exon I whose expression is dependent on ongoing protein synthesis. For comparison a probe directed to the total BDNF gene was also used.

Groups of six male Sprague-Dawley rats (225-250g) were administered with either paroxetine (5 mg/kg), TCP (5 mg/kg), or saline (control). After 4 hours rats were killed, their brains removed and frozen in isopentane prior to being processed for *in-situ* hybridisation using ^{35}S labelled oligonucleotide probes specific to the relevant BDNF exon mRNA (Zetterström et al, 1998). Autoradiograms were quantified by computer-aided densitometry using NIH imaging programme.

Administration of paroxetine or TCP significantly inhibited expression of the exon IV transcript as well as that for the total BDNF gene in parts of the hippocampus (DG, exon IV and total BDNF mRNA; CA3, only total BDNF mRNA). In contrast, no significant changes were seen for exon I, see table below.

Drug /Area	Total BDNF	BDNF exon I	BDNF exonIV
Parox/CA1	100±5	102±2	92±4
TCP/CA1	94±3	91±3	85±3
Parox/CA3	81±7*	105±2	89±4
TCP/CA3	79±3*	101±4	84±4
Parox/DG	79±3*	101±4	75±3*
TCP/DG	57±4**	87±4	70±5**

Data are mean ±s.e.m. Values (n=6) and expressed as % of saline controls (100%). *$p<0.01$; **$p<0.001$ versus controls (Bonferroni post-hoc test following ANOVA).

In conclusion, we confirm here previous findings that some antidepressant drugs decrease total BDNF gene expression at 4 h after a single administration (Coppel and Zetterström, 2000) An effect which could be mediated by changes in exon IV (ieg type gene) rather than exon I (protein synthesis dependent type).

Coppel, A. L. and Zetterström T.S.C. (2000). *Br. J. Pharmacology.* **129**: 134
Timmusk, T. P. K., Palm, K., Metsis, M *et* al (1993) *Neuron* **10**: 475-489.
Zetterström T.S.C., Pei, Q., Grahame-Smith, D.G. *et al* (1998) *Molecular Brain Research*, **57**:106-110.

123P 1-METHYL-4-PHENYLPYRIDINIUM (MPP^+) UPTAKE BY THE JAR HUMAN PLACENTAL CHORIOCARCINOMA CELL LINE

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The aim of this work was to characterize the uptake of MPP^+, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in a model of the placental barrier, the human choriocarcinoma cell line, Jar. MPP^+ is a substrate for several distinct plasmalemmal transporters, including the serotonin transporter (SERT), the extraneuronal monoamine transporter (EMT) and the organic cation transporters type I and II (OCT1 and OCT2) (Gründemann *et al.*, 1999; Sitte *et al.*, 2001). As the placenta, as well as Jar cells, express SERT, a comparison between the uptake of ^{3}H-MPP^+ and ^{3}H-serotonin (^{3}H-5HT) was made.

Jar cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose and 10% fetal calf serum. For the experiments, the cells were seeded on 24-well plastic cell culture clusters (2 cm^2) precoated with 0.1 g/l poly-L-ornithine. After 2-4 days in culture (90-100% confluence), the cells were used in uptake experiments. Except for IC_{50}'s, results are presented as means±SEM. IC_{50}'s are presented as means with 95% confidence limits. Data were analysed by one-way ANOVA followed by Dunnett test.

^{3}H-5HT (0.2 μM) uptake by Jar cells was found to be temperature-dependent (uptake at 4°C was reduced to 14±1% of control; n=4), as well as Na^+- and potential-dependent (replacement of NaCl by LiCl, choline chloride or KCl reduced uptake to <20% of control; n=2).

The SERT inhibitors desipramine and fluoxetine concentration-dependently inhibited ^{3}H-5HT uptake (their IC_{50}'s were found to be 0.17 μM (0.04-0.67 μM; n=4) and 0.05 μM (0.02-0.1 μM; n=4), respectively). Decynium22 and corticosterone are specific inhibitors of EMT/OCT1/OCT2. Decynium22 showed no effect on ^{3}H-5HT uptake (up to 1 μM; n=4) and corticosterone produced a slightly inhibitory and concentration-independent effect (at 100 μM, it reduced uptake to 76±11% of control; n=4). In the presence of MPP^+ (0.02 or 1 mM), ^{3}H-5HT uptake was reduced to 72±1% and 19±2% of control, respectively (n=6).

A component of ^{3}H-MPP^+ (0.2 μM) uptake was temperature-dependent (uptake at 4°C was reduced to 64±9% of control; n=9), as well as Na^+- and potential-dependent (replacement of NaCl by LiCl, choline chloride or KCl reduced uptake to 84±9%, 76±6% and 61±6% of control; n=6). Desipramine and fluoxetine significantly inhibited ^{3}H-MPP^+ uptake (300 μM desipramine and 100 μM fluoxetine reduced uptake to 62±4% (n=5) and 82±11% (n=6) of control, respectively). Decynium22 (up to 1 μM; n=6) and corticosterone (up to 100 μM; n=6) had no effect. In the presence of 5HT (0.02 or1 mM), ^{3}H-MPP^+ uptake was reduced to 64±6% and 66±2% of control, respectively (n=4).

In conclusion, the results suggest that: (1) both ^{3}H-5HT and ^{3}H-MPP^+ are taken up by Jar cells through SERT; (2) EMT, OCT1 and OCT2 are not functionally present at the apical membrane of Jar cells.

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Gründemann, D. *et al.* (1999) *Mol. Pharmacol.* 56: 1-10.
Sitte, H.H. *et al.* (2001) *Mol. Pharmacol.* 59: 1129-1137.

124P ACUTE AND CHRONIC PCP-INDUCED CHANGES IN PSD95 mRNA EXPRESSION IN RAT PREFRONTAL CORTEX: LACK OF MODULATION BY CLOZAPINE AND HALOPERIDOL

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Phencyclidine (PCP) is a NMDA receptor antagonist which has been shown to produce a metabolic hypofunction (Cochran *et al.*, 2001) similar to that observed in schizophrenia (Wu *et al.*, 1991). Post density protein 95 (PSD95) is known to bind to NMDA receptor subunits and to be involved in synaptic plasticity. Human postmortem studies have shown alterations PSD95 mRNA in the prefrontal cortex of schizophrenic brains (Ohuma, *et al.*, 2000).

The aim of this study was to investigate the effect of acute and chronic PCP administration on PSD95 mRNA expression and to examine if any PCP-induced changes could be altered by acute and chronic clozapine or haloperidol treatment

For the acute PCP treatment, adult male hooded Long Evans rats were treated with PCP (2.58mg/kg i.p.) or vehicle (sterile saline) for 23 hours then treated for 1 hour with clozapine (20mg/kg i.p), haloperidol (1mg/kg i.p.) or vehicle (0.5% glacial acetic acid) (n=6 for all groups) then sacrificed. For the chronic treatment regime, PCP (2.58 mg/kg i.p.) was administered intermittently over a 26 day period (Cochran *et al.*, 2001). On day 8 of the treatment regime osmotic minipumps were implanted under halothane anaesthetia to deliver a dose of 1mg/kg/day haloperidol or 20mg/kg/day clozapine or vehicle (3% glacial acetic acid in 0.9% saline) (n=6 for all groups). 72 hours after the final exposure to PCP the animals were sacrificed. In *situ* hybridisation was carried out according to Simpson and Morris *(1994)*. Data were analysed by one way ANOVA followed by the post hoc test.

$p < 0.05$ was defined as significant. Chronic intermittent PCP treatment produced a significant increase (14%) in PSD95 mRNA expression in the prelimbic region of the prefrontal cortex ($F(3,23) = 3.128$, $P < 0.05$) which was not reversed by chronic clozapine or haloperidol (Figure 1). Acute administration of PCP failed to show any significant alterations in PSD95 mRNA expression.

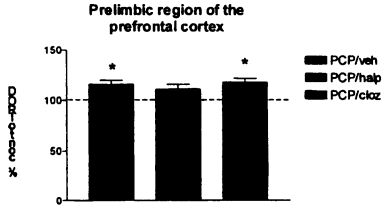

Figure 1 Effect of chronic PCP treatment in the prelimbic region of the prefrontal cortex on PSD95 mRNA expression. No reversal with clozapine or haloperidol. *=p<0.05 compared to veh/veh. Data expressed as % control (ROD = Relative Optical Density).

These results suggest that repeated exposure to PCP leads to neuroadaptive changes in the NMDA receptor complex and adds further weight to the use of chronic PCP as a model for mirroring prefrontal cortex dysfunction in schizophrenia.

Wu, J.C., Buchsbaum, M.S. and Bunney, W. E. *et al.*, (1991), *Japanese Journal of Psychopharmacology*, **1**, 47-8.
Cochran, S.M., McKerchar, C. E., Pratt. J.A. *et al.*, (2001) *Society of Neuroscience Abstract* No 733.10.
Wisden and Morris (1994.) *In situ hybridisation protocols for the brain*, 9-34 Academic Press
Ohnuma, T., Kato, H., Arai, H., *et al.*, (2000). Gene expression of PSD95 in prefrontal cortex and hippocampus in schizophrenia. *Neuroreport*, **11**, 3133-7.

125P GABAERGIC NEURONE GENE MARKERS; MODULATION OF EXPRESSION IN THE RAT PREFRONTAL CORTEX BY ACUTE AND SUBCHRONIC PCP

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There is increasing evidence that defective GABAergic neurotransmission is likely to play a role in schizophrenia. Several studies on human post mortem tissue show changes in expression of GABA linked genes such as GAD67 (Akbarian *et al* 1995) The drug phencyclidine (PCP) has been shown to produce a metabolic hypofunction and reduced parvalbumin expression in GABAergic interneurones in the prefrontal cortex (Cochran *et al* 2001), these changes in the rat brain are similar to those reported in human schizophrenics (Wu *et al* 1991). The overall objectives of the study were to determine if acute and subchronic exposure to PCP induced changes in expression levels of GABA neurone marker genes.

Adult male hooded Long Evans rats were administered i.p. injections of either vehicle (sterile saline) or PCP 2.58mg/kg. animals receiving acute PCP were administered one i.p. injection and then killed 24hrs later, whereas animals receiving subchronic PCP were administered 5 daily i.p. injections then killed 72 hrs later (for each group n=7-9) Brains were sectioned at the level of the prefrontal cortex and *in situ* hybridisation carried out according to Wisden and Morris (1994), sections were analysed using computer based densitometry and statistical analysis was performed using independent T tests. Subchronic exposure to PCP caused a decrease in mRNA expression levels in three of the five genes examined. GAD65 mRNA expression was increased after both acute and subchronic PCP

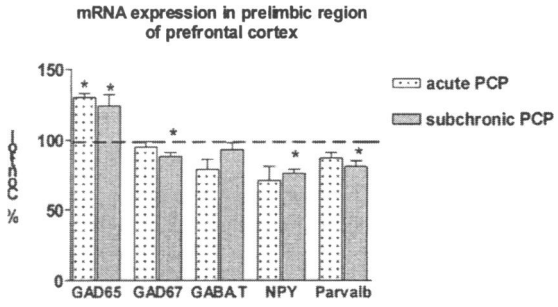

Figure 1 mRNA expression of GABA marker genes in prefrontal cortex (PFC) after acute and subchronic exposure to PCP. Values are expressed as % control ± SEM. *=P<0.05 compared to vehicle control

The PFC is implicated in the cognitive deficits and negative symptoms of schizophrenia. The changes observed were similar to those reported in schizophrenics. (Akbarian et al 1995). It is possible that reversal of the PCP-induced changes in expression of these genes may serve as a useful screening tool for identification of compounds with potential antipsychotic activity.

Akbarian Kim, J.J. and Potkin, S.G. *et al* (1995) *Arch Gen. Psych.* **52**, 258 – 266. Cochran et al (2001) *Society of Neuroscience* Abstract No 733.10
Wisden & Morris (1994.) *In situ hybridisation protocols for the brain,* 9-34 Academic Press
Wu, J.C., Buchsbaum, M.S. and Bunney, W.E. (1991), *Japanese Journal of Psychopharmacology*, **1**, 47-8.

126P METHYLPHENIDATE INDUCES ARC mRNA EXPRESSION DIFFERENTIALLY IN ADULT AND JUVENILE RAT BRAIN

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The central stimulant methylphenidate (MPH, Ritalin) is currently a widely prescribed drug for the treatment of attention deficit hyperactivity disorder (ADHD) in children. MPH is known to produce psychomotor stimulant behaviour in adults. In contrast, a paradoxical calming effect is seen in young children diagnosed with ADHD, however the mechanism for this effect remains unknown. In common with other central stimulants, MPH is known to block the dopamine transporter (DAT), resulting in enhanced dopamine transmission. (Solanto, 1998). This can be monitored by increased expression of Arc mRNA, an immediate early gene whose mRNA expression is induced by stimulation of D_1 receptors (Fosnaugh *et al*, 1995).

Male Sprague Dawley adult (340g, 9-10 weeks old) and juvenile (80g, 4 weeks old) rats were administered (i.p.) either saline or MPH (4mg/kg). Rats were killed 2 hours later and the brains isolated and flash-frozen in isopentane. Arc mRNA expression was analysed by in situ hybridisation histochemistry using a [^{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 data was made using one and two-way ANOVA and Newman-Keuls post-hoc test.

Systemic injection of MPH increased gene expression for Arc in specific brain regions of both adult and juvenile rats, including the orbital and cingulate frontal cortex, striatum and parietal cortex (table 1). Within the hippocampus MPH-induced Arc expression was restricted to the CA1 region of adult rats, while juveniles showed a non-significant increase. MPH-induced Arc mRNA expression in juvenile rats was significantly less than that in adult rats ($p<0.001$) in both cingulate and parietal cortex, according to 2-way ANOVA.

	Striatum	CA1	Cingulate Cortex	Parietal Cortex
Juvenile	24±6 *	23±10	49±8 *	80±14 **
Adult	49±12 **	37±10 *	123±11 **†	192±11 **†

Table 1. Increase in Arc expression induced by MPH in adult and juvenile brain areas expressed as a percentage of control values. Comparison with control; * $p<0.05$, ** $p<0.001$,
Comparison with juvenile † $p<0.001$.

In conclusion, MPH induced Arc mRNA expression is generally significantly higher in adult versus juvenile rat brain regions. This highlights important neurodevelopmental effects of central stimulants.

Fosnaugh, J. S., Bhat, R. V., Yamagata, K., *et al.* (1995). *J. Neurochem* **64**, 2377-80.
Pei, Q., Lewis, L., Sprakes, M.E., *et al.* (2000). *Neuropharmacol.*, **39**, 463-470
Solanto, M.V. (1998). *Behav. Brain Res.* **94**, 127-152

127P CHRONIC RESPONSES TO METHYLENEDIOXYMETHAMPHETAMINE ADMINISTRATION: EFFECTS ON LOCAL CEREBRAL BLOOD FLOW AND GLUCOSE UTILISATION IN THE RAT

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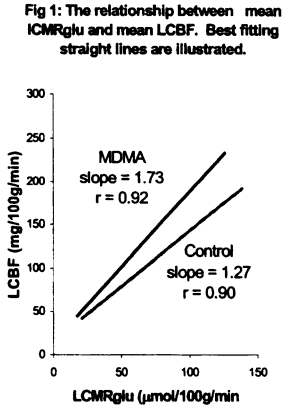
A single dose of 3,4,-methylenedioxymethamphetamine (MDMA) in Dark Agouti (DA) rats induces degeneration of 5-HT nerve terminals (O'Shea *et al.* 1998). This may have consequences for both cerebral function and cerebrovascular control. In this study we investigated the persistent effects of a neurotoxic dose of MDMA on the relationship between cerebral blood flow (lCBF) and metabolism (lCMRglu).

Adult DA rats were injected with either 15mg/kg *i.p.* MDMA (n=11) or saline (n=9). Three weeks later lCBF (saline, n=4; MDMA, n=6) or lCMRglu (saline, n=5; MDMA, n=5) was measured using quantitative [^{14}C]-iodoantipyrine and [^{14}C]-2-deoxyglucose autoradiography respectively (Kelly *et al.*, 1995). Membranes were prepared from frontal poles of each brain for saturation analysis of [^{3}H]-paroxetine binding (Battaglia *et al.*, 1987).

[^{3}H]-paroxetine binding was decreased by 47% in the MDMA-treated group. MDMA also produced significant increases in lCBF in 38 brain areas ($P < 0.05$; t-test), and there was a global trend towards increased lCBF. Most marked increases were found in sensorimotor cortex (from 119±7 to 140±8 s.e.m.), amygdala (80±6 to 104±9), hypothalamus (88±3 to 109±12) and paramedian raphe (125±3 to 142±10). Significant decreases in lCMRglu were observed in 7 brain areas, including sensorimotor cortex (93±4 to 77±3), paramedian raphe (83±4 to 67±3) and medial striatum (91±4 to 72±1).



A global analysis of the relationship between lCBF and lCMRglu (Fig. 1) revealed a close correlation ($r = 0.90$; $r=0.92$) for both groups. However the divergence of lCBF (increases) and lCMRglu (decreases) in the MDMA-treated group resulted in a shift in the relationship. The ratio of lCBF to lCMRglu was significantly increased ($P < 0.0001$; Mann-Whitney U-test).

This study provides direct evidence that exposure to MDMA has the potential to disrupt cerebrovascular control mechanisms. The uncoupling of lCBF from lCMRglu could result from the loss of 5-HT-mediated vasoconstrictor tone.

Battaglia, G., Yeh, S.Y., O'Hearn, E. *et al.* (1987) *J. Pharmacol. Exp. Ther.*, **242**, 911-916.
Kelly, P.A.T., Ritchie, I.M., McBean, D.E. *et al.* (1995) *J. Cereb.Blood Flow Metabol.*, **15**, 706-713.
O'Shea, E., Granados, R., Esteban, B., *et al,* (1998) *Neuropharmacol.*, **37**, 919-926.

128P [^{3}H]DOPAMINE RELEASE FROM RAT STRIATUM EVOKED BY ELECTRIC FIELD STIMULATION OF CORTEX IN A COMPLEX CORTICOSTRIATAL SLICE PREPARATION IN VITRO

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Glutamate, released from corticostriatal axon terminals, modulates the release of dopamine in the striatum which affects the release of GABA (Harsing & Zigmond, 1997). The effect of glutamate receptor activation, however, is contradictory: several reports have suggested facilitation (Krebs et *al.*, 1991), while others inhibition (Wu et *al.*, 2000) of dopamine release.

We have developed a method to study [^{3}H]dopamine release from the striatum in response to cortical stimulation *in vitro*. Horizontal slices (400-600 µm) containing the striatum and the adjacent prefrontal cortex of male rat (Crl:CD®BR, 200-250 g) brain were cut with a Vibratome in a plane that maintains corticostriatal connections (Kawaguchi *et al.*, 1989). After loading with [^{3}H]dopamine (1 µM, for 45 min) a slice was submerged into a two-compartments bath so that the cortical part was contained entirely in one compartment, the corpus callosum passed through a silicone greased slot, and the striatal part was contained in the other compartment. Tissue was superfused with Mg^{2+}free Krebs solution at room temperature. A cannula was placed just above (500 µm) the striatal part of the submerged slice and effluent was collected in 3 min fractions. Released [^{3}H] activity was counted with a liquid scintillation counter. Stimulation of cortex (40 V, 2 ms, 20 Hz, 3 min) increased the release of [^{3}H]dopamine in the striatum (Fig. 1). 1 mM bicuculline increased the basal and stimulated release of [^{3}H]dopamine in the striatum (Fig. 1).

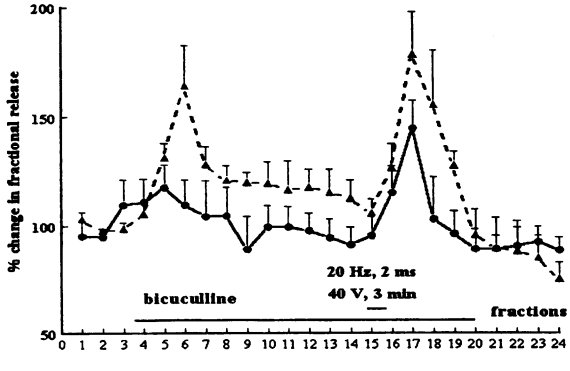

Fig. 1. Effect of cortical stimulation on [^{3}H]dopamine release in striatum. Control (circles): 96 ± 9 % to 145 ± 13 % ($p < 0.05$, Student t-test, n=6). 1 mM bicuculline (triangles): 106 ± 6 % to 179 ± 19 % ($p < 0.05$, Student t-test, n=3). Area under curve values were compared from 16th to 19th fractions (364 ± 36 vs 590 ± 60, $p < 0.05$, Student t-test).

These results suggest that cortical stimulation increased the release of [^{3}H]dopamine in the striatum through the activation of corticostriatal afferentation. The further increase in [^{3}H]dopamine release in the presence of bicuculline indicates the decrease of GABA-erg inhibition on dopamine release.

Harsing L.G. & Zigmond M.J. (1997) Neuroscience, 77, 419-429.
Kawaguchi Y. et al. (1989) J. Neurophysiology, 62, 1052-1067.
Krebs M.O. et al. (1991) J. Neurochemistry, 56, 81-85.
Wu Y. et al. (2000) Neuroscience, 96, 65-72.
Supported by NSF-OTKA (N31162)

129P RELATIONSHIP BETWEEN DOPAMINE D_2 RECEPTOR OCCUPANCY AND THE BEHAVIOURAL EFFECTS OF HALOPERIDOL IN THE MALE RAT

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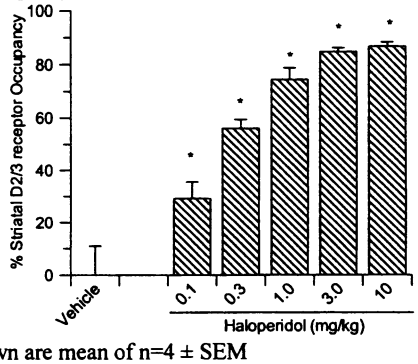
Blockade of D_2 receptors has been a suggested primary mode of action for anti-psychotics for some time (Seeman *et. al.*, 1998). Potential antipsychotic drugs have been tested in the catalepsy test (CAT) to determine extra-pyramidal symptoms (EPS), and in tests such as the reversal of amphetamine induced locomotor activity (amph. LMA) and suppression of conditioned avoidance response to estimate *in vivo* blockade of D_2 receptors. It has previously been reported that these drugs only produce a cataleptic response at doses that yield >80% D_2 receptor occupancy whilst activity in efficacy models occurs at lower occupancy levels (Wadenberg *et. al.*, 2000). The aim of this study was to determine *in vivo* occupancy of test compounds at rat striatal D_2 receptors using [^{3}H]raclopride, a commercially available radioligand that selectively labels D_2/D_3 receptors, and to compare this with degree of efficacy and catalepsy as measured by amph. LMA and CAT respectively. CD male rats, (~300g), were dosed p.o. with either vehicle or haloperidol (D_2 receptor antagonist) 30 min prior to i.v. bolus dose of 8μCi [^{3}H]raclopride. Rats were sacrificed after 30 min, and striatum and cerebellum dissected out. Tissue was dissolved using soluene, and 0.5M HCl and scintillation fluid added. The radioactivity in each sample was determined by scintillation spectrometry and corrected for tissue weight. % occupancy at striatal D_2 receptors was calculated according to Wadenberg *et. al.*, 2001. Amph. LMA and CAT were carried out as described in Reavill *et. al.*, 2000. % occupancy of D_2 receptors by haloperidol is shown in Fig. 1. Haloperidol (p.o.) gave an ED_{50} of 2.3mg/kg in CAT studies (n=6), and 0.2mg/kg in amph. LMA studies (n=8). These values correlate to ≥75% and approx. 40% striatal D_2 receptor occupancy as determined using [^{3}H]raclopride.

Fig.1. Occupancy of Rat Striatal D_2 Receptors by Haloperidol.



Data shown are mean of n=4 ± SEM

* indicates $p<0.01$ vs Vehicle : Dunnetts test

These studies show that haloperidol occupies rat striatal D_2 receptors in a dose dependent manner. Moderate occupancy of D_2 receptors is associated with efficacy in pharmacodynamic models whereas higher occupancy introduces EPS. These studies confirm that *in vivo* [^{3}H]raclopride binding can be utilised to investigate the therapeutic window for potential anti-psychotic compounds and the ED_{50} dose in the amph. LMA assay equates to approximately 40% occupancy of striatal rat dopamine D_2 receptors.

Reavill C., Taylor S., and Wood M.D., *et.al.*, (2000). *JPET*, **294**, 1154-1165.

Seeman P. and Tallerico T. (1998). *Mol. Psych.*, **3**, 123-134.

Wadenberg M., Kapur S., and Soliman A., *et. al.*, (2000). *Psychopharm.*, **150**, 422-429.

Wadenberg M., Soliman A., and VanderSpek S.C., *et. al.*, (2001). *Neuropsychopharm.*, **25**, 633-641.

130P BINDING OF THE A_{2A} ADENOSINE RECEPTOR ANTAGONIST RADIOLIGAND [^{3}H]-ZM241385 TO PARTICULATE PREPARATIONS FROM THE PORCINE PUTAMEN AND NUCLEUS ACCUMBENS

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The antagonist ZM241385 (4-(2-[7-amino-2-triazolo-triazin-5-yl-amino]ethyl)phenol) has proved useful as a means of identifying A_{2A} adenosine receptor-mediated responses both *in vivo* and *in vitro* (Ralevic & Burnstock, 1998). Binding of a tritiated version of this ligand has recently been examined in the rat brain (Alexander & Millns, 2001), where a high density of binding was observed in the caudate putamen and nucleus accumbens. We have previously reported to the Society that this radioligand bound to particulate preparations from the porcine striatum with high affinity and specificity (Alexander *et al.*, 1999). Subsequently, however, we have identified that solely the caudate nucleus had been used in preparing tissue for these binding experiments (Felix *et al.*, 1999). Here, we report an investigation of binding of [^{3}H]-ZM241385 to particulate preparations from other porcine striatal elements, the putamen and nucleus accumbens.

Particulate preparations from porcine striatum (dissected from whole brain rapidly transported in ice from the abattoir) were obtained and used in binding assays, as previously described (Alexander *et al.*, 2001). Saturation analysis was conducted over the nominal radioligand concentration range of 0.1 - 3.4 nM; competition curves (antagonist range 10 fM to 10 μM; agonist range 4 nM to 2 μM) at 0.3-0.5 nM. Data reported are means ± SEM of 3-6 separate experiments.

Analysis of [^{3}H]-ZM241385 saturation isotherms in particulate preparations from porcine putamen and nucleus accumbens showed the radioligand to have a K_d of 0.34 ± 0.09 nM and 0.50 ± 0.03 nM, respectively. B_{max} values in these tissues were 403 ± 63 fmoles.mg^{-1} protein and 640 ± 177 fmoles.mg^{-1} protein, respectively.

Competition analysis of [^{3}H]-ZM241385 binding was conducted using the A_{2A}-selective antagonist SCH58261 and the non-selective agonist NECA (Ralevic & Burnstock, 1998). For both ligands, data were fitted better to a single-site model compared to a two-site model (Prism). For the antagonist SCH58261, pK_i values of 7.89 ± 0.02 and 8.10 ± 0.04 with Hill slopes of -0.92 ± 0.09 and -0.72 ± 0.16 were estimated using preparations from putamen and nucleus accumbens, respectively. For the agonist NECA, pK_i values of 7.13 ± 0.04 and 7.10 ± 0.03 with Hill slopes of -0.99 ± 0.05 and -1.14 ± 0.01, respectively, were estimated.

In comparison with the caudate nucleus (Alexander *et al.*, 1999), binding of [^{3}H]-ZM241385 was of similar affinity (0.26 nM) as were the affinities of SCH58261 and NECA (pK_i values of 8.39 and 7.20, respectively). Density of binding in the caudate nucleus, however, was greater (941 fmol.mg^{-1} protein, Alexander *et al.*, 1999). Taken together, therefore, these data show a lower, but appreciable, density of A_{2A} adenosine receptors in striatal regions of the pig brain other than the caudate nucleus. It is notable that agonist competition curves in these other striatal regions are also monophasic. Given the ready availability of large quantities of porcine brain, these regions may be useful for future study of A_{2A} receptor function *in vitro*.

LKH is a BBSRC Research Committee Student.

Alexander S.P.H., Hamilton E.L., Pollock A.C., *et al.* (1999) *Br.J.Pharmacol.* 128:182P

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

Felix B., Leger M.E., Albe-Fessard D., *et al.* (1999) *Brain Res.Bull.* 49, 1-137

Ralevic V. & Burnstock G. (1998) *Pharmacol.Rev.* 50, 413-493

131P A_{2A} ADENOSINE RECEPTOR INHIBITION OF DOPAMINE RELEASE IN THE RAT NUCLEUS ACCUMBENS IN VITRO

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A_{2A} adenosine receptors are found in the brain to high density in the dopamine-rich areas, such as the caudate, putamen and nucleus accumbens (Ferre, 1997). In the rat dorsal striatum, there are contradictory reports of the effects of A_{2A} receptors on dopamine (DA) release (Jin *et al.*, 1993; Lupica *et al.*, 1990; Okada *et al.*, 1996). Here, we have studied the effects of A_{2A} receptors on [^{3}H]-DA release in the rat nucleus accumbens (NAc) *in vitro*.

Nucleus accumbens tissue from male Lister Hooded rats (300-400 g) was sliced and pre-labelled with [^{3}H]-DA (Cadogan *et al.*, 1997) in the presence of 200 μM pargyline. Slices were washed and then subjected to two periods of electrical stimulation (S1, S2) preceded by two periods in the absence of stimulation (C1, C2). Data were expressed as ratios of the second and first periods of electrical stimulation (S2/S1), or absence of stimulation (C2/C1) from 3 separate experiments. Statistical analysis was performed using one-way ANOVA with post-hoc Bonferroni multiple comparison test.

[^{3}H]-DA release was maintained over the control period (C2/C1 97 ± 1 %), but was reduced during the second period of stimulation (S2/S1 79 ± 2 %). Inclusion of 10 μM cocaine in the suprafusion buffer prior to S2 led to a significant enhancement of [^{3}H]-DA release in both the absence and presence of electrical stimulation (122 ± 5%, $P<0.01$ and 125 ± 4 %, $P<0.001$, respectively). The presence of the A_{2A} antagonist, SCH58261 (300 nM), also significantly increased [^{3}H]-DA release in both control and stimulated phases (C2/C1 115 ± 7 %, $P<0.05$ and S2/S1 132 ± 13 % $P<0.01$), while an A_{2A} agonist CGS21680 (300 nM) led to a significant decrease (C2/C1 75 ± 3%, $P<0.001$ and S2/S1 57 ± 1%, $P<0.001$).

[^{3}H]-DA release in the presence of the $GABA_B$ receptor antagonist 2-hydroxysaclofen (10 μM) alone was similar to that in control conditions (C2/C1 92.5 ± 2%; S2/S1 83 ± 3%). The presence of SCH58261 blocked the inhibitory effect of subsequent exposure to CGS21680 (C2/C1 82 ± 3% and S2/S1 99 ± 9%), however, 2-hydroxysaclofen failed to block inhibition of [^{3}H]-DA release by CGS21680 (C2/C1 61 ± 1%; S2/S1 49 ± 3%).

These results indicate that activation of A_{2A} receptors leads to an inhibition of DA release in the rat NAc. Furthermore, it appears that tonic activation of these receptors by endogenous adenosine is sufficient to inhibit DA release in this tissue. Although these results do not support the hypothesis that A_{2A} adenosine receptor effects may be mediated by the $GABA_B$ receptor, a role for the receptor in A_{2A} adenosine receptor modulation of DA release should not be ruled out. Future experiments could investigate the effects of an alternative $GABA_B$ antagonist or a higher concentration of 2-hydroxysaclofen.

Given the association between dopamine release in the nucleus accumbens and reward mechanisms, an investigation into the effects of A_{2A} receptor antagonists in reward and DA release *in vivo* may give further insight into the physiological role of these receptors.

LKH is a BBSRC Research Committee Student.

Cadogan A.K., Alexander S.P.H., Boyd E.A., and Kendall D.A. (1997) *J. Neurochem.* 69(3), 1131-1137
Ferre S. (1997) *Psychopharmacol.* 133, 107-120
Jin S. Y., Johansson B. and Fredholm B.B. (1993) *J.Pharmacol.Exp. Ther.* 267, 801-808
Lupica C. R., Cass W. A., Zahniser N. R. and Dunwiddie T.V. (1990) *J.Pharmacol.Exp.Ther.* 252, 1134-1141
Okada M., Mizuno K., and Kaneko S. (1996) *Neurosci.Lett.* 212, 53-56

132P A COMPARISON OF THE BINDING AFFINITIES OF ENDOTHELIN (ET) RECEPTOR ANTAGONISTS AT RECOMBINANT AND NATIVE ET_A RECEPTORS

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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

133P ANTI-ABSENCE EFFECT OF ETHOSUXIMIDE ADMINISTERED VIA REVERSE MICRODIALYSIS INTO THE VENTROBASAL THALAMUS (VB) OF THE GENETIC ABSENCE EPILEPSY RAT FROM STRASBOURG (GAERS)

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Ethosuximide (ETX) is used clinically for its selective effect on absence seizures. Generation of the spike and wave discharges (SWD) that characterise absence epilepsy requires functional connectivity of thalamocortical circuitry. We have recently shown that direct infusion of ETX into the VB or RTN (reticular thalamic nucleus) (Manning *et al.*, 2002) did not elicit an immediate reduction in seizure activity, like that observed after systemic administration. This study aimed to investigate whether delivery of ETX to a larger volume of the VB, using reverse micro dialysis may produce a more immediate response.

GAERS (316±44g) were anaesthetised with medetomidine/ketamine (0.5 & 75 mg/kg i.p. respectively) and implanted with a bipolar EEG electrode in the frontal cortex, inclined from the front at an angle of 20° from the vertical (AP, +3.2; L, -2.4; V, 2.8) and, bilaterally, with concentric microdialysis probes (2mm active regenerated cellulose membrane) into the VB (AP, -2.1; L, ±2.2; V, 6.0). The following day the microdialysis probes were perfused with artificial CSF (mM: NaCl, 125; KCl, 2.5; $MgCl_2$, 1.18; $CaCl_2$, 1.26; NaH_2PO_4, 0.2, pH adjusted to 7.0) at a flow rate of 1µl/min with samples being collected consecutively every 15mins for 3 hours. After 1 hour the perfusate was switched to one containing ETX (see below) for an hour before reverting back to artificial CSF. The EEG signal was amplified, filtered and recorded (Neurolog NL 824/820/135/530) for a 30min basal period during samples 3 & 4 and for two post drug periods during samples 5 & 6 and 7 & 8. There were 9 experimental groups; ETX 100mg/kg ip, ETX perfused at 0.2, 1, 5, 25, 50, 200mM and 1M and an artificial CSF perfusate control. Concentrations of aspartate, glutamate, glutamine, glycine, citrulline and GABA were determined by HPLC with fluorescence detection after derivatisation with o-phthaldialdehyde/ mercaptopropionic acid. SWD are expressed as the percentage of each 30 min period. Effects were assessed by one-way ANOVA, with post-hoc comparison to basal values using Dunnett's test when significant ($p<0.05$) differences were found.

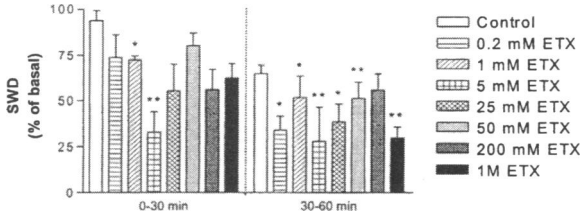

Figure 1. Effect of the administration of ETX into VB, by bilateral reverse microdialysis, on SWD in GAERS.

Administration of ETX produced no significant alteration in any of the amino acids at any of the doses investigated. A finding consistent with *in vitro* studies using ETX (Crowder and Bradford, 1987), inferring that amino acid variations are not implicated in either the initiation or termination of seizure activity. The SWD data (Fig. 1) indicate a marked reduction of SWDs within the first 30 min, but only at 5 mM, with indications of a reduction within this time period at 1 and 25 mM. This biphasic characteristic of the dose-response relationship may be explained by the fact that at lower doses, the concentration of ETX was insufficient to mediate an anti-absence action, whilst at higher levels there may have been local perturbation of pH and osmolarity around the probe. From these results it is possible to hypothesise that ETX may have a narrow effective window. It may also indicate the need to simultaneously target both thalamic and cortical areas for there to be immediate and substantial cessation of seizures.

Crowder J.M., Bradford H.F. (1987) *Epilepsia* **28**,378-382
Manning J-P.A. Barnes D. et al., (2002) *Br.J.Pharmacol.***135**,102P

134P MODULATION OF EPILEPTIFORM BURSTING ACTIVITY IN HIPPOCAMPAL SLICES BY NICOTINIC ACETYLCHOLINE RECEPTOR ACTIVATION

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Nicotinic acetylcholine receptors (nAChRs) are expressed throughout the central nervous system where they regulate neuronal excitability (Jones et al., 1999). nAChRs have also been implicated in a number of disease states including several forms of idiopathic epilepsy (Steinlein, 2001). Previous studies have identified a role for nAChRs in regulating physiological patterns of neuronal activity (Williams & Kauer, 1997). In the present study we have examined the effects of nAChR ligands on epileptiform activity induced by the convulsant compound 4-aminopyridine (4-AP) in order to assess the role of nAChRs during pathological network states.

Transverse horizontal hippocampal slices (400µm thick) were prepared from male Wistar rats (2-6 weeks old, 50-270g) as described previously (Morton & Davies, 1997). Slices were placed in a recording chamber at an artificial cerebrospinal fluid / humidified oxygen (95% O_2/ 5% CO_2) interface. Extracellular recordings were obtained from stratum pyramidale of area CA3 using KCl filled glass microelectrodes. Electrical potentials were recorded under current clamp conditions, digitised and stored onto a PC for off-line analysis using pClamp8. Drugs were added to the perfusion medium from frozen stock solutions. All values are expressed as means ± S.E.Mean and statistical significance determined by ANOVA with Tukey HSD post test.

Epileptiform bursts were induced following bath application of the convulsant and potassium channel blocker 4-AP (10-50 µM, n=140). Bursts were very stereotyped lasting 257±15ms (range 0.2-0.4s) and occurred at regular intervals (mean frequency 0.4 ± 0.02 Hz). The basal frequency of 4-AP induced bursting under control conditions varied from slice to slice and therefore all data were normalised to control (pre-nAChR agonist) conditions. Subsequent co-application of the selective nAChR agonists dimethylphenylpiperazinium iodide (DMPP, 0.3-300µM, n=31 of 37), choline (1-3000µM, n=27 of 36) and lobeline (1-30µM, n=8 of 10) produced a dose dependent increase in burst frequency with a mean maximal frequency potentiation of 137±5%, 127±5% and 124±11%, respectively ($P<0.05$). These effects were reversed upon subsequent washout of nAChR agonist or upon co-application of selective nAChR antagonists. In the presence of DMPP (30µM), the nAChR antagonists dihydro-β-erythroidine (20 µM, n=6 of 8), α-bungarotoxin (100nM, n=4) and the nAChR channel blocker mecamylamine (200µM, n=3 of 4) produced a reduction of burst frequency of 26.6±6.2%, 30.5±1.5% and 26.3±10.9%, respectively (all $P<0.05$).

These results demonstrate that nAChRs modulate epileptiform activity in the hippocampus.

Jones, S., Sudweeks, S. & Yakel, J. (1999). *TINS.* **22**, 555-561.
Morton, R. & Davies, C., (1997) *J. Physiol.* **502**, 75-90.
Steinlein, O. (2001). *Am. J. Med. Genet.* **16**, 139-145
Williams J. & Kauer, J. (1997). *J. Neurosci.* **78**, 2631-2640.

Thanks to SHERT, ERF and CRF for support.

135P REPEATED ADMINISTRATION OF SUBCONVULSANT DOSES OF 4-AMINOPYRIDINE (4-AP) INDUCES DECREASED SEIZURE INCIDENCE TO HIGHER DOSES OF 4-AP BUT NOT OTHER CONVULSANT DRUGS

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Repeated administration of sub-convulsant 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 include pentylenetetrazole (PTZ) or FG 7142, both of which act at the GABAA/Benzodiazepine receptor complex. We recently demonstrated that nicotine also induced kindling (Bastlund *et al.*, 2002). In the following experiments we investigated whether repeated dosing with subconvulsant doses of the potassium channel blocker, 4-aminopyridine (4-AP), could also induce kindling.

Separate groups of 8-25 male NMRI mice (22 g at the start of the experiment) were injected with 4-AP (7 mg/kg, IP) every day (Mon to Fri) for 2 weeks. Control groups received saline injections. Three days after the last injection, convulsion incidence was measured following IP injection of various convulsant drugs (table 2). Convulsant drugs were also infused via a lateral tail-vein in these two groups, using bicuculline (0.05 mg/ml, 1 ml/min), 4-AP (6 mg/ml, 0.7 ml/min), PTZ (5 mg/ml, 0.5 ml/min) or DMCM (0.25 mg/ml, 1 ml/min) (parameters chosen so that the first clonic convulsion was seen at around 25s in control animals). The threshold dose of the infused convulsant was then calculated (mg/kg) and expressed as mean ± SE. Results were compared by Student's t-test (thresholds) or Fishers Exact probability test (incidences).

Table 1 shows that the thresholds to IV infusion of convulsant drugs in 4-AP treated animals and control animals were not significantly different.

Table 2, however, shows that 4-AP treated animals became "tolerant" to seizures induced by IP injection of convulsant doses of 4-AP ($P < 0.01$) but not other convulsant drugs.

These results clearly demonstrate that kindling did not occur to repeated administration of 4-AP, in fact there was a decrease in seizure susceptibility with time. Furthermore, the "tolerance" that occurred to the convulsive action of 4-AP was not seen with the other convulsant drugs. Finally, this study has also illustrated that changes in thresholds to infused drugs, do not necessarily reflect changes in convulsion incidence. This may reflect pharmacokinetic changes or imply that the different methods used may detect different adaptations in the processes of seizure development.

Bastlund, J.F., Watson, W.P. & Sánchez, C. (2002) *Brit. J. Pharmacol*, **135**, 363P.

Table 1: Threshold convulsive doses (mean ± s.e.) of 4-AP, bicuculline, DMCM or PTZ measured 3 days after daily dosing with 4-AP or saline (‡ P = 0.06 cf saline group)

Infused Drug	Theshold in saline group (mg/kg)	Theshold in 4-AP group (mg/kg)
4-AP	58.9 ± 1.5	61.2 ± 1.7
Bicuculline	0.74 ± 0.04	0.81 ± 0.05
DMCM	3.16 ± 0.17	3.54 ± 0.10 ‡
PTZ	38.7 ± 2.0	41.3 ± 1.5

Table 2: Incidence of convulsions induced by various drugs measured 3 days after repeated dosing with 4-AP or saline (* P < 0.05 cf saline group)

Injected Drug	Incidence in saline group	Incidence in 4-AP group
4-AP (10 mg/kg)	16 out of 25	2 out of 17 *
Bicuculline (5 mg/kg)	6 out of 10	6 out of 10
DMCM (6 mg/kg)	9 out of 10	8 out of 8
PTZ (70 mg/kg)	10 out of 10	8 out of 8

136P BRAIN CATALASE ACTIVITY IN A MODEL OF CLOSED HEAD TRAUMA IN THE RAT: EFFECT OF GADOLINIUM AND AMILORIDE

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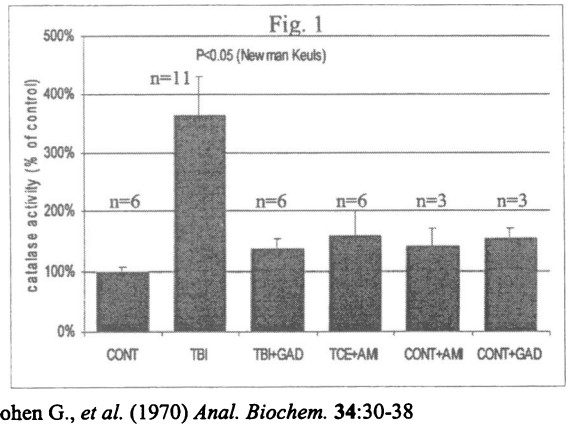
Brain trauma increases intracellular Ca^{2+} concentration activating enzymes that cause cellular damage by the formation of nitrogen and oxygen reactive species (Siesjo B., 1993). Mechanogated membrane cation channels elicit an increase in intracellular Ca^{2+} concentration in neurons after mechanical stimulation, which is inhibited by gadolinium (Gschossmann J., 2000). Catalase (CAT) is one of the major defense enzymes against superoxide radicals, acting in concert with superoxide dismutase (Islekel *et al*, 1999). Our aim was to evaluate the effect of gadopentetate dimeglumine (GAD) and amiloride (AMI) on brain CAT activity after brain trauma. Male Wistar rats, weighing 340-360g, were i.p. anaesthetised with diazepam, ketamine and atropine (6, 60 and 0.5 mg.kg-1, respectively) and allowed to breathe spontaneously. Marmarou's closed head trauma was used (Marmarou A. *et al.*, 1994). Thirty minutes after traumatic brain injury (TBI) one group of rats (TBI+GAD, n=6) was administered GAD (70 $mg.kg^{-1}$ i.v.), another group (TBI+AMI) was administered AMI (20 $mg.kg^{-1}$ i.p.) and another group (TBI) received saline i.v. A control group (CONT) was sham operated and received no weight drop. Two additional groups (CONT+GAD; CONT+AMI) were sham operated, received no weight drop and were administered GAD (70 $mg.kg^{-1}$ i.v.) or AMI (20 $mg.kg^{-1}$ i.p.) respectively. Twenty-four hours later, the animals were anaesthetized again and the brain was removed. Procedures were carried out in accordance with EU guidelines for animal experiments. Catalase activity was evaluated according to the method of Cohen *et al.*, 1970. Results of CAT activity (mean±SEM) are presented in Figure 1 and expressed as percentage of control. TBI increased CAT activity ($p<0.05$, Newman-Keuls test), this effect being reversed by GAD and AMI. These compounds showed no effect when administered to control rats. Post-traumatic administration of GAD or AMI reduced CAT activity to values similar to those of the control group. This suggests that these drugs may protect against oxidative stress after brain trauma.



Cohen G., *et al.* (1970) *Anal. Biochem.* **34**:30-38
Gschossmann J. (2000) *Brain Res.* **856**:101-110
Islekel S., *et al.* (1999) *Res. Exp. Med.***199**:167-176
Marmarou A., Foda M.A., van den Brink W., *et al. J. Neurosurg* (1994) **80**:291-300.
Siesjo B.K. (1993) *Ann. Emerg. Med.* **22**:959-969.
Supported by FCT and FEDER

137P GLIAL ANTIOXIDANT NETWORK PREVENTS H_2O_2-INDUCED OXIDATIVE DAMAGE IN GUINEA PIG BRAIN SLICES

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Hydrogen peroxide (H_2O_2) reversibly inhibits the evoked population spike (PS) in rat hippocampal slices. Recovery of the PS after washout of 1.5 mM H_2O_2 washout, however, is accompanied by epileptiform activity (Avshalumov & Rice, 2002). 1.5 mM H_2O_2 also causes oedema in rat brain slices (Brahma *et al.* 2000). We report here that; these consequences of H_2O_2 exposure are absent in guinea pig (GP) brain slices and we hypothesised protection is a consequence of the higher glia-to-neurone ratio in GP brain.

Vibratome-cut brain slices (400 μm) were prepared from male Hartley GPs (150-250g) and recovered for 1 hour at 24°C in oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 124 NaCl, 3.7 KCl, 26 $NaHCO_3$, 1.5 $CaCl_2$, 1.3 $MgSO_4$, 1.3 KH_2PO_4 and 10 glucose). Electrophysiological experiments using hippocampal slices were performed as described in Avshalumov & Rice; (2002). Oedema experiments were performed as described in Brahma *et al.*; (2000) and water content expressed as g H_2O (g dry weight)$^{-1}$. Data indicate mean ± s.e.m., n = number of slices, and statistical analysis was performed by ANOVA with a Student-Newman-Keuls post test, with significance assumed when $p<0.05$.

Although 1.5 mM H_2O_2 applied for 15 min reversibly depressed the PS in GP hippocampus (control = 2.1 ± 0.2 mV, depressed to 18% n=8) as in rats, no pathological activity was seen upon H_2O_2 washout. Similarly, 3 h exposure to 1.5 mM H_2O_2 did not enhance oedema in GP slices (control: 5.4 ± 0.1, n=19 and 1.5 mM H_2O_2: 5.5 ± 0.1 g H_2O (g dw)$^{-1}$, n=21). Inhibition of glutathione (GSH) synthesis with buthionine sulfoximine (BSO, 5 mM), present during the recovery phase, had no effect on control water content (5.4 ± 0.1 g H_2O (g dw)$^{-1}$, n=11), but did significantly enhance the effect of H_2O_2 (5.8 ± 0.1 g H_2O (g dw)$^{-1}$, n=12, $p<0.01$). Likewise, inhibition of GSH peroxidase (GSHPx) with mercaptosuccinate (MCS, 1 mM) or catalase with 3-amino-1,2,4-triazole (ATZ; 10 mM) during last 30 min of recovery and throughout the incubation had no effect on control water content (5.5 ± 0.1, n=9 and 5.6 ± 0.1 g H_2O (g dw)$^{-1}$, n=9 respectively). In MCS and ATZ, however, H_2O_2 did cause oedema in GP slices (MCS, 6.0 ± 0.1 g H_2O (g dw)$^{-1}$, n=11, $p<0.001$; ATZ, 6.2 ± 0.1 g H_2O (g dw)$^{-1}$, n=18, $p<0.001$). Ascorbate (400 μM) prevented the ATZ potentiation of H_2O_2 (5.5 ± 0.1 g H_2O (g dw)$^{-1}$, n=9, $p<0.001$). Treatment with BSO, MCS or ATZ led to epileptiform activity in GP hippocampal slices during H_2O_2 washout. This pathology was prevented by the inclusion of 400 μM ascorbate during washout.

We conclude that: 1) GP brain slices are less vulnerable than rat slices to H_2O_2 toxicity because of glia-mediated antioxidant protection (GSH synthesis, GPx, and catalase); 2) loss of any of these components of the antioxidant network can be compensated for by ascorbate. Supported by: NIH grants NS 34115 and NS-36362.

Avshalumov, M.V., & Rice M.E. (2002). *J. Neurophysiol.* **87**, 2896-2903.
Brahma, B., Forman, R.E., Stewart, E.E. et al., (2000). *J. Neurochem.* **74**, 1263-1270.

138P MECHANISMS UNDERLYING RAT CEREBRAL OEDEMA FORMATION IN *IN VITRO* ISCHAEMIA

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Clinically, cerebral oedema in stroke patients is associated with poor prognosis and increased mortality. *In vitro* models of ischaemia have been used to investigate mechanisms of ischaemic damage, but have been rarely used to address mechanisms of oedema formation. Here we describe an *in vitro* model of ischaemia-reoxygenation-induced oedema and investigate the mechanisms involved in oedema formation.

Coronal brain slices (400 μm) of male Long Evans rats (7-9 weeks old) were prepared as in Brahma *et al.*, (2000). After the recovery period, the slices were equilibrated in normal artificial CSF (ACSF) at 35°C for 30 min, then incubated in ischaemic-ACSF (zero glucose, osmotically balanced with increased NaCl, bubbled with 95% N_2 / 5% CO_2) for 5-30 min followed by reoxygenation in normal ACSF ± drugs (0-145 min). Water content was determined using the method of Brahma *et al.*, (2000) and expressed as g H_2O (g dry tissue weight)$^{-1}$, (g H_2O (g dw)$^{-1}$). Data indicate means ± s.e.mean, n = number of slices, minimum of 3 animals per experiment and statistical significance was determined by one-way ANOVA with the Student-Newman-Keuls post test and a limiting probability of $p<0.05$. In some experiments, the drugs were present before, during and after ischaemia.

Slice oedema increased with increasing duration of the ischaemic insult and reoxygenation. Because significant water gain was observed after only 5 min of ischaemia, with 145 min of reoxygenation (control slices: 5.7 ± 0.1 g H_2O (g dw)$^{-1}$, n=23 and ischaemic slices 6.3 ± 0.0 g H_2O (g dw)$^{-1}$, n=79, $p<0.001$), this duration of insult and reoxygenation was used in all further studies. To investigate mechanisms involved in oedema formation, we examined oedema prevention by antioxidants, glutamate receptor antagonists and inhibition of mitochodrial permeability transition. All these agents gave a small but significant decrease in water content after the ischaemic insult ($p<0.01$-0.001): ascorbate (400 μM, 6.0 ± 0.0 g H_2O (g dw)$^{-1}$, n=14), dimethylthiourea (5 mM, 6.0 ± 0.1 g H_2O (g dw)$^{-1}$, n=18), tempol (1 mM, 6.0 ± 0.1 g H_2O (g dw)$^{-1}$, n=15), Trolox® (1 mM, 6.00 ± 0.1 g H_2O (g dw)$^{-1}$, n=11), DL-2-amino-5-phosphonopentanoic acid (AP5) + 6-cyano-7-nitroquinoxaline- 2,3-dione (CNQX), (100 μM + 25 μM), (5.9 ± 0.1 g H_2O (g dw)$^{-1}$, n=17), Cyclosporin A (CsA) (1 μM, 5.9 ± 0.1 g H_2O (g dw)$^{-1}$, n=23), using FK506 (1 μM) as a control, 6.3 ± 0.1 g H_2O (g dw)$^{-1}$, n=12). A combination of CsA, tempol and AP5/CNQX during reoxygenation provided greater protection (5.8 ± 0.0 g H_2O (g dw)$^{-1}$, n=24) than any one treatment ($p<0.05$). If the cocktail was also present before and during ischaemia, significantly ($p<0.05$) greater protection was observed (5.6 ± 0.0 g H_2O (g dw)$^{-1}$, n=15).

We conclude that in this model of ischaemia-reoxygenation, oedema results from multiple mechanisms including glutamate receptor activation, oxidative stress, and mitochondrial dysfunction. Moreover, the data are consistent with the view that combination therapy provides greater benefits than single agent treatment alone. Supported by: NIH grant NS 34115.

Brahma, B., Forman, R.E., Stewart, E.E. *et al.*, (2000). *J. Neurochem.* **74**, 1263-1270.

139P NEUROPEPTIDE-CONTAINING CELLS IN THE CORTEX AND STRIATUM OF MICE WITH CEREBRAL MALARIA

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Cerebral malaria (CM) is a poorly understood and life-threatening complication of malaria caused by the parasite *Plasmodium falciparum*. One of the most useful animal models of CM is the *Plasmodium berghei* ANKA (PbA) infection in C57 mice. Interestingly, a 40-fold increase in the activity of indoleamine 2,3-dioxygenase and a significant elevation in the concentration of quinolinic acid have been reported in the brains of these mice (Sanni *et al.*, 1998). As a high local concentration of quinolinic acid in the brain could lead to neuronal damage (Stone, 2001), we compared the neurochemical characteristics of PbA-infected and control mice to examine whether any changes found resemble those reported to be mediated by quinolinic acid.

Female C57BL/6J mice weighing between 20 and 25g were inoculated by intravenous injection of $5x10^4$ parasitised erythrocytes. The mice developed behavioural changes at approximately day 5 post-inoculation (p.i.) and progressed to coma and death at approximately days 6 to 8 p.i. On day 7 p.i., the mice had their brains removed following asphyxiation with CO_2 and perfusion with 4% (wv^{-1}) paraformaldehyde in PBS, pH 7.4. Controls were uninfected C57BL/6J mice of the same weight and sex. Floating frozen 20μm thick coronal sections were immunostained by the avidin-biotin-peroxidase method. Three animals were studied in each group.

Substance P-containing neurones were almost completely lost from the cortex and striatum of PbA-infected mice compared with uninfected controls. The intensity of calbindin immunolabelling was increased in the cortex and striatum of day 7 p.i. mice, and this appeared to be due to an increase in neuropil immunostaining rather than an increase in cell number. Neuropeptide Y- and somatostatin-containing neurones were dramatically reduced in number in the cortex of day 7 p.i. mice, and although the cell number appeared to be unchanged in the striatum, their morphology was markedly different, being smaller with fewer processes.

This neurochemical pattern in mice with CM is similar to that previously reported to be produced in rats by quinolinic acid (Beal *et al.*, 1986). Therefore, these results are consistent with a role for quinolinic acid in the production of brain damage in fatal murine CM. Indeed, this may also be the case in humans, as a significant increase in the level of quinolinic acid has been reported in the CSF of Kenyan children (Dobbie *et al.*, 2000) and Vietnamese adults (Medana *et al.*, 2002) with CM.

Beal, M.F., Kowall, N.W., Ellison, D.W., *et al.* (1986) *Nature*, **321**, 168-171.
Dobbie, M., Crawley, J., Waruiru, C., *et al.* (2000) *Am. J. Trop. Med. Hyg.*, **62**, 284-290.
Medana, I.M., Hien, T.T., Day, N.P., *et al.* (2002) *J. Infect. Dis.*, **185**, 650-656.
Sanni, L.A., Thomas, S.R., Tattam, B.N., *et al.* (1998) *Amer. J. Pathol.*, **152**, 611-619.
Stone, T.W. (2001) *Progr. Neurobiol.*, **64**, 185-218.

140P THIOPERAMIDE REDUCES HISTAMINE-INDUCED SCRATCHING IN BALBC MICE

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Pruritus (itch) is important clinically and we are using a murine model of experimentally induced scratching to investigate the mechanism(s) of action of putative mediators of itch. Histamine has a recognised role in clinical pruritic conditions, yet in many disorders, such as eczema, traditional H_1 and H_2 histamine receptor antagonists are largely ineffective at relieving itching (Hagermark, 1992). We have investigated the possible role of H_3 receptors in histamine-induced itch.

'Itch' was induced in mice by an intra-dermal (i.d.) injection of a sub-maximal dose of histamine (1mg in 100μl; 26G needle), into the back of the neck, of female BalbC mice. Itch was estimated by recording scratching of the neck, by the hind limbs, during the 20 min period directly following the injection. Six mice (20-23g) were given three successive histamine injections and the number of bouts of scratching (3 or more individual scratch movements) evoked were recorded. Histamine was injected 30mins before thioperamide (20mgkg^{-1}, i.p.), and again 30 and 90 mins post-thioperamide. In a separate control group, six mice (20-22g) received 100μl saline, i.p. (vehicle for thioperamide). Experiments lasted for up to 3 hours and all mice were later killed with an overdose of sodium pentobarbitone (24mg i.p.).

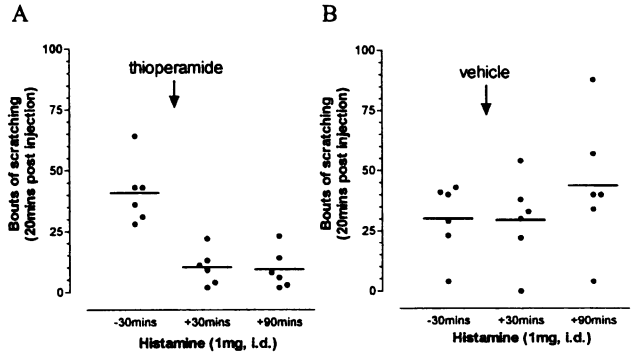

Figure 1 shows histamine-induced scratching in A) six mice: 30mins before thioperamide, 30mins after thioperamide and 90mins after thioperamide, and, B) six mice: 30mins before vehicle, 30mins after vehicle and 90mins after vehicle. Bars show mean value.

Thioperamide significantly reduced scratching induced by histamine at 30mins and 90mins post-thioperamide ($P<0.05$, repeated-measures one-way ANOVA). Scratching was unchanged in the vehicle-treated control group ($P>0.05$, repeated-measures one-way ANOVA).

In summary, we have shown that thioperamide reduces histamine-induced scratching in BalbC mice and we are investigating the action of H_3 agonists. Further studies in mice will help to characterize pruritic mechanisms and provide a useful model for developing specific and effective treatments for itch.

Hagermark, O. (1992). *Skin Pharmacology*, **5**, 1-8.

141P INVOLVEMENT OF HYPOTHALAMIC MELANOCORTIN RECEPTORS IN ROSIGLITAZONE-INDUCED WEIGHT GAIN

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Thiazolidinediones (TZDs) are insulin-sensitising agents used in the treatment of type 2 diabetes. Administration of TZDs to experimental animals results in significant increases in fat and body weight (Fujiwara *et al.*, 1988), which could potentially counteract the compounds' clinically beneficial effects. Previous studies have shown that TZD-induced weight gain is associated with hyperphagia (Wang *et al.*, 1997). However, the mechanisms involved in these hyperphagic effects have not been elucidated. Melanocortin receptors (MC-R), which mediate the appetite-suppressing effects of the hypothalamic peptide α-MSH, are important in the physiological regulation of food intake. In this study, we investigated the involvement of the hypothalamic melanocortin system in TZD-induced hyperphagia, by utilising quantitative receptor autoradiography with 50 pM [^{125}I] NDP-MSH (and 3 μM α-MSH to define the non-specific binding) to measure the density of MC-R (both MC3-R and MC4-R) in key appetite-regulating regions. Thirty male Wistar rats (175 g) were fed a highly palatable diet to induce obesity and compared with 10 chow-fed controls. After 10 weeks, the diet-fed animals were divided into 3 groups (each n=10): [1] placebo (methylcellulose) given by gavage; [2] rosiglitazone (3 mg/kg) by gavage with *ad libitum* access to the palatable diet and [3] rosiglitazone-treated but pair-fed to the intake of the placebo group. These treatment regimes were maintained for 2 further weeks, during which time the freely-fed treated animals demonstrated significant hyperphagia, as compared with the placebo group (451±4 *vs* 410±12 kJ/day; $p<0.05$ Student's *t*-test). Consistent with previous studies (Harrold *et al.*, 1999), specific [^{125}I] NDP-MSH binding in untreated controls was highest in the hypothalamic ventromedial, dorsomedial and arcuate nuclei (ARC), median eminence and medial habenular nucleus. The diet-fed placebo group showed selective decreases in MC-R binding in all hypothalamic areas (e.g ARC: 0.053±0.006 [placebo] *vs* 0.092±0.005 fmol/mg tissue [chow-fed]; $p<0.001$). This also confirms previous results (Harrold *et al.*, 1999) and is consistent with increased MC-R activation and thus enhanced satiety signalling. Despite the significant hyperphagia displayed by the rosiglitazone freely-fed group, a comparable degree of MC-R down-regulation was observed in these animals (e.g. ARC: 0.053±0.006 [placebo] *vs* 0.063±0.004 fmol/mg tissue [rosiglitazone freely-fed]; $p>0.05$). By contrast, the pair-fed group showed a selective increase in binding in all hypothalamic areas compared with the placebo group, with receptor density being restored to control levels (e.g. ARC: 0.086±0.005 [pair-fed] *vs* 0.090±0.004 fmol/mg tissue [chow-fed]; $P>0.05$). This is consistent with reduced satiety signalling, as previously reported in food-restricted animals (Harrold *et al.*, 1999). Notably, in the pair-fed treated animals MC-R were up-regulated even though food intake and body weight were comparable to the placebo group. We conclude that rosiglitazone interferes with the normal regulation of MC-R mediated satiety and suggest that this may help to drive the hyperphagia and weight gain observed in TZD treated animals.

Fujiwara T, *et al.*, (1988) *Diabetes* 37, 1549-58.
Harrold J.A., *et al.*, (1999) *Diabetes* 48, 267-271
Wang Q, *et al.*, (1997) *Br. J. Pharmacol.* 122, 1405-10

142P INCREASED MELANIN-CONCENTRATING HORMONE (MCH) LEVELS IN THE ARCUATE (ARC), AND PARAVENTRICULAR (PVN) NUCLEI OF DIETARY OBESE RATS

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Melanin-concentrating hormone (MCH) acts as an appetite-stimulating (orexigenic) neurotransmitter in the mammalian brain (Qu *et al.*, 1996). MCH-immunoreactive fibres predominantly originate from a small population of neurones in the lateral hypothalamic area (LHA) and project throughout the brain, with extensive terminations in the hypothalamus (Bittencourt *et al.*, 1992). We have previously reported increased expression of MCH mRNA in dietary obese (DIO) rats (Elliott *et al.*, 2002). In this study we examined how these changes in mRNA relate to MCH peptide levels. We also investigated the relationship between MCH and leptin, as previous studies have suggested that MCH is regulated by this appetite-reducing hormone: rodents deficient in the leptin receptor overexpress MCH, while leptin can inhibit the increase in food intake generated by centrally administered MCH (Sahu, 1998). Twenty-four male Wistar rats (200 g) were given *ad libitum* access to a highly palatable diet, whilst control rats (n=9) received standard rodent chow. Following 13 weeks of feeding, diet-fed rats were sorted in to 2 groups (high and low gainers) according to their weight increase during this period. Rats were sacrificed by CO_2 inhalation and blood was collected for determination of plasma leptin levels by radioimmunoassay (RIA; Linco Research, U.S.A.). MCH concentrations were measured in the paraventricular (PVN), ventromedial (VMH), dorsomedial (DMH) and arcuate (ARC) hypothalamic nuclei and the lateral hypothalamus (LHA), using an RIA (Phoenix Pharmaceuticals, USA). At termination, the high gainers weighed 683±15 g, the low gainers 599±10 g, and the controls 583±15 g. Significantly higher MCH levels were observed in the ARC of the high gainers compared with the low gainers (9.6±0.7 *vs* 7.1±0.7 ng mg^{-1} $p=0.05$, Student's *t*-test) and controls (6.9±0.5 ng mg^{-1}, $p=0.02$). MCH levels were also significantly higher in the PVN of the low gainers (7.4±0.5 ng mg^{-1}, $p=0.02$) and high gainers (7.1±0.4 ng mg^{-1}, $p=0.01$) compared with their lean counterparts (5.6±0.5 ng mg^{-1}). MCH concentrations were not significantly altered in the DMH, VMH or LHA in any group. Circulating levels of leptin were increased in the high gainers (6.8±0.2 ng mg^{-1}), but not in the low gainers (5.5±0.2 ng mg^{-1}), as compared with controls (5.7±0.4 ng mg^{-1} , $p<0.01$ and $p=0.2$, respectively). However, leptin levels did not correlate with MCH concentrations in any of the five nuclei examined. These results indicate that MCH is not globally increased in the hypothalamus of dietary obese rats, but is restricted to discrete areas. The PVN and ARC are both involved in the regulation of energy homeostasis, and MCH receptors are located here (Hervieu *et al.*, 2000). The previous finding that MCH mRNA is also increased in dietary obese rats suggests that a rise in MCH synthesis is occuring in this model. This increase in the orexigenic peptide may be driving the hyperphagia and weight gain observed in (DIO) rats. MCH levels in the ARC and PVN were not correlated with leptin - unlike MCH mRNA levels – which positively correlates with plasma leptin in dietary obese rats. This suggests that the translation of MCH is regulated by other factors.

Bittencourt JC, *et al.*, (1992) J Comp Neurol. 319:218-45
Elliott J, *et al.*, (2002) Brain Res. (In press)
Hervieu GJ, *et al.*, (2000) Eur J Neurosci. 12:1194-216.
Qu D, *et al.*, (1996) Nature 380:243-7
Sahu A, (1998) Endocrinology 139:4739-42

143P THE INFLUENCE OF REPETITIVE STRESS ON HIPPOCAMPAL 5-HT OVERFLOW AND PLUS-MAZE ACTIVITY IN THE RAT

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5-HT projections to the hippocampus have been implicated in the psychopathology underlying both depressive and anxiety disorders (Graeff *et al* 1996). The primary aim of this study was to test the hypothesis that repeated exposure to an unavoidable stressor exerts influences on 5-HT overflow in the dorsal hippocampus and behavioural responses to a novel anxiogenic stimulus

Male Sprague-Dawley rats (200-250g N=6 per gp) were placed on an elevated platform (Balfour & Reid 1979) for 60 min per day for 10 days. Control animals remained in their home cages. In experiment 1, dialysis probes were located stereotaxically in the dorsal hippocampus (AP –5.2mm from bregma; lateral +2.9mm; depth –3.1mm from the brain surface) on day 8. Dialysis experiments were performed on day 10. Control dialysate samples (4x30 min) were collected. All animals were then exposed to the platform stress for 60 min before being returned to their home cage and a further 4x30 min dialysate samples collected. The samples were analysed for 5-HT and 5-HIAA by HPLC with electrochemical detection. In experiment 2, one group was exposed to the platform for 10 days prior to being tested in an elevated plus maze 24h after the last session; a second group was placed on the platform once 24h prior to the maze test; the third group were not stressed prior to the maze challenge (N = 6 per gp).

Exposure to the platform on the test day had no significant effect on 5-HT or 5HIAA overflow in either group of rats. Repetitive exposure to the stressor, however, increased ($P<0.05$) baseline extracellular 5-HT from 1.08±0.17 to 1.67±0.15pg $20\mu l^{-1}$ of dialysate and baseline extracellular 5-HIAA from 389±66 to 587±43 pg $20\mu l^{-1}$ measured prior to exposure to the stress. Repetitive, but not acute exposure to the platform stress also increased ($P<0.01$) the ratio of open to enclosed arm entries in the elevated plus maze from 0.16±0.04 to 0.30±0.02 when compared with unstressed controls. The time spent in the open arms was also increased ($P<0.01$) from 27±8 to 82±13sec. Neither acute nor chronic stress influenced total activity or the number of closed arm entries. The data suggest that repetitive exposure to an unavoidable stressor causes a sustained increase in extracellular 5-HT and 5-HT turnover in the dorsal hippocampus and also elicits 'anxiolytic-like' activity in the plus-maze test. However, previous studies suggest that increased 5-HT release in dorsal hippocampus has anxiogenic consequences (File *et al* 1996). Thus the putative relationship between the effects of repeated stress on hippocampal 5-HT and its anxiolytic-like effects remains to be established.

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Balfour, D.J.K. & Reid, A. (1979) *Arch. int. Pharmacodyn. Ther*,.**237**,67-74.

File, S.E., Gonzalez, L.E. & Andrews, N. (1996) *J. Neurosci.*, **16**,4810-4815

Graeff, F.G, Guimaraes, F.S., De Andrade, T.G., *et al* (1996) *Pharmacol. Biochem. Behav*,.**54**,129-141

144P REGIONALLY-SELECTIVE UPREGULATION OF HIPPOCAMPAL GLUCOCORTICOID RECEPTORS IN RESPONSE TO CHRONIC UNAVOIDABLE STRESS IN THE RAT

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Exposure to a chronic unavoidable stress leads to an increase in hippocampal glucocorticoid receptors (GR). This effect has been implicated in the mechanisms underlying habituation to stress and impaired adaptation may be a factor in the psychopathology of depressive disorder (Holmes *et al* 1997). The primary aim of this study was to investigate the time course and regional selectivity of this response.

Male Sprague-Dawley rats (250g N=6 per group) were subjected to elevated platform stress (Balfour & Reid 1979) for 1 hour per day for 1, 10, 20 or 30 days. Control animals were kept in their home cages, but were removed to the experimental room for the 1-hour stress period. Immediately following the final session on the platform, the animals were humanely killed and blood was taken for measurement of plasma corticosterone and brain tissue was taken for analysis of GR and mineralocorticoid receptor (MR) in the hippocampus, cerebral cortex and hypothalamus. Plasma corticosterone was measured using a radioimmunoassay. GR and MR were measured in solubilised membrane extracts using Western blot analysis. The protein content was determined using a Bio-Rad protein assay kit. The results were quantified using scanning and densitometry. One-way analysis of variance was used to detect any statistical significance. *Post hoc* analyses were performed using Duncan's test.

Corticosterone levels were elevated by acute, 1-day stress (from 2.0±0.31 to 55.3±15.80$\mu g.100ml^{-1}$; $P<0.01$). Repetitive exposure to the stressor resulted in habituation of the response (10 days = 22.3±3.64; 20 days = 5.5±1.12; 30 days = 1.33± 0.25 $\mu g.100ml^{-1}$). The density of GR in the hippocampus was significantly increased (from 80.2±9.2 in unstressed rats to 181.2±22.8 density units.$100\mu g^{-1}$ of protein; $P<0.01$) by 20 days exposure to the stressor. After 30 days exposure to the stressor, the density (106.7±25.4 density units.$100\mu g^{-1}$ protein) was no longer significantly different from control. No significant differences in GR density were observed in the other brain regions investigated. MR density was not significantly influenced in any of the brain regions studied.

Our findings indicate that repetitive exposure to this unavoidable stressor is associated with a regionally selective upregulation of GR density in the hippocampus. The response peaks after 20 exposures to the stressor and, thereafter, returns to levels close to control. The putative relationship between upregulation of the receptor and habituation of the plasma corticosterone response to the stressor remains to be established.

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Balfour, D.J.K. & Reid, A. (1979) *Arch. Int Pharmacodyn. Ther.***237**, 67-74.

Holmes, M.C., French, K.L. & Seckl, J.R. (1997) *J. Neurosci.* **17(11)**; 4056-4065.

145P SUPPRESSION OF THE PRESYNAPTIC EFFECTS OF ADENOSINE BY METABOTROPIC GLUTAMATE RECEPTORS IN RAT HIPPOCAMPAL SLICES

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Interactions between adenosine and metabotropic glutamate receptors have been reported (de Mendonca & Ribeiro, 1997). The aim of this work is to determine whether those interactions are specific for adenosine receptors and whether they occur primarily at presynaptic or postsynaptic sites.

Hippocampal slices from male Wistar rats (150-200g) were maintained in artificial cerebrospinal fluid (ACSF) gassed with 95% O2 and 5% CO2 and containing (in mM): KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 10. Stimulation was delivered to the Schaffer collaterals and evoked field excitatory postsynaptic potentials (EPSPs) were recorded from the CA1 area. Paired-pulse inhibition is expressed as the change in the second response of a pair compared with the first response. Significance was calculated with repeated measures ANOVA followed by the Student-Newman-Keuls test.

Before application of glutamate receptor agonists, adenosine depressed the EPSP size by 59.35% ± 3.39 (n=4; $p<0.001$). Following application of the non-selective mGluR receptor agonist (1S,3R)-1-amino-cyclopentane-dicarboxylic acid (ACPD), adenosine was less effective, reducing the EPSPs by 38.7% ± 5.59 (n=4; $p<0.01$) after 20 minutes. Responses obtained 40 and 60 minutes after ACPD were still significantly reduced ($p<0.05$). The effect of baclofen was also significantly reduced after ACPD. Baclofen 2μM depressed single EPSP slope by 59.86%±2.64 ($P<0.001$; n=4) whereas after ACPD, baclofen reduced the EPSP slope by only 41.66%±.3.57 (n=4, $p<0.01$), 43.61%±1.69 ($p<0.01$) and 47.57%±3.42 ($p<0.05$) after 20, 40 and 60 minutes. A depression of adenosine responses was also produced in the paired pulse experiments. All these effects of ACPD were reproduced by the mGluR1a receptor agonist (R,S)-3,5-dihydroxyphenylglycine (DHPG). (S)-(+)-α-amino-4-carboxy-2-methylbenzene acetic acid (LY367385 100μM), an antagonist at mGLU1a receptors, prevented the depression by DHPG of adenosine sensitivity but the mGLU5 receptor antagonist 2-methyl-6-(2-phenylethenyl) pyridine (SIB 1893) had no effect. Responses to the A_1 receptor agonist N6-cyclopentyladenosine (CPA) were reduced after perfusing DHPG and adenosine deaminase and this reduction was not altered by the adenosine A_{2A} receptor selective antagonist 4-(2-[7-amino-2-(2-furyl)-1,2,4]-triazolo[2,3a] [1,3,5]triazin-5-ylamino ethyl phenol (ZM241385).

In conclusion, activation of mGluR1a metabotropic glutamate receptors is responsible for the suppression of adenosine sensitivity, which is mediated selectively via adenosine A_1 receptors, but is also able to suppress responses mediated by $GABA_B$ receptors. The paired-pulse experiments indicate that the interactions occur at presynaptic sites.

Budd, D.C. and Nicholls, D.C. (1995). *J. Neurochem.* 65, 615-621.
De Mendonca, A. and Ribeiro, J.A (1997). *Brit. J. Pharmacol.* 121, 1541-1548.

146P WINFLUOR - AN INTEGRATED SYSTEM FOR THE SIMULTANEOUS RECORDING OF CELL FLUORESCENCE IMAGES AND ELECTROPHYSIOLOGICAL SIGNALS ON A SINGLE COMPUTER SYSTEM.

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An integrated computer system for recording fluorescence images simultaneously with membrane current and voltage signals from single patch-clamped cells will be demonstrated. Combining the data acquisition within one computer in this way greatly simplifies the experimental recording and subsequent data analysis, as well as ensuring accurate synchronisation of images and analogue signals.

The system is based around a Nikon TE 300DV inverted microscope (Nikon, UK), Princeton I-PentaMAX intensified CCD camera (Roper Scientific, Trenton, NJ, USA), Optoscan computer-controlled monochromator (Cairn Research, Faversham, UK) and National Instruments (Austin, TX, USA) data acquisition and timing hardware, running on a 1.6 GHz Pentium IV computer under Microsoft Windows 2000.

Using a mechanically masked section of the I-PentaMAX camera's CCD sensor and its specialised "virtual chip" operating mode, image capture rates as high as 140 frames per second at a 160x160 pixel spatial resolution can be achieved. Up to 8 analogue signal channels can be acquired simultaneously with images at a maximum rate of 5,000 samples per second per channel. Image/signal capture at these rates can be sustained for long periods limited only by computer storage disc capacity.

The wavelength of the fluorescence excitation light can be switched within the range 300-600 nm in less than 1 ms on a frame by frame basis, with the CCD camera being shuttered during wavelength changes by a gating pulse applied to the intensifier unit attached to the camera. This permits the acquisition of the image pairs required for ratiometric calcium imaging, using dual excitation fluorophores such as fura-2, at the frame rate of the camera (70 ratios per second max.). In addition, substitution of the microscope's standard tungsten transmission light source with a digitally controlled high intensity white light emitting diode (Lumileds, San Jose, CA, USA) permits rapidly acquired transmission light images to be interspersed with fluorescence images, for applications such as cell length measurement. Six TTL digital pulses can also be generated during recording to control perfusion flow valves or apply electrical stimuli.

The WinFluor software used to control the system displays live images and analogue signal traces within the same window during an experiment. Analysis features permit the plotting of the time course of the average intensity within multiple regions of interest within the images along with selected analogue signal channels. Ion concentration time courses can also be computed from fluorescence ratio images using the standard binding equations for ratiometric fluorophores.

The WinFluor software is available free of charge to academic and non-commercial users and can be downloaded from the web site www.strath.ac.uk/Departments/PhysPharm/ses.htm.