171P ANALYSIS OF OSTEOPROTEGERIN LIGAND EFFECTS ON THE FLG 29.1 PRE-OSTEOCLASTIC CELL LINE

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Osteoprotegerin (OPG) and OPG ligand (OPGL) regulate osteoclastogenesis, with OPGL promoting osteoclast differentiation, and OPG acting as a soluble decoy receptor neutralizing osteoclast formation and activity (Simonet *et al.*, 1997; Lacey *et al.*,, 1998). Since OPGL in combination with macrophage colony-stimulating factor (CSF)-1 can induce osteoclast–like cell formation from blood monocytes, spleenocytes and macrophages, we investigated the effects of these cytokines on the pre-osteoclastic cell line FLG 29.1 (Gattei *et al.*, 1992). Blood mononuclear cells (PBMC) were used as control.

FLG 29.1 cells were routinely grown in RPMI-1640 culture medium, supplemented with 10% foetal calf serum (FCS), and 100 μg/ml gentamicin, in an atmosphere of 5% CO_2/95% air at 37 °C. PBMC were isolated from human blood sample by separation on a gradient of Histopaque 1119/1077; The mononuclear cell population (~20% monocytes) was kept in RPMI-1640 culture medium as for the FLG 29.1 cells. Osteoclast differentiation was monitored by measuring tartrate resistant acid phosphatase (TRAP) activity. In both cases, cells were plated in 6 well plates ($5x10^4$/well) in 2ml RPMI-1640 medium with 1% FCS in the presence or in the absence of various concentrations of stimuli including OPGL (0.1-30 $ngml^{-1}$), CSF-1 (3 $ngml^{-1}$) and calcitonin (20 pM). Cell numbers were counted with a Neubauer hemocytometer after 3 and 6 days of culture. For TRAP activity, cell lysates were tested using a kit based upon hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Co., Poole, UK). Briefly, 500μl of substrate solution, 500μl of tartrate acid buffer solution and 200μl of the sample were incubated at 37 °C for exactly 30 min. The samples were read using a spectrophotometer (Labsystem Multiskan Bicromatic, Basingstoke Hants, UK) with a measurement filter of 405 nm. Bone resorption, was determined using the bone-slice assay. Cells were incubated in 24 well plates coated with a film of calcium phosphate apatite (OAAS™, OCT Inc). After 24h cells were removed by bleaching, the number of excavation pits counted for each well, and the pit area measured with an Argus-10 image-processing system (Hamamatsu Photonics, Enfield, UK). The resorbed area was calculated as total areas of individual excavations and expressed as a percentage of control values. All experiments were carried out in triplicate and results (mean ± s.e.mean) were analysed by analysis of variance followed by the Dunnett's test.

FLG 29.1 cells responded to OPGL with a decrease in cell replication and an increase in TRAP activity. These effects were observed after 3 days of culture but were more pronounced after 6 days: OPGL (30 $ngml^{-1}$) led to a significant ($P<0.01$) decrease in cell number (-59±5.5%, n=3 experiments). This was associated with a marked increase in TRAP activity: +77±24.8%, n=3, $P<0.01$ vs. non-stimulated cells. Co-addition of CSF-1 (3 $ngml^{-1}$) did not modify OPGL effect on cell replication, while produced a shift to the left of the concentration-response curve to the cytokine with respect to FLG 29.1 differentiation as measured by TRAP activity: an approximate ED_{50} of 1 $ngml^{-1}$ (vs. a value between 10 and 30 $ngml^{-1}$ in the absence of CSF-1) could be calculated. Interestingly the addition of calcitonin (a potent inhibitor of osteoclast activity) completely abolished the increase in TRAP activity caused by OPGL 30 $ngml^{-1}$ while it had minor effects on cell replication (n=3, $P<0.01$ vs. OPGL alone). Treatment of PBMC with OPGL 10 $ngml^{-1}$ and CSF-1 3 $ngml^{-1}$ for 6 days increased TRAP activity by 55±7% above the values measured in untreated cells, however a similar effect was measured with CSF-1 alone. Treatment of FLG 29.1 cells with either combination of stimuli led only to some morphological changes (cells become bigger and multinucleated), without producing resorptive activity. PBMC differentiation (9 d) with OPGL 10 $ngml^{-1}$ and CSF-1 3 $ngml^{-1}$, but not with either stimuli alone, led to induction of bone resorptive activity: 2220 ± 250 μm^2 (n=3).

In conclusion, FLG 29.1 cells respond to OPGL and a synergism between this cytokine and CSF-1 was observed. FLG 29.1 cells are a useful model for studying osteoclast precursors, but not to extrapolate mature osteoclast behavior.

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172P CARDIOVASCULAR EFFECTS OF ANANDAMIDE AND OF 5-HT IN THE ABSENCE AND PRESENCE OF THE $5HT_3$-RECEPTOR ANTAGONIST, AZASETRON IN CONSCIOUS RATS

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Intravenous administration of a high dose of anandamide in conscious rats causes initial, short-lived, pronounced bradycardia and hypotension (Lake *et al.*, 1997), reminiscent of the $5\text{-}HT_3$ receptor-mediated, chemosensitive (von Bezold-Jarisch) reflex (see Veelken *et al.*, 1993). This raises the possibility that some of the cardiovascular effects of anandamide may be secondary to 5-HT release. We have, therefore, measured the cardiovascular responses to anandamide and to 5-HT in conscious rats, in the absence and presence of the $5\text{-}HT_3$-receptor antagonist, azasetron (Fukuda *et al.*, 1991).

Under anaesthesia (fentanyl and meditomidine, 300μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1mg kg^{-1} of each s.c.), 9 male, Sprague-Dawley rats (350-400g) had pulsed Doppler flow probes and intravascular catheters implanted, in a 2 stage procedure, separated by at least 2 weeks. Measurements of heart rate (HR), mean arterial blood pressure (BP) and renal (R) mesenteric (M) and hindquarters (H) vascular conductances (VC) began 24h after catheterisation (Gardiner & Bennett, 1988). On Day 1, animals were given increasing i.v. doses of anandamide (75μg kg^{-1} to 2.5mg kg^{-1}), and a single dose of 5-HT (25μg kg^{-1}). On Day 2, the anandamide and 5-HT doses were repeated in the presence of azasetron (10μg kg^{-1}, 10μg kg^{-1} h^{-1}). Data are expressed as mean ± s.e.mean.

At the lower doses (up to 750μg kg^{-1}), anandamide caused initial (at 10s), dose-dependent, increases in BP and vasoconstriction (e.g., anandamide 750μg kg^{-1}, BP + 18±2 mmHg, RVC - 18±3%, MVC - 19±3%, HVC - 20±2% at 10s), which were not significantly affected by azasetron (e.g., anandamide 750μg kg^{-1} after azasetron, BP + 20±3 mmHg, RVC - 15±3%, MVC - 21±4%, HVC - 20±9% at 10s),. There was no significant change in HR at that time, in either the absence or the presence of azasetron.

At the highest dose (2.5mg kg^{-1}), anandamide caused an early (5±1s), pronounced, fall in HR (-245±35 beats min^{-1}), and BP (-43±8 mmHg), associated with falls in RVC (-32±5%), MVC (-61±6%) and HVC (-70±7%), which were not significantly affected by azasetron (after azasetron, HR -251±22 beats min^{-1}, BP -40±9 mmHg, RVC -39±9%, MVC -66±7%, HVC -83±4% at 6±1s).

In contrast, the bradycardic and hypotensive responses to 5-HT (-113±45 beats min^{-1}, -31±4 mmHg at 7±3s) were abolished by azasetron (-14±21 beats min^{-1}, -5±3 mmHg at 9±1s), although the associated changes in VC were not significantly affected (before *vs* after, RVC -10±9 *vs* -8±4%, MVC -29±7 *vs* -31±7%, HVC -27±9 *vs* -27±9%).

The present results, therefore, do not support the hypothesis that the initial cardiovascular effects of anandamide are secondary to 5-HT release.

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173P THE CONTRIBUTION OF NITRIC OXIDE (NO) TO ANGIOTENSIN II-INDUCED TACHYPHYLAXIS IN RAT AORTA

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Repeated administration of angiotensin AII to isolated vascular preparations frequently leads to tachyphylaxis (Gruetter *et al.*,1987, Robertson *et al.*,1994). This tachyphylactic phenomenon has been attributed to either an endothelium–dependent (Kornfeld *et al.*, 1997) or independent mechanism (Gruetter *et al.*, 1987) or the activation of protein kinase C (PKC) (Balmforth *et al.* 1997). In this experiment, we studied the involvement of NO and PKC in AII-induced tachyphylaxis in the rat aorta.

Endothelium intact aortic rings 3-5mm wide, prepared from male Hooded Lister rats (250-300g), were set up under 2g tension in Krebs' solution containing 10μM indomethacin (37°C, 95% O_2, 5% CO_2). The presence of endothelium was confirmed by relaxant responses to acetylcholine (1μM) following contractions induced by KCl (60mM). Each ring was repeatedly exposed to a maximal concentration of AII (1μM) for 5 min followed by washing and a 10 min recovery period before further exposure. Paired preparations were used: in one either a constitutive NO synthase inhibitor (L-NAME,100 or 300μM), a NO synthase substrate (L-arginine,1mM) or a selective/non-selective PKC inhibitor bisindolylmaleimide (BIM, 2μM) or chelerythrine (Che, 10μM) were administered for 20 min prior to application of AII. The other ring served as a time-matched control. L-NAME (100 or 300μM) was also administered to rings following induction of tachyphylaxis to AII.

A single exposure of the tissue to AII (1μM) induced a tension of 0.76±0.01g. Subsequent challenges with AII induced smaller increases in tension, approximately 15% of the first challenge. L-NAME (100μM) increased initial AII-induced tension to 0.9±0.03g and, when administered prior to each AII challenge, modulated the development of tachyphylaxis compared to the vehicle–treated paired-control. L-arginine enhanced tachyphylaxis (Table 1). PKC inhibitors (BIM and Che) did not prevent tachyphylaxis to AII (Table 1). In further experiments where tachyphylaxis to AII (1μM) was established, L-NAME (100μM) partly reversed (29 ± 6%) and L-NAME (300μM), fully restored (116 ± 8%) contractions to AII.

Table 1. Contractile response (as % of control Emax) to AII (1μM) in the absence or presence of L-NAME (100 or 300μM) or L-arginine (1mM) or BIM (2μM) or Che (10μM) in rat aortic rings (n = 6), *p<0.05, **p<0.01 and ***p<0.001. Treatment Vs control (100%). Student's paired t-test.

Treatment	Angiotensin II (1μM) challenges		
	2nd	3rd	4th
Control	19.7±2.0	15.3±3.0	14.3±3.1
L-NAME (10μM)	51.1±10.0**	52.2±8.3**	50.0±5.5***
L-NAME (300μM)	136±7.0***	136±11.0***	146±12.1***
L-Arginine (1mM)	46±8.5	2.2±2.0*	2.3±2.0*
BIM (2μM)	18.7±5.0	15.6±3.0	15.6±3.4
Che (10μM)	19.7±8.2	14.9±6.0	14.9±6.0

We conclude that in this preparation, L-NAME prevents and reverses tachyphylaxis to AII, L-arginine enhances it while PKC activation does not appear to be involved.

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174P EFFECTS OF INSULIN AND TYROSINE KINASE INHIBITION ON ISOLATED VASCULAR MUSCLE

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A relationship appears to exist between metabolic diseases such as obesity and diabetes and cardiovascular conditions such as hypertension, common features being insulin resistance and vascular endothelial dysfunction. Chronic complications of diabetes are dominated by cardiovascular disorders and the link between diabetes and cardiovascular disease may involve loss of a modulating role of the endothelium. Vasodilation involving endothelium-derived nitric oxide (NO) was impaired in animal models of diabetes and diabetic patients (Consentino & Luscher, 1998). Acute administration of insulin was reported to cause either NO-mediated vasodilation (Sartori *et al*, 1999) or NO/endothelium-independent relaxation by Izhar *et al* (2000). In the present experiments we studied effects of insulin in intact and endothelium-denuded (ED) rat aortic rings and the possible involvement of tyrosine kinase.

Endothelium intact or denuded rings 3-5mm wide, prepared from male Hooded Lister rats (250-300g) were set up under 2g tension in Krebs' solution containing 10μM indomethacin (37°C, 95%, O_2, 5% CO_2). The presence of endothelium was confirmed by relaxant responses (>30%) to acetylcholine (1μM) following contraction by KCl (60mM). Concentration-response curves were constructed to KCl (10-110mM) alone and in the presence of insulin (INS 0.1, 1.0, 10mU ml^{-1} or the tyrosine kinase inhibitor, genistein (GEN 20-40μM) or daidzein (DAD, an inactive analogue of GEN, 20μM) or combinations of INS and GEN or DAD. N = 4-6. One concentration of insulin was used in each ring; no vehicle effects were observed.

In intact rings INS (0.1, 1.0, 10mU ml^{-1}) caused rightward shifts in the KCl concentration-response curve and reduced E_{max} by 8.8±1.7, 10.2±0.4, 19.7±5.6% respectively (P<0.01). However in ED rings INS (0.1, 1.0, 10.0mU ml^{-1}) enhanced contractile responses to KCl: E_{max} increased by 15.9±2.6. 25.3±3.3, 46.1±3.4% respectively (P<0.01) (Student's paired t-test). In the presence of intact endothelium GEN (20, 40μM) caused rightward shifts of the KCl concentration-response curve and reduction in E_{max} of 13.3±4.0 and 32.0±3.6% respectively. DAD (20μM) caused little change. Combination of INS and GEN (20, 40μM) caused large concentration-related reductions in responses to KCl, e.g. 40μM GEN + 10mU ml^{-1} INS reduced E_{max} by 40.3±4%. Combination of INS and DAD (20μM) showed similar effects.

In ED rings GEN and DAD (20μM) alone had little effect on KCl concentration-response curves. GEN (40μM) reduced responses to low concentrations of KCl but did not affect E_{max}. However, both GEN and DAD reversed the enhancement of KCl-induced contractile responses produced by insuln.

We conclude that effects of insulin on aorta are markedly affected by the endothelium; contractile responses were reduced when endothelium was present and enhanced when absent. These actions of insulin are unlikely to involve tyrosine kinase activity since, in intact rings, the tyrosine kinase inhibitor, genistein, did not reverse but potentiated relaxant effects of insulin. Daidzein, an analogue devoid of tyrosine kinase inhibitory activity, had similar actions. In denuded rings both genistein and daidzein reversed insulin-induced increases in contractile response, suggesting that properties of these phytoestrogens, other than specific tyrosine kinase inhibition, are responsible for the effects observed.

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175P N^G-NITRO-L-ARGININE METHYL ESTER ATTENUATES THE VASORELAXATIONS TO DY-9708, BUT NOT CROMAKALIM, IN RAT ISOLATED AORTIC RINGS

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The chemical diversity of ATP-sensitive potassium channel openers (K_{ATP}COs) has suggested the involvement of multiple sites on the target membrane (Lawson, 1996). N^G-nitro-L-arginine methyl ester (L-NAME) differentially inhibits vasorelaxant responses to K_{ATP}COs suggesting that pinacidil and aprikalim activate a vasorelaxant mechanism in rat aorta independent of the mechanism(s) operated by the benzopyran, cromakalim (Carr & Lawson, 1999). We therefore studied the vasorelaxations to the benzopyrans, DY-9708 (Horino *et al.*, 2000) and SKP-450 (Lee *et al.*, 1998), in rat aorta to determine if L-NAME insensitivity is a common feature to all agents in this chemical family of K_{ATP}COs.

Aortic rings, devoid of endothelium, from male Wistar rats (200-250g) were suspended under a resting tension of 2g in Kreb's bicarbonate solution (gassed with 95%O_2/5%CO_2 at 37° C). After 60 min equilibration, cumulative concentration response curves (CRC) to cromakalim (0.05-12.8μM), DY-9708 (0.001-0.256μM) or SKP-450 (0.001-0.0512μM) were constructed 30 min after incubation with L-NAME (100 or 300μM), N^G-nitro-L-arginine (L-NNA 100μM), N^G-monomethyl-L-arginine (L-MMA 100μM), L-N^5-(l-iminoethyl)ornithine (L-NIO 100μM) or vehicle (control) in phenylephrine (1.0μM)-contracted tissues. EC_{50} (concentration of K_{ATP}CO to evoke 50% relaxation of phenylephrine contraction) and Emax values are reported as means±s.e.m. (n = 4-10). Concentration ratios with 95% confidence limits CR(cl) were determined from EC_{50} values of paired preparations.

Concentration related relaxations were produced by cromakalim (EC_{50} 0.40±0.24μM, Emax 84±6%), DY-9708 (EC_{50} 0.016±0.002μM, Emax 74±5%) and SKP-450 (EC_{50} 4.1±0.5nM, Emax 100%). L-NAME (100μM) significantly displaced to the right of controls the CRC to DY-9708 (CR(cl) 1.93 (1.36-2.51)), but not to cromakalim (EC_{50} 1.32±0.90μM) or SKP-450 (EC_{50} 7.0±2.9nM). L-NAME (300μM) did not further attenuate the relaxations to DY-9708 (EC_{50} 0.037±0.006μM) and did not modify the CRCs to cromakalim or SKP-450. The DY-9708-induced vasorelaxations were also attenuated by the L-arginine analogues, L-MMA (CR(cl) 2.48 (1.42-3.53)) and L-NNA (CR(cl) 2.96 (0.38-5.54)). In contrast, L-NIO (100μM) failed to modify the CRC to DY-9708 (EC_{50} 0.020±0.001μM) relative to control.

In conclusion, L-NAME can selectively modify the vasorelaxant responses to DY-9708 relative to cromakalim and SKP-450. The effects of L-NAME appear to be independent of nitric oxide synthase (NOS) inhibitory properties. These findings suggest that the benzopyran K_{ATP}COs may not possess a common pharmacophore and interact with the 'K_{ATP}CO receptor' on the K_{ATP} channel differently.

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176P INTERACTIONS BETWEEN ISOPRENALINE AND LEVCROMAKALIM IN THE RELAXATION OF RAT ISOLATED MESENTERIC ARTERY

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We have shown that activation of either the cyclic GMP or cyclic AMP systems can modulate the relaxant effects of the ATP-sensitive K^+ channel (K_{ATP}) activator, levcromakalim, even though activation of K_{ATP} does not contribute to the relaxant effects of the cyclic nucleotides in rat mesenteric arteries (White & Hiley, 1998; Omar *et al.*, 2000). The aim of the present study was to examine whether or not the β-adrenoceptor agonist, isoprenaline, also modulates levcromakalim-induced relaxation.

Segments (2 mm) of third generation mesenteric arteries from male Wistar rats (250-300g) were mounted in a wire myograph under a normalised tension in oxygenated Krebs-Henseleit buffer at 37°C as described by White & Hiley (1998). Vessels were precontracted with methoxamine (10 μM, a submaximal concentration) and >90% relaxation to 10 μM carbachol was taken as showing the endothelium was functional. In some experiments vessels were denuded of endothelium by rubbing the intimal surface with a human hair. Interactions between vasodilators were investigated according to protocols described previously (White & Hiley, 1998). Briefly, vessels were precontracted with methoxamine, and a concentration of isoprenaline added to give approximately 30 or 50% relaxation. The vessel tension was then returned to the initial level of tone by further addition of methoxamine, and a concentration-response curve to levcromakalim was then established from the restored tone level. Data were fitted to a logistic equation to determine EC_{50} and the maximal response (R_{max}) and comparison between values was by Student's t-test (White & Hiley, 1998).

Isoprenaline (at a concentration causing ~30% relaxation of tone induced by methoxamine) potentiated relaxation to levcromakalim (EC_{50}: control, 0.45±0.04 μM; in the presence of isoprenaline, 0.17±0.03 μM; n = 10; $P < 0.001$); higher concentrations of isoprenaline exerted no additional effect. Relaxation to isoprenaline was antagonised by the β_1-adrenoceptor antagonist, atenolol (10 μM, which gave a 16-fold shift in the EC_{50}), and atenolol abolished the modulatory effect on the levcromakalim response (EC_{50}: control, 0.32±0.03 μM; in the presence of isoprenaline + 10μM atenolol, 0.28±0.04 μM; n = 8 for both). However, the K_{ATP} inhibitor glibenclamide did not inhibit relaxations to isoprenaline.

The protein kinase A inhibitor, Rp-cAMPS (50 μM), abolished modulation by isoprenaline of levcromakalim relaxation (EC_{50}: control, 0.13±0.04 μM; with isoprenaline + Rp-cAMPS, 0.13±0.02 μM; n = 6 for both). However relaxation to isoprenaline was unaffected by Rp-cAMPS (up to 100 μM) and only slightly inhibited by H-89, another protein kinase A inhibitor (maximal relaxation to isoprenaline: control, 100±1%; with 5 μM H-89, 84±3%; n = 5). Nevertheless Rp-cAMPS (50 μM) effectively inhibited relaxations induced by forskolin (a direct activator of adenylyl cyclase).

Isoprenaline relaxed methoxamine-precontracted mesenteric arteries without endothelium in a concentration-dependent manner (EC_{50} = 69±10 nM, R_{max} = 102±5%; n = 4) but it did not relax arteries precontracted with 60 mM KCl (R_{max} = 7±5%; n = 7). In contrast, forskolin relaxed arteries contracted with either methoxamine (EC_{50} = 38±2 nM, R_{max} = 97±1%; n = 4) or 60 mM KCl (EC_{50} = 0.22±0.02 μM, R_{max} = 78±2%; n = 5).

We conclude that isoprenaline modulates the actions of levcromakalim through β_1-adrenoceptors and protein kinase A, even though K_{ATP} channels do not contribute to its relaxant effect. It is likely that the mechanism by which isoprenaline modulates K_{ATP} is distinct from its relaxant mechanism.

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177P INFLUENCE OF THE ET_A RECEPTOR ANTAGONIST, SB 234551, ON HAEMODYNAMIC RESPONSES TO LIPOPOLYSACCHARIDE (LPS) IN CONSCIOUS RATS

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In conscious rats, the nonselective (ET_A/ET_B) endothelin receptor antagonist, SB209670, enhances LPS-induced hypotension and mesenteric and hindquarters vasodilatation, but, surprisingly, not the accompanying hyperaemic renal vasodilatation (Gardiner *et al.*, 1995), possibly because of concurrent inhibition of ET_A-mediated vasoconstriction and ET_B-mediated vasodilatation in the kidney. Since it has been shown that the selective ET_A receptor antagonist, SB 234551, unmasks endothelin-induced renal vasodilatation under certain conditions in the dog (Brooks *et al.*, 1998), we hypothesised that this compound might enhance the renal vasodilator effects of LPS infusion in conscious rats.

Male, Long Evans rats (350-450g) were chronically instrumented with renal, mesenteric and hindquarters Doppler probes and intravascular catheters (all surgery under anaesthesia, (Hypnorm (0.126mg kg^{-1} fentanyl citrate, 4mg kg^{-1} fluanisone) and midazolam, 5mg kg^{-1} i.p.) at least 24 h before experiments). On the first experimental day animals were given 3 increasing i.v. doses (25, 50 and 250 pmol kg^{-1}) of endothelin-1 (ET-1) separated by at least 1h. Thereafter, animals were given an i.v. infusion of vehicle (n=8, 0.5% Na_2CO_3 diluted 1:4 with 5% dextrose, 0.4 ml h^{-1}) or SB 234551 (n=8, 500nmol kg^{-1}, 500nmol kg^{-1} h^{-1}) beginning 2h before being re-challenged with ET-1 (as above). On the following day, animals that had received vehicle or SB 234551 on the first day were given the same treatment beginning 1 h before onset of co-infusion of LPS (*E coli* serotype 0127 B8, 150 μg kg^{-1} h^{-1}) for 6 h. SB 234551 significantly ($P<0.05$, Mann-Whitney U test) inhibited the rise in blood pressure (BP) and falls in renal (R) and mesenteric (M) vascular conductances (VC) following ET-1 (e.g., vehicle/SB 234551, ET-1 at 50 pmol kg^{-1} 0-10 min area under or over curve = ΔBP, + 167±21/+101±11 mmHg min, ΔRVC, –421±19/–239±20% min, ΔMVC, –404±27/–310±31% min) but not the increase in hindquarters (H)VC (+48±11/+50±16% min). Table 1 summarises some of the results with LPS infusion.

Table 1. Integrated (0-6h area under or over curve (AUC, AOC)) changes during LPS infusion in the presence of vehicle or SB 234551 in conscious rats. Values are mean ± s.e.mean. *$P<0.05$ Mann Whitney U test

		Vehicle	SB 234551
ΔBP	(AOC; mmHg h)	–63±13	–103±10*
ΔRVC	(AUC; % h)	+245±40	+229±36
ΔMVC	(AUC; % h)	+63±12	+199±37*
ΔHVC	(AUC; % h)	+103±18	+185±23*

Although SB 234551 enhanced the hypotensive and mesenteric and hindquarters vasodilator responses to LPS, the renal vasodilator response was unaffected. This contrasts with the ability of SB 234551 to inhibit the renal vasoconstrictor effect of exogenous ET-1. The failure of either SB209670 (Gardiner *et al.*, 1995) or SB234551 to affect the renal vasodilator responses to LPS infusion may indicate that endogenously produced endothelin, in this condition, does not affect renal vascular tone.

SB234551 was a gift from Dr E. Ohlstein (SKB, USA)

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178P ANTI-ARRHYTHMIC AND ELECTROPHYSIOLOGICAL EFFECTS OF THE ENDOTHELIN RECEPTOR ANTAGONISTS, BQ-123 AND PD161721

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The ET_A receptor antagonist BQ-123, and the mixed $ET_{A/B}$ receptor antagonist PD161721, have previously been shown to be anti-arrhythmic in the setting of myocardial ischaemia *in vivo* (Sharif *et al.*, 1998). The aims of this study were to determine if these antagonists also protected against ischaemia-induced arrhythmias *in vitro* and to assess if they had direct cardiac electrophysiological activity.

Male Sprague-Dawley rats (300 to 450g) were anaesthetized with sodium pentobarbitone, and the hearts removed for Langendorff perfusion. Ischaemia was induced by left main coronary artery occlusion for 30 min. Coronary perfusion pressure (CPP) and an electrocardiogram were recorded. Vehicle (n=12), BQ-123 ($5x10^{-7}$; n=8 and 10^{-6}M; n=8) or PD161721 (10^{-7}; n=8 and 10^{-6}M; n=7) was infused 10 min prior to and throughout the ischaemic period. The incidences of ventricular tachycardia (VT) and fibrillation (VF) were compared using Fischer's exact (X^2) test. Left atrial muscle, from male Dunkin-Hartley guinea-pigs (400 to 450g) was stimulated, at a frequency of 4 Hz, in an organ bath containing Krebs Henseleit solution with a concentration of either 5.4 or 8.0 mM KCl. The maximum following frequency was measured and the effective refractory period (ERP) calculated. BQ-123 (10^{-8} to 10^{-6}M; n=6), PD161721 (10^{-8} to 10^{-6}M; n=6), lignocaine (0.43 to $3.4x10^{-4}$M; n=10) or vehicle (deionised water; n=3) was added cumulatively to the organ bath and ERP determined 10 min after the addition of each concentration. CPP, heart rate and ERP were expressed as mean ± SEM and compared within groups using one-way ANOVA and Dunnett's post-hoc test. $P\leq0.05$ was taken as statistically significant.

The % incidence of ischaemia-induced irreversible VF was reduced significantly from 58%, in control rat hearts, to 0% (at both concentrations of PD161721 and 10^{-6}M BQ-123). BQ-123, at $5x10^{-7}$M, did not affect the incidence of VF (50%). Neither antagonist reduced the % incidence of VT from 58% in the control group. CPP (61±4 mmHg) and heart rate (305±21 beats min^{-1}) were not significantly modified by either antagonist before occlusion. In guinea-pig left atrial muscle, in the presence of normal extracellular [K^+], BQ-123 and PD161721, at 10^{-6}M, significantly increased ERP from 114±8 to 147±3 and from 121±6 to 150±6 ms respectively. These effects were not potentiated by raising the extracellular [K^+]; e.g. ERP values of 128±6 and 145±7 ms were recorded before and after 10^{-6}M PD161721. In contrast, lignocaine's ability to prolong ERP was greater in elevated (e.g. at $1.7x10^{-4}$M from 120±4 to 556±144 ms) than normal [K^+] (from 112±4 to 229±26 ms).

In conclusion, both ET receptor antagonists, BQ-123 and PD161721, had an anti-fibrillatory effect in isolated rat hearts that may be due, at least in part, to an ability to prolong ERP. This latter effect is unlikely to be due to Na^+ channel blockade since it was not potentiated by elevation of the extracellular K^+ concentration.

TRC is supported by the British Heart Foundation.

SHARIF, I., KANE, K.A. & WAINWRIGHT, C.L. (1998). *Cardiovasc. Res.*, **38**, 625-632.

179P ANGIOTENSIN II GIVES CONCENTRATION-DEPENDENT REDUCTIONS IN INFARCT SIZE IN RAT ISOLATED PERFUSED HEARTS SUBJECTED TO ISCHAEMIA/REPERFUSION

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Angiotensin II (AngII), used as a preconditioning stimulus, reduces ischaemia/reperfusion (IR)-associated infarct size (Liu et al., 1995). However, a number of AngII responses are potentially harmful in a setting of IR. It is not clear what effect AngII might have on the severity of IR injury when present throughout IR. We have therefore examined the effect of graded AngII concentrations, present throughout IR, on post-ischaemic infarct size and contractile recovery of isolated hearts.

Hearts from male Wistar rats (250-350g) were perfused via the aorta at constant pressure (75 mmHg) with Krebs solution (mM: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; Mg_2SO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; D-glucose, 11) containing 100 mU l^{-1} insulin and saturated with 95% O_2/5% CO_2. Left ventricular developed pressure (LVDP) was measured with a pressurised (5-10 mmHg) balloon inserted into the ventricle and connected to a pressure transducer. Coronary flow (CF) was measured with a Transonic flow probe on the aortic inflow cannula connected to a T206 flow meter. Baseline mechanical function was recorded during an initial 30 min aerobic perfusion. Hearts were then subjected to 25 min global, normothermic (37°C), no-flow ischaemia followed by 30 min reperfusion. They were electrically paced at 5 Hz when being perfused. Infarct size (% of the left ventricle) was determined from triphenyltetrazolium chloride staining. Vehicle (n = 13) or Ang II (final concentrations of 0.3, 1, 10 & 100 nM, n = 5, 15, 9 & 8, respectively) was infused for a 10 min period before ischaemia and throughout reperfusion. Values (mean ± s.e.mean) obtained after 30 min of reperfusion were compared among vehicle and Ang II groups by analysis of variance with Dunnett's *post hoc* test.

LVDP (mmHg) did not vary during the baseline perfusion between the groups (control, 103±5; AngII, 0.3 nM, 101±5; 1 nM, 111±5; 10 nM, 111±4; 100 nM, 96±6). Additionally, baseline coronary flow (ml min^{-1}) was not significantly different between the groups (control, 14.5±3.0; AngII 0.3 nM, 15.5± 1.6; AngII, 1 nM, 14.0±0.8; AngII, 10 nM, 13.8±2.6; AngII, 100 nM, 13.0±1.0).

Addition of AngII before ischaemia significantly reduced coronary flow (0.3 nM, 6%; 1 nM, 16%; 10 nM, 22%; 100 nM, 13%) but had no effect on LVDP (Student's paired t-test). After 30 min reperfusion, LVDP in control hearts was depressed (53±4 mmHg) compared to that obtained after 30 min baseline perfusion (102±5 mmHg). Coronary flow in control hearts was also depressed after 30 min reperfusion (5.6±2.5 ml.min^{-1}) compared to that obtained before ischaemia (14.0±1.4 ml.min^{-1}). Compared with controls, none of the concentrations of AngII used had any significant effect on the recovery of LVDP or coronary flow after 30 min reperfusion. Compared to control, 1, 10 and 100 nM AngII decreased infarct size whereas 0.3 nM AngII had no significant effect (Figure 1).

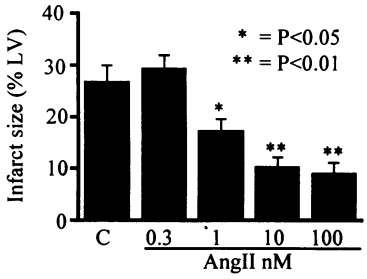

Figure 1. Effects of Krebs vehicle or increasing concentrations of AngII on infarct size in rat hearts subjected to IR.

Thus, increased exogenous AngII during IR reduces infarct size in a concentration-dependent manner and has no adverse effects on mechanical function. Although no conclusions can be made about the specific receptor subtype(s) involved, these data suggest that the rationale for using blockers of the renin-angiotensin system to reduce IR injury needs to be reassessed.

Liu, Y. et al. (1995) *J. Mol. Cell. Cardiol.*, 27, 883-892.

180P RELEASE OF A VASODEPRESSANT FACTOR FROM THE RAT ISOLATED HEART: EFFECT OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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We recently reported studies indicating the possible release from isolated rabbit hearts of a transmissible factor modulating the release of the vasoconstrictor peptide, endothelin-1 from isolated peripheral vasculature (Chokkukanan, et al, 1998). We have now studied the potential release of a similar transmissible modulating factor from isolated perfused rat hearts.

Hearts from male Sprague Dawley rats (370-530g) were perfused (Langendorff, 10ml.min^{-1}) with modified Krebs solution (mM: NaCl 118, KCl 2.0, $CaCl_2$ 1.23, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 11.0). Perfusate draining from the hearts was directly pumped (4ml.min^{-1}) through isolated hind-limbs via femoral artery. Perfusate entering both hearts and hind-limbs was gassed with O_2:CO_2 (95:5%). Hind limb perfusion pressure (PP) was measured to monitor vascular tone. Following 85 min stabilisation, the hearts were subjected to 30 min left main coronary artery occlusion followed by 120 min reperfusion (Isch/Rep). The two comparator groups consisted of sham operated (SO, i.e. without coronary occlusion) sequentially perfused organs, and non-sequentially perfused hind limbs (NS). Data were analysed using two-way ANOVA.

PP in the non-sequentially perfused limbs showed a continuous gradual increase between the start and the end of the experiment (Figure 1). This increase was greatly reduced ($p<0.0001$) when the limbs were supplied with effluent from normally perfused hearts. The increase was not reduced when the limbs were supplied with effluent from hearts which had been subjected to ischaemia and reperfusion. The mean PP in the NS group at the end of the experiment was 58.3% greater than that of the SO group, that of the Isch/Rep group was 82.7% greater. However, the difference between Isch/Rep and NS groups was not significant. These observations suggest that the hearts were releasing a transmissible factor which either was a vasodilator or was capable of inhibiting ongoing vasoconstrictor mechanisms in the peripheral vasculature. Following myocardial ischaemia and reperfusion (MIR), this transmissible factor was no longer detectable.

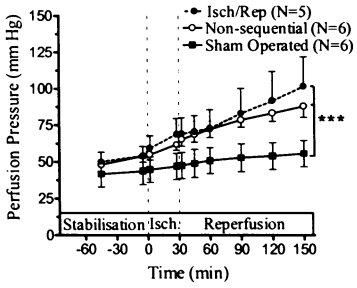

Figure 1. Comparison between the effect of perfusate from normally perfused and MIR rat hearts on vascular resistance in sequentially perfused rat hind-limbs. Results from non-sequentially perfused control hind-limbs are shown (mean ± SEM). Stars show $p<0.0001$ for differences between the sham operated and the other two groups.

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Chokkukanan J., Zeitlin I.J. & Wainwright C.L. (1998). *J. Cardiovasc. Pharmacol.* 31, S427-S430

181P ALTERED RESPONSIVENESS TO β_2 ADRENOCEPTOR AND NPY Y_2 RECEPTOR STIMULATION DURING PROGRESSION OF CARDIOMYOCYTE HYPERTROPHY

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Increased plasma levels of catecholamines and neuropeptide Y (NPY) occur in hypertension and correlate with severity of left ventricular hypertrophy (LVH) *in vivo* (Hulting *et al.*, 1990). Neither NPY nor the β-adrenoceptor agonist, isoprenaline, initiate protein synthesis in freshly isolated cardiomyocytes *in vitro*, but have been found to augment protein synthesis in re-differentiated cardiomyocytes, a cellular model relevant to established hypertrophy (Millar *et al.*, 1994). The spontaneously hypertensive rat (SHR) displays many similarities to human essential hypertension including the development of LVH (Pfeffer *et al.*, 1976).

To determine whether NPY Y_2 receptor-related and β-adrenoceptor-related signalling pathways become activated with progression of the hypertrophic process *in vivo*, cellular protein mass and *de novo* protein synthesis, as evidenced by incorporation of ^{14}C-phenylalanine, (Phe, 0.1 $\mu Ci.ml^{-1}$) were assessed in cardiomyocytes obtained from SHRs (12, 16 and 20 weeks of age) and from age-matched normotensive WKY animals. Cardiomyocytes were isolated by Langendorff perfusion of the excised hearts with collagenase ($0.4mg.ml^{-1}$) and cultured for 24 hours in the presence of the appropriate agonists (Millar *et al.*, 1994). Data were analysed using a 1 or 2 way analysis of variance using SPSS-PC. If $p<0.05$, a multiple range test (Scheffe) (> than 2 conditions), or an unpaired Student's t-test (2 conditions) was applied as appropriate.

Hypertension was already evident in SHRs at 12 weeks: systolic blood pressure, assessed by tail cuff sphygmomanometry, was 190±3 mmHg in SHRs versus 132±6 mmHg in WKYs, n=10, $p<0.05$. Heart: body mass ratios were not different at 12 weeks, but were 6.7±0.9% and 14.0±1.9% greater in SHRs at 16 and 20 weeks, respectively, compared to age-matched WKYs ($p<0.05$). At cellular level, basal incorporation of ^{14}C-phe was elevated in cardiomyocytes from SHRs (531.8±63.1 dpm.μg DNA^{-1}, n=6) compared to WKYs (378.5±41.7 dpm.μg DNA^{-1}, n=6) at 16 weeks ($p<0.05$) and, to a lesser extent, at 20 weeks (476.6±32.5 7 dpm.μg DNA^{-1} versus 369.0±25.8 7 dpm.μg DNA^{-1}, n=10; $p<0.05$). No difference was observed between strains at 12 weeks of age. Isoprenaline (10^{-7}M) increased cellular protein mass (10.7±2.9%, n=6) and *de novo* protein synthesis (11.6±3.5%, n=6) above basal levels in SHRs at 16 weeks ($p<0.05$) but elicited negligible increases in SHRs at 12 and 20 weeks and was devoid of activity in WKYs at all ages. The β_2-adrenoceptor agonist, procaterol (10^{-7}M), also increased these parameters by 13.6±7.1% and 10.7±3.4%, respectively, (n=6; $p<0.05$) in SHRs at 16 weeks. NPY (10^{-7}M) did not increase cellular protein mass significantly in cardiomyocytes from SHRs at any age (n=4) and was devoid of any effect in WKY cells at all ages. However, de *novo* protein synthesis was increased in SHRs at 12 and 16 weeks ($p<0.05$) in response to NPY (10^{-7}M) by 10.9±2.8% and 14.1±5.9%, respectively. A smaller, non-significant increase of 4.5±2.6% was observed in SHRs at 20 weeks while no increases were evident in WKY cells at any age. The Y_2-receptor agonist, PYY_{3-36} (10^{-7}M), increased cellular protein mass and *de novo* protein synthesis by 18.0±6.3% and 16.2±5.1%, respectively (n=4: $p<0.05$) in SHRs at 16 weeks.

In conclusion, these data confirm that cardiac hypertrophy occurs in the SHR subsequent to the onset of hypertension. β_2-adrenoceptor and NPY Y_2 receptor-associated signalling pathways are not associated with the initiation of hypertrophy at cellular level but become coupled to the hypertrophic response during the most active phase of hypertrophic growth and contribute to the magnitude of the hypertrophic adaptation.

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182P EVIDENCE FOR INVOLVEMENT OF ET_B RECEPTORS AND ASSOCIATED INVOLVEMENT OF PKC-DEPENDENT MECHANISMS IN ENDOTHELIN-1 MEDIATED CARDIOMYOCYTE HYPERTROPHY

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Increased plasma levels of endothelin-1 (ET-1) correlate with the severity of left ventricular hypertrophy (LVH) *in vivo* (Levin, 1995). Although there is emerging evidence that ET_A receptor antagonism can attenuate LVH *in vivo*, it is unclear if this occurs as a direct result of blockade of receptors for the peptide present on cardiomyocytes, or represents an indirect effect, mediated by reduction in pressure overload (Ito *et al.*, 1994). The role of ET_B receptors in the initiation of cardiac hypertrophy in response to ET-1 is less clear.

The aim of the study was to (i) establish the requirement for *de novo* protein synthesis, as evidenced by the incorporation of *l*-U-[^{14}C]-phenylalanine (phe) ($0.1\ \mu C_i.ml^{-1}$) and increased abundance of total cell RNA, for increased protein mass elicited directly in response to ET-1 in ventricular cardiomyocytes, isolated from 12 week old male Sprague-Dawley rats by Langendorff perfusion of excised hearts with collagenase ($0.4mg.ml^{-1}$) (Bell *et al.*, 1995), and maintained in culture for 24 hours; (ii) determine the relative contribution of stimulation of ET_A and ET_B receptors and (iii) provide evidence for involvement of protein kinase C (PKC) in the response elicited by the peptide. Data were analysed using a 1 or 2 way analysis of variance using SPSS-PC. If $p<0.05$, a multiple range test (Scheffe) (> than 2 conditions), or an unpaired Student's t-test (2 conditions) was applied as appropriate.

ET-1 increased the total mass of cellular protein and incorporation of *l*-U-[^{14}C]-phe maximally at 10^{-7} M to 26% and 25% greater ($p < 0.05$) than basal values which were 53.1±5.7 μg.μg DNA^{-1} (n=6), and 733.3±85.4 dpm.μg DNA^{-1} (n=12), respectively. ET-1 (10^{-8}M) increased the total content of RNA to 23.0% ($p<0.05$) greater than the basal value of 0.56±0.09μg. μg DNA^{-1} (n=4). The incorporation of 2-[^{14}C]-uridine ($0.1\ \mu C_i.ml^{-1}$) into cell RNA in the presence of ET-1 (10^{-8} M) was 21.0% greater than the basal value at 8 hours (1392±120 dpm.μg DNA^{-1}, n=6; $p<0.05$). Actinomycin D (5×10^{-6}M), an inhibitor of transcription, abolished both the incorporation of *l*-U-[^{14}C] phe and the increased cell protein mass elicited by ET-1 (10^{-10}M-10^{-8}M).

The selective agonists at the ET_B receptor, sarafotoxin 6c (S6c) and ET-3, increased the incorporation of *l*-U-[^{14}C] phe maximally at 10^{-7} M to 13% greater than basal values (504.0±44.8 dpm.μg DNA^{-1} and 518.9±27.0 dpm.μg DNA^{-1}, n=6 and n=10, respectively). The incorporation of *l*-U-[^{14}C] phe in response to ET-1 (10^{-7}M) was decreased by 50% ($p<0.05$) by the selective antagonist at ET_A receptors, ABT-627 (10^{-9}M) (Douglas, 1997), while the response to S6c was not attenuated. Conversely, the selective antagonist at ET_B receptors, A-192621 (10^{-10}M), abolished the response to S6c (10^{-7}M) and attenuated the response to ET-1 (10^{-7}M) by 43% ($p<0.05$). The incorporation of *l*-U-[^{14}C] phe in response to ET-1 (10^{-11}M -10^{-7} M) was attenuated ($p<0.05$) by the selective inhibitor of PKC , bisindolylmaleimide (BIM, 5×10^{-6} M) (Toullec *et al.*, 1991). BIM attenuated the response to S6c (10^{-7} M) by 78% ($p<0.05$) and to ET-1 (10^{-7} M), elicited in the presence of A-192621 (10^{-10}M), by 52% ($p<0.05$).

In conclusion, these data implicate ET_B receptors, in addition to ET_A receptors, in cardiomyocyte hypertrophy elicited in response to ET-1 and provide evidence for the involvement of protein kinase C, at least in part, in the hypertrophic signalling pathways associated with activation of each receptor sub-population.

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183P INVESTIGATING THE EFFECTS OF ADENINE NUCLEOTIDES AND RELATED COMPOUNDS ON CARDIAC RYANODINE RECEPTOR ACTIVATION USING [^{3}H]RYANODINE BINDING

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It has previously been shown that ATP can almost fully activate cardiac ryanodine receptor (RyR) channels in the presence of activating levels of cytosolic Ca^{2+} (Kermode *et al.*, 1998). To investigate the specificity of the RyR binding sites for ATP we have examined the ability of a variety of adenine nucleotides to stimulate [^{3}H]ryanodine binding to isolated sarcoplasmic reticulum (SR) membrane vesicles. Ryanodine binds only to the open state of RyR and therefore changes in [^{3}H]ryanodine binding may be used as an index of RyR open probability.

Sheep hearts were obtained from an abattoir and the heavy SR membrane fraction was isolated as previously described (Kermode *et al.*, 1998). SR membrane vesicles were incubated in the presence of 5 nM [^{3}H]ryanodine for 24 hr in 250 mM HEPES, 125 mM Tris, pH 7.2 at 21±2°C. Non-specific binding was determined by the addition of 5 μM unlabelled ryanodine. The free [Ca^{2+}] of the solutions was maintained at 10 μM by buffering with EGTA and $CaCl_2$.

10 μM Ca^{2+} alone stimulated [^{3}H]ryanodine binding to 0.18±0.02 pmol/mg protein (s.e.m.; n=19). Addition of ATP to the incubation medium stimulated [^{3}H]ryanodine binding to an optimal value of 0.85±0.20 pmol/mg protein; an increase of 470±100% (s.e.m.; n=6) at 20 mM ATP. The related compounds, AMP-PCP, ADP, AMP, adenosine, adenine and guanosine were much less effective at stimulating [^{3}H]ryanodine binding and produced optimal increases in binding to 177±39%, 257±41%, 179±39%, 178±36%, 284±51% and 111±7%. (s.e.m.; n=4-7) of control levels respectively. To investigate the role of the phosphate groups in the adenine nucleotide-induced stimulation of [^{3}H]ryanodine binding, we examined how phosphate (P_i) and diphosphate (PP_i) affected binding in the presence and absence of ADP and AMP respectively. P_i and PP_i both failed to significantly stimulate [^{3}H]ryanodine binding. In addition, 20 mM P_i had no effect on ADP-induced stimulation of binding (optimal binding was 258±22% of control (s.e.m.; n=7)). 20 mM PP_i potentiated the effects of AMP, increasing optimal binding to 259±10% (s.e.m.; n=4) of control although this increase in binding was still much lower than that seen with ATP.

In conclusion, as guanosine produces no stimulation of [^{3}H]ryanodine binding this suggests that the adenine ring structure is important for stimulation of [^{3}H]ryanodine binding and therefore for activation of cardiac RyR channels. The levels of [^{3}H]ryanodine binding obtained with AMP or ADP plus the extra phosphate groups are not as high as those obtained with ATP. This indicates that the structural conformation of ATP is very important for the ability of ATP to increase P_o.

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184P NOVEL EFFECTS OF DIDS ON THE GATING AND CONDUCTANCE OF THE SHEEP CARDIAC RYANODINE RECEPTOR

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We have previously demonstrated that, with Ca^{2+} as the permeant ion, cytosolic application of diisothiocyanostilbene-2, 2'-disulphonic acid (DIDS) to cardiac ryanodine receptor (RyR2) channels causes an abrupt and irreversible increase in unitary conductance to a fully open state from which no closings can be resolved (Sitsapesan, 1999). Evidence suggests that DIDS binds to the suramin binding sites on RyR to cause these effects (Sitsapesan, 1999). To investigate if the permeant ion can influence the effects of DIDS we have now studied the DIDS-induced modification of RyR2 function using K^+ as the permeant ion. Purified RyR2 were isolated from sheep hearts obtained from an abattoir as described by Lindsay and Williams (Lindsay & Williams, 1991). Channels were then incorporated into planar phosphatidylethanolamine lipid bilayers under voltage-clamp conditions and current fluctuations through the channel were recorded in symmetrical solutions of 210 mM KCl, 20 mM HEPES, pH 7.2 as previously described (Sitsapesan & Williams, 1994).

Addition of 1 mM DIDS to the cytosolic face of the channel irreversibly increased open probability (Po) to near unity (n=38) and conductance from 702±4 pS to 760±2 pS (SEM; n=4). This was an 8% increase in conductance in comparison to the 20% increase observed with Ca^{2+} as the permeant ion (Sitsapesan, 1999). Additionally, in contrast to the experiments where Ca^{2+} was the permeant ion, resolvable closings were observed in symmetrical K^+ after irreversible modification by DIDS. The frequency and duration of these closings was shown to be voltage dependent, with both parameters increasing with increasing magnitude of holding potential. The duration of closing events increased 4 fold between both +20 mV and +80 mV and between –20 mV and –80 mV. The frequency showed a different trend increasing more between +20 mV and + 80 mV (35 fold) than between –20 mV and –80 mV (25 fold). This slight assymetry in the voltage dependence of closing event frequency gives rise to the slightly higher Po seen at negative than at positive potentials (0.953±0.007 at +80 mV compared to 0.973±0.02 at –80 mV (SEM; n=5)).

DIDS also irreversibly altered voltage-dependent inactivation of the channel. At negative holding potentials the DIDS modified channel can enter an inactivated state. The inactivation could always be reversed by switching to a positive holding potential (n=35). At increasingly negative holding potentials, a decrease in time to inactivation and an increase in the number of channels inactivating was observed. Inactivation was never seen at positive potentials (n=35).

Our results demonstrate that DIDS-induced modifications to RyR2 function depend on the permeant ion, as both gating and conductance of the DIDS-modified channel were shown to be altered by changing the permeant ion.

This work was supported by the BHF.

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185P PHARMACOLOGICAL CHARACTERISATION OF HUMAN ADENOSINE A_{2B} RECEPTORS CO-EXPRESSED WITH $G_{\alpha 16}$ IN CHO-K1 CELLS

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The adenosine A_{2B} receptor is relatively poorly characterised. It is ubiquitously expressed in the brain and is activated by the endogenous nucleoside adenosine. In this study we have characterised the pharmacology of this receptor when co-expressed with $G_{\alpha 16}$ in CHO-K1 cells using both cytosensor microphysiometry and FLIPR (FLuorometric Imaging Plate Reader).

FLIPR assays were performed as described previously (Porter *et al.*, 1999). Briefly, cells were plated at a density of 30,000 per well, 24 hours before assay into black walled clear bottomed 96 well plates. Dye loading with 4 μM Fluo-3-AM was followed by washing and, for antagonist studies, pre-incubation offline for 10 min. Agonist additions were at a rate of 50 μl/sec and the peak fluorescence signal within a 60 sec time-frame was exported and used to construct concentration-dependent response curves. Microphysiometry was performed largely as described previously (Coldwell *et al.*, 1999). Briefly, cells were seeded into 12 mm transwell inserts at 300,000 cells per well, 24 hours prior to assay. The cells were loaded onto the microphysiometer and the chambers perfused with modified RPMI 1640 medium at a rate of 100 μl/min. Agonists were applied to the cells for a 2 min period and the maximal increase in extracellular acidification rate was recorded. Pre-incubation with the antagonist occurred for 20 min prior to subsequent agonist addition.

The agonist profiles obtained in FLIPR were generally at least one order of magnitude greater than those obtained using microphysiometry and the agonists also displayed greater efficacy in FLIPR (Table 1).

Table 1. Agonist potencies at the human A_{2B} receptor (n=3)

	FLIPR		Microphysiometry	
Agonist	pEC_{50}	Rel Eff	pEC_{50}	Rel Eff
NECA	7.4	1.00	6.2	1.00
N^6-Benzyl NECA	7.0	0.97	5.7	0.94
Adenosine	6.7	1.00	5.6	0.90
R-PIA	6.5	0.90	5.6	0.89
CPA	6.0	0.88	5.2	1.01
S-PIA	5.7	0.84	5.1	0.31
CHA	5.6	0.91	4.9	0.84
CGS 21680	4.8	0.88	3.7	(1.28)

Relative efficacy (Rel Eff) values are corresponding fractions of the response elicited by the maximum NECA response.

The rank order of potencies obtained (Table 1) agrees well with adenylyl cyclase activity studies (Klotz *et al.*, 1998). ZM-241385 was the most potent antagonist examined, with a pA_2 of 8.0 obtained from FLIPR and 7.9 from microphysiometry. Further characterisation of the antagonist profiles in FLIPR yielded a rank order of ZM-241385 (pA_2: 8.0) > CGS-15943 (pA_2: 7.9) > 8-cyclopentyl-1, 3-dipropylxanthine (pA_2: 7.4) > KW-6002 (pA_2: 6.9) > SCH 58261 (pA_2: 6.3) > theophylline (pA_2: 5.9).

This study further defines the pharmacology of the human A_{2B} receptor and indicates that differences in the potency and efficacy of agonist responses can be obtained from different methodologies, but with preservation of the respective rank orders of agonist affinity.

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186P COMPARISON OF THE EFFECTS ON ADENOSINE METABOLISM BY CULTURED ENDOTHELIAL CELLS OF INHIBITORS OF ADENOSINE DEAMINASE AND NUCLEOSIDE TRANSPORT

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Adenosine has both vasodilatory and anti-inflammatory actions that are mediated, at least in part, by the endothelium. Adenosine produces endothelium-dependent vasodilatation in a number of vascular preparations (Prentice & Hourani, 1996; Hiley *et al.*, 1995). Its anti-inflammatory actions include a decrease in cytokine release from stimulated human umbilical vein endothelial cells (Bouma *et al.*, 1996), and a reduction in neutrophil-endothelial cell interactions (Felsch *et al.*, 1995). The endothelium is also a key site of adenosine metabolism with high levels of nucleoside transporters and large amounts of adenosine deaminase (Shyrock & Belardinelli, 1997). Hence, rapid metabolism may limit its therapeutic potential for modulating endothelial function. The aim of this study was to investigate the relative importance of nucleoside uptake and enzymatic degradation in the metabolism of adenosine by bovine aortic endothelial cells (BAEC).

Clonal BAEC were cultured as described (Corder & Barker, 1999). Confluent cultures were incubated for 1 h at 37°C in a 5% CO_2 atmosphere with serum free DMEM containing 100 μM adenosine alone, and with inhibitors of uptake or metabolism (NBTI 1 μM; lidoflazine 1, 10 μM; dipyridamole 1, 10 μM; erythro-9-(2-hydroxy-3-nonyl)adenine, EHNA, 0.01 – 100 μM). After incubation medium was collected and frozen at –20°C. Adenosine levels were measured by reverse phase HPLC using a TSK-ODS column equilibrated with 10 mM KH_2PO_4 (pH 5) and eluted with a gradient of acetonitrile (0 – 20% over 15 min). Absorbance was measured at 260 nm and adenosine levels were quantified by comparison to a reference solution.

Inhibition of adenosine deaminase with EHNA caused a concentration-dependent decrease in adenosine metabolism (IC_{50} 69 nM, 95% confidence limit 56 – 85 nM). Dipyridamole (1, 10 μM), lidoflazine (1, 10 μM) and NBTI (1 μM), inhibited adenosine metabolism by 74 ± 2, 83 ± 2, 52 ± 5, 75 ± 4 and 69 ± 3 % respectively (n = 12, P <0.001, ANOVA). Inhibition with EHNA (10 μM) was 79 ± 2%. Combination of EHNA with NBTI (1 μM) did not increase the inhibition.

These results show that ≈ 20% adenosine metabolism by cultured BAEC is insensitive to either inhibition of adenosine deaminase or NBTI-sensitive nucleoside transport. Observations that nucleoside transport inhibitors caused similar maximal effects to adenosine deaminase inhibition indicate that the two mechanisms are closely associated so that nucleoside transport inhibition simultaneously prevents metabolism by adenosine deaminase.

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187P ANTI-INFLAMMATORY ACTIVITY OF MYELOPEROXIDASE INHIBITORY MOLECULES ISOLATED FROM RESIN EXTRACTS OF *COMMIPHORA KUA*

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We have recently reported the purification of myeloperoxidase (MPO) inhibitory molecules from resin extracts of *C. kua*, family Burseraceae, a traditional Kenyan anti-inflammatory medicinal plant (Battu *et al.*, 1999). We now report the anti-inflammatory effects of these molecules on carrageenan-induced rat paw inflammation.

Drugs were administered *p.o.* in acacia mucilage to male Sprague Dawley rats (200 – 250g) 18h and 2h prior to the induction of inflammation by subplantar injection of saline with/without carrageenan (1%) in hind paws. The inflammation was evaluated by monitoring paw swelling as dorso-ventral thickness and leukocyte infiltration by measuring neutrophil elastase (NE), a marker for neutrophils (Tanaka *et al.*, 1990). Paw thickness was measured hourly for 6h. After 6h, the animals were killed and hind paws were removed, skinned, homogenised with 0.5% hexadecyl trimethyl ammonium bromide in 50 mM phosphate buffer (pH 8.3), centrifuged and the supernatants stored for NE assay. NE was assayed by incubating paw extracts with the specific chromogenic substrate S-2484 (CN Biosciences UK) at pH 8.3 and the cleavage of *p*-nitroaniline was measured as the increase in optical density at 405 nm (Tanaka *et al.*, 1990). Statistical analysis was carried out using the paired *t*-test (Two-tailed).

Anti-inflammatory activity was evaluated for the purified MPO inhibitory molecules including the terpenes, mansumbinone (M_1; Provan and Waterman, 1986) mansumbinoic acid (M_2; Duwiejua *et al.*, 1993), 16(S),20(R)-Dihydroxydammar-24-en-3-one (M_3) and 3β,16(S),20(R)-Trihydroxydammar-24-ene (M_4), the lignan, picropolygamain (PP), and including indomethacin as a positive control. Of the molecules tested, M_2 was equiactive with indomethacin at equimolar concentrations in suppressing carrageenan-induced acute oedema (Fig.1). Figure 2 shows the effect of these molecules on neutrophil infiltration in the inflamed paws.

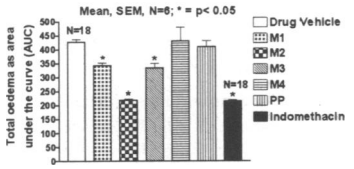

Figure 1: Effects of test molecules (1.3×10^{-5} mol.kg^{-1}) on carrageenan-induced rat paw total oedema response during 6h as area under the time-course curve.

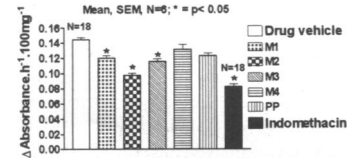

Figure 2: Neutrophil infiltration in inflamed paws of rats treated with test drugs (1.3×10^{-5} mol.kg^{-1}) measured as tissue NE content.

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188P PYRIDOXAL-5-PHOSPHATE IS A SLOWLY DISSOCIATING $P2X_7$ RECEPTOR ANTAGONIST

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We have previously reported the P2-receptor antagonist, pyridoxal-5-phosphate (P5P), to be a $P2X_7$ receptor antagonist (Bowers *et. al.*, 1997). However, this antagonism was not consistent with simple competition, with P5P causing depression of the agonist concentration-effect (E/[A]) curve asymptote. The present study investigates the mechanism of this depression.

Human pre-monocytic (THP-1) cells, which express $P2X_7$ receptors (Spranzi *et. al.*, 1993), were maintained using standard tissue culture techniques. $P2X_7$ receptor activation was measured by uptake of Ethidium Bromide (EthBr; 100 μM) in a 96-well plate SpectraMax Gemini fluorimeter. E/[A] curves were constructed to 2' or 3'-O-(4-benzoyl, benzyol)-ATP (BzATP; 0.3-300 μM) by incubating agonist for either 30 or 90 min with EthBr added for the last 15 min of the incubation. P5P (3-30 μM) was added 15 or 60 min before, or simultaneously with BzATP. Results shown are the mean ± s.e.mean, n=4. Differences were assessed by paired *t* test and considered significant at the level of $P<0.05$.

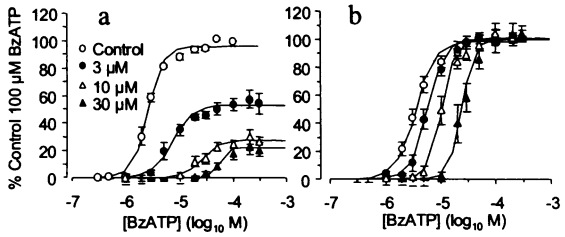

Figure 1: The effect of 15 min pre-incubation with P5P on BzATP-induced EthBr uptake with either a) 30 min or b) 90 min BzATP incubation.

BzATP caused time- and concentration-dependant increases in EthBr uptake. The kinetics of uptake were linear over 15 min. Agonist potencies ($p[A]_{50}$s) were estimated using EthBr uptake at 15 min since $p[A]_{50}$s estimated using initial rate of uptake or fluorescence at 15 min were not significantly different (5.72 ± 0.11 and 5.64 ± 0.01 respectively). With 15 min pre-incubation of antagonist and a subsequent 30 min incubation of BzATP, P5P caused concentration-dependant depression of asymptote and rightward shifts of the BzATP E/[A] curve (figure 1a). Increasing P5P pre-incubation to 60 min did not produce any further increase in antagonist potency. When the BzATP incubation period was increased to 90 min, keeping P5P pre-incubation at 15 min, rightward shifts of the BzATP E/[A] curve were observed with no depression (figure 1b). Clark analysis of the data in figure 1b yielded a pK_B value of 5.3 ± 0.13 (slope of 0.96 ± 0.03). Simultaneous addition of both ligands for either 30 or 90 min also produced surmountable antagonism by P5P. Clark analyses of these data yielded pK_B values of 5.0 ± 0.04 and 5.0 ± 0.07 respectively, with identical slopes of 1.00 ± 0.01.

Insurmountable antagonism can occur when an antagonist dissociates slowly from its receptor and behaves as a 'pseudo-irreversible' antagonist (Kenakin, 1993). If the agonist is allowed sufficient time to equilibrate after antagonist pre-incubation the asymptote depression should disappear. Similarly, co-incubation of antagonist and agonist should favour the agonist on-rate and produce surmountable characteristics for P5P. The results obtained in this study are consistent with P5P behaving as a pseudo-irreversible antagonist in this system.

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189P $P2X_7$-INDUCED PORE FORMATION IN A POPULATION OF THP-1 CELLS

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$P2X_7$ receptors are ligand-gated ion channels that form large transmembrane pores in response to 2'- or 3'-O-(4-benzoyl)benzoyl-adenosine-5'–triphosphate (BzATP) (Nuttle & Dubyak, 1994). In the present study, we have used the promonocytic THP-1 cell line and the entry of the DNA-binding probe, ethidium bromide (EthBr) as a measure of $P2X_7$ receptor activation. We have obtained a kinetic profile of EthBr uptake using 96-well plate fluorimetry and analysed cell population responses using flow cytometry.

THP-1 cells expressing $P2X_7$ receptors (Bowers *et al.*,1996) were suspended at $2.5X10^6$ cells.ml^{-1} in a high potassium buffer containing EDTA. Agonist concentration-effect curves to BzATP (0.2-20μM) were constructed using quarter-log increments. Uptake of EthBr (100μM) was assessed over 90 minutes at 37°C using a Spectromax Gemini plate reader (excitation 520nm, emission 595nm). In parallel, EthBr uptake was measured at 90 minutes by flow cytometry as an increase in FL-3 fluorescence. Plate fluorimetry data were expressed as % 20μM BzATP response whilst flow cytometer data were expressed as % cells responding (mean ± s.e.mean, n=4).

BzATP-induced EthBr uptake into THP-1 cells (Figure 1) initially increased in a linear fashion with time before reaching a plateau; the magnitude of which was concentration-dependent and corresponded to an increase in the % cells responding as measured by flow cytometry. $p[A]_{50}$ values for BzATP determined by the intial rate (5.87±0.04) and at 90 minutes (5.93±0.03) using plate fluorimetry were not significantly different to the $p[A]_{50}$ value determined by flow cytometry (6.03±0.04) ($p > 0.05$, one-way ANOVA).

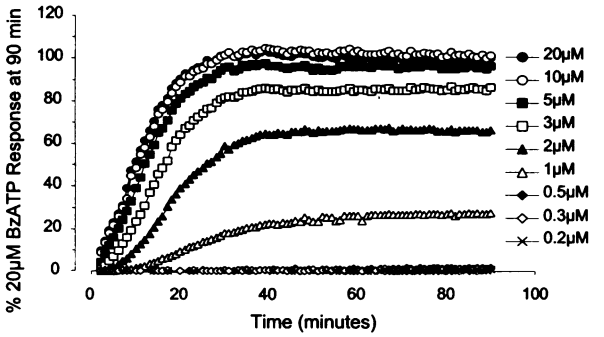

Figure 1. Time course of BzATP-induced EthBr uptake into THP-1 cells (Representative trace, consistent with 4 replicates)

In summary, we have demonstrated that the $p[A]_{50}$ values for BzATP-induced EthBr uptake are independent of initial rate, fixed time point measurement and method of analysis. We further propose that the magnitude of the agonist concentration-dependent plateau of the EthBr signal can be ascribed to the number of cells responding to BzATP in a population of heterologously sensitive cells.

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190P *EX VIVO* BINDING WITH [^{125}I]SB-258585 TO ESTIMATE *IN VIVO* 5-HT_6 RECEPTOR OCCUPANCY BY 5-HT_6 SELECTIVE COMPOUNDS AND TYPICAL AND ATYPICAL ANTIPSYCHOTICS.

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[^{125}I]SB-258585 (4-Iodo-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl] benzene-sulfonamide) is a selective, high affinity radioligand for 5-HT_6 receptors, which displays high levels of specific binding in membranes from rat striatum (Hirst *et al.*, 2000). Whilst unsuitable for direct assessment of 5-HT_6 receptor occupancy *in vivo*, primarily due to limited blood-brain barrier penetration, we now demonstrate that this ligand can be used for *ex vivo* binding studies in crude striatal homogenates. This is possible because 5-HT_6 receptors have relatively low affinity for 5-HT and high affinity for [^{125}I]SB-258585.

Male Sprague-Dawley rats (200-250g) (n = 3 per dose) received vehicle, the selective 5-HT_6 receptor antagonists; SB-271046 (5-Chloro-3-methyl-benzo[*b*]thiophene-2-sulfonic acid (4-methoxy-3-piperazin-1-yl-phenyl)-amide) (1–100 mg kg^{-1} p.o.), SB-258510 (5-chloro-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-2-benzothiophenesulfonamide) (1–100 mg kg^{-1} p.o.), SB-357134 (N-[2,5-dibromo-3-fluorophenyl]-4-methoxy-3-piperazin-1-ylbenzenesulfonamide) (0.1–50 mg kg^{-1} p.o.), or known psychoactive compounds; clozapine (1–10 mg kg^{-1} i.p.), olanzapine (1–10 mg kg^{-1} i.p.), chlorpromazine (0.1–10 mg kg^{-1} i.p.), octoclothepin (0.1–10 mg kg^{-1} p.o.) or haloperidol (0.1–5 mg kg^{-1} i.p.). All drugs administered i.p. were in saline and those given p.o. were in 1% methyl cellulose. Animals were sacrificed 1–6 hours later and striatal tissue was removed and frozen. The striata (approx. 100 mg) were each homogenised in 4 ml of buffer containing 50 mM Tris, 5 mM $MgCl_2$, 5 mM ascorbate and 0.5 mM EDTA.

The crude homogenate was incubated for 45 minutes at 37°C with 0.1 nM [^{125}I]SB-258585. Non-specific binding was defined with 10 μM methiothepin. The assay was terminated by rapid filtration through Whatman GF/B filters, pre-treated with 0.3% polyethyleneimine, and washed with 9 ml of ice cold buffer. Radioactivity was determined by gamma spectrometry using a Packard Cobra II gamma counter.

SB-271046, SB-258510 and SB-357134 all completely prevented specific [^{125}I]SB-258585 binding with ED_{50} values of 11 mg kg^{-1}, 0.8 mg kg^{-1} and 8 mg kg^{-1}, respectively. All three compounds display similar affinity for 5-HT_6 receptors *in vitro*, with pK_i values of 8.45 ± 0.03, 8.34 ± 0.11 and 8.44 ± 0.05, respectively. The higher potency of SB-258510 probably reflects greater blood brain-barrier penetration. The atypical antipsychotics, clozapine and olanzapine, both showed clear inhibition of specific [^{125}I]SB-258585 binding at 10 mg kg^{-1} (71 ± 6% and 60 ± 2%, respectively). Similarly, 10 mg kg^{-1} of the typical antipsychotics, chlorpromazine and octoclothepin also inhibited specific [^{125}I]SB-258585 binding (65 ± 7% and 72 ± 2%, respectively). However, haloperidol (5 mg kg^{-1}), a dose which has previously been shown to display clear CNS activity (Reavill *et al.*, 1999), failed to inhibit specific [^{125}I]SB-258585 binding.

These data demonstrate that *ex vivo* [^{125}I]SB-258585 binding is a useful indication of *in vivo* 5-HT_6 receptor occupancy. The results show that a number of 5-HT_6 receptor selective compounds and many commonly prescribed antipsychotics, at therapeutically relevant doses, bind to the 5-HT_6 receptor *in vivo*.

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191P EVIDENCE FOR NICOTINIC CHOLINERGIC RECEPTORS ON A MONOCYTIC CELL LINE (THP-1)

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Nicotine has anti-inflammatory actions in a rat model of colitis (Sykes *et al* 2000). However, the mechanism by which it mediates these effects are poorly understood and it is not clear whether they are a direct or indirect effect on immune function. In this study we investigated the presence of nicotinic cholinergic receptors (nAChR) in an acute monocytic cell line (THP-1 cells obtained from ECACC) by radioligand binding studies and RT-PCR techniques.

Whole THP-1 cells were incubated with [^{3}H]-(-)-nicotine (0.1-40nM) in a modified Hanks Balanced Salt Solution (HBSS) buffer (pH 7.4) for 20minutes at 4^{0}C. Reactions were terminated by the filtration of cells onto Whatman GF/C filters using a Skatron cell harvester with subsequent washing with the HBSS buffer. Non-specific binding was determined by incubating cells with [^{3}H]-(-)-nicotine in the presence of 10μM unlabelled (-)-nicotine. Competition studies were performed on whole cells using nicotine, cytisine, epibatidine and hexamethonium. All values (B_{max}, Kd and pK_i) are the mean ± S.E.M. of at least three triplicate determinations. For RT-PCR studies mRNA was isolated from settled THP-1 cells using RNAstat. 1μg of mRNA was used for the template for RT-PCR (Promega) with primers that had been used successfully in the amplification of nAChR mRNA previously (Sato *et al* 1999) or designed by a computer programme (Generunner). Products were subjected to electrophoresis on 1.1% agarose gels.

Saturation studies on THP-1 whole cells revealed two binding sites for nicotine as determined by a non-linear regression curve fit (Prism, Graphpad software). The higher affinity site displayed displayed a cell receptor number of (B_{max1}) 4100±560 sites/cell and an affinity to nicotine of (Kd_1) 3.5±2.1nM (n=6) whereas the lower affinity site had a receptor number of (B_{max2}) 11600±630 sites/cell with an affinity of (Kd_2) 27±9.2nM (n=6). Competition studies revealed that nicotine displayed a biphasic competition curve with two calculated affinity constants pKi_1=9.47±0.81 and pKi_2 = 7.51 ± 0.59 (n=4). pKi values for competing ligands were epibatidine = 8.5 ± 0.2 (n=4), cytisine = 7.5 ± 0.4 (n=4) and hexamethonium = 5.6 ± 0.6 (n=3).

Saturation studies in the presence of epibatidine ($1x10^{-6}$M) revealed a one site saturation curve with a B_{maxE} of 4030±800 sites/cell and a Kd_E of 5.7 ± 3.3nM (n=6). These values were not significantly different from the high affinity site ($p<0.05$, Student's t-test) indicating that the lower affinity nicotine site was cholinergic in character. RT-PCR studies detected the presence of the nAChR subunits α7 and α2 in the mRNA of resting cells as confirmed by product size and restriction digests of the products.

These results provide evidence that THP-1 cells express nAChR subunits shown by binding data and RT-PCR. THP-1 cells also express a second nicotine-binding site that is not typically cholinergic, as it was not competed for by any of the ligands tested. However the nature of this site is unknown and more work is needed to elucidate whether it is an allosteric conformation of nAChR or a unique receptor.

These data supports the concept that nAChRs are found on monocytes and adds to the previous finding that nAChR may play a modulatory role on the immune system (Borovikova *et al* 2000).

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192P INVESTIGATION OF THE ROLE OF NICOTINIC RECEPTORS IN THE FEMALE RAT ISOLATED URETHRA

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The role of nicotinic receptors in the local control of tracheal (Kannan & Johnson, 1992), vascular (Toda, 1976) and bladder (Hisayama *et al.*, 1988) smooth muscle has been investigated. The present experiments were carried out to study the role of such receptors in the local control of the urethra. The effects of nicotine and the selective neuronal nicotinic receptor agonists, DMPP and epibatidine (Holladay et al., 1997), were examined on isolated female rat urethra.

Female Sprague-Dawley rats (200-300 g) were killed by cervical dislocation and the urethras were removed and mounted longitudinally under 1g resting tension in 5 ml organ baths. The tissues were bathed in Krebs solution, maintained at 37 °C and aerated with 95% O_2 and 5% CO_2. Each concentration of agonist was applied to separate tissues. Agonist-evoked changes in basal urethral tone following administration of antagonists were expressed as percentages of control responses, and compared with vehicle controls by unpaired Students t-test. $P<0.05$ was considered statistically significant. All values are mean ± s.e.mean.

Nicotine (10, 30 & 100 μM, n = 4-5), epibatidine (0.03, 0.1 & 0.3 μM, n = 4-5) and DMPP (30, 100 & 300 μM; n = 4-5) evoked urethral relaxations (all tissues relaxed by 0.2 – 0.7g), that were abolished by hexamethonium (100 μM).

These relaxations were significantly attenuated by pretreatment with L-NAME (100 μM), being reduced by 51 ± 12 % (P = 0.042) at 100 μM nicotine, 63 ± 6 % (P = 0.034) at 0.3 μM epibatidine and 80 ± 15% (P = 0.001)at 300 μM DMPP. Nicotine- and epibatidine-evoked relaxations were TTX (1 μM) insensitive, however DMPP (30 μM)-evoked relaxations were significantly attenuated by 37 ± 9% after TTX treatment, but higher concentrations of DMPP were not attenuated. Interestingly, DMPP-evoked relaxations were followed by maintained urethral contractions. These contractions were unaffected by hexamethonium, TTX, atropine sulphate (300 μM), prazosin (24 μM) and tubocurarine (100 μM).

The present results demonstrate that activation of neuronal nicotinic receptors activate nitrergic pathways by TTX-resistant mechanisms. This suggests that these nicotinic receptors are located on the nitrergic nerve terminals (Wonnacott, 1997). The alternate mechanisms by which DMPP produce varied responses in the female rat urethra remain to be determined.

A.W. is a MRC (collaborative) student with Pfizer.

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Wonnacott (1997) *Trends Neurosci.* **20**, 92-98.

193P DETERMINANTS OF EPIBATIDINE SELECTIVITY AT MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR COMPLEXES

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Recent studies have demonstrated that epibatidine selects strongly between the agonist binding sites of muscle nicotinic acetylcholine receptors (AChRs) (Prince and Sine, 1998). At the foetal muscle receptor (subunit composition $\alpha_2\beta\gamma\delta$), epibatidine binds with high affinity to the agonist site formed by the α and γ subunits but with low affinity to the site formed by the α and δ subunits. As the two α subunits are identical, it is likely that differences in epibatidine affinity at the $\alpha\gamma$ and $\alpha\delta$ interfaces are due to different contributions of the γ and δ subunits to the agonist binding domains. The aim of the present study was to use γ/δ subunit chimeras to identify determinants of epibatidine selectivity at the muscle AChR.

To simplify interpretation of our data we used intracellular "dimer" complexes as a model of the resting AChR binding sites. Intracellular complexes, which have the subunit stoichiometry αX (where X is γ, δ or chimera), have similar pharmacology to the corresponding binding sites in the resting state of the native receptor, but do not appear to be able to enter desensitised or open-channel conformational states. Intracellular complexes were expressed by transiently transfecting HEK 293 cells with human α subunit and mouse γ, δ or chimera as previously described (Prince & Sine, 1996). We used the human α subunit because it increases expression levels by ~10-fold compared with its mouse counterpart but does not alter selectivity (Prince & Sine, 1996). γ/δ subunit chimeras were constructed as previously described (Prince & Sine, 1996). The notation $\gamma n\delta$ denotes a chimera that contains γ sequence for the first n amino acids and then δ sequence thereafter. Epibatidine affinity was measured by competition against the initial rate of [^{125}I]-labelled α-bungarotoxin binding as previously described (Prince & Sine, 1996). Binding data are summarised in Table 1. Data were analysed by one-way ANOVA followed by Tukey's post-hoc test.

Table 1. Epibatidine affinities of intracellular complexes. Data are the mean ± SEM of (n) determinations.

	Ki Epibatidine (nM)
$\alpha\gamma$	16.7 ± 1.5 (26)†
$\alpha\delta$	318 ± 23 (24)*
$\alpha\gamma82\delta$	316 ± 22 (7)*
$\alpha\gamma100\delta$	120 ± 7.4 (11)*†
$\alpha\gamma104\delta$	21.84 ± 6.7 (3)†
$\alpha\gamma112\delta$	58.5 ± 3.7 (6)*†
$\alpha\gamma115\delta$	78.5± 5.2 (7)*†
$\alpha\gamma117\delta$	14.4 ± 1.7 (5)†

*Log K_i significantly different ($P<0.05$) from Log K_i $\alpha\gamma$
†Log K_i significantly different ($P<0.05$) from Log K_i $\alpha\delta$

Our results indicate that at least three regions of the γ and δ subunits influence epibatidine selectivity: 82-100; 100-104 and 104-117 (γ numbering). The region 111-117 encompasses known determinants of carbamylcholine, d-tubocurarine and conotoxin M1 selectivity (Prince & Sine, 1996; Sine 1993; Sine et al. 1995) and it is possible that the same residues may also influence epibatidine selectivity. However, to our knowledge, this is the first study to implicate residues in the region 82-104 as ligand selectivity determinants.

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Sine, S.M. (1993) Proc. Natl. Acad. Sci. USA 90: 9436-9440
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194P CYTISINE AND DIHYDRO- β-ERYTHROIDINE SELECTIVITY AT MUSCLE NICOTINIC RECEPTOR COMPLEXES

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Muscle nicotinic acetylcholine receptors (AChRs) are heteropentamers with the subunits compositions $\alpha_2\beta\delta\gamma$ (foetal) or $\alpha_2\beta\delta\varepsilon$ (adult). Both types of receptor contain two binding sites for acetylcholine (ACh) located at the interfaces of the α subunits with their neighbouring δ, γ or ε subunits.
Many compounds that compete with ACh distinguish between the $\alpha\delta$, $\alpha\gamma$ and $\alpha\varepsilon$ binding sites (Taylor et al., 1998). As the AChR α subunits are identical, such affinity differences between the AChR agonist sites must be due to different contributions of the ε, δ and γ subunits to the binding interfaces. Identification of sequence differences between ε, δ and γ that mediate ligand selectivity can therefore be very useful in determining how these subunits participate in the formation of the ligand binding sites.

In the present study we investigated the binding site selectivity of the agonist cytisine and competitive antagonist dihydro-β-erythroidine (DHβE) at AChRs. To simplify interpretation of our results, we transiently expressed AChR subunits in HEK 293 cells as intracellular complexes of the form $\alpha\gamma$, $\alpha\delta$ or $\alpha\varepsilon$. Intracellular complexes exhibit similar pharmacology to the corresponding binding site of native receptors but do not undergo the conformation changes that correspond to channel opening or desensitisation (Prince & Sine, 1996). To increase expression levels of intracellular complexes we used the human α subunit and mouse γ, δ and ε subunits (Prince & Sine, 1996). We use the notation $\gamma n\varepsilon$ to signify a chimera that contains γ sequence for the first n amino acids and ε sequence thereafter. Chimeric subunits were generously provided by Dr. S.M. Sine. Cytisine and DHβE affinities were determined by competition against the initial rate of [^{125}I]-labelled α-bungarotoxin binding, as previously described (Prince & Sine, 1996). To allow access of ligands to the intracellular binding sites, cells were permeabilised with saponin. Data are presented as the mean ± SEM of (n) determinations. Log Ki values were compared using one-way ANOVA followed by Tukey's post-hoc test.

DHβE and cytisine both bound to intracellular complexes with the rank order of affinity $\alpha\varepsilon > \alpha\delta > \alpha\gamma$ (Table 1).

Table 1. Affinities for cytisine and DHβE.

	Cytisine Ki (μM)	DHβE Ki (μM)
$\alpha\gamma$	19.7 ± 3.7 (10) †	150 ± 21 (5) †
$\alpha\delta$	1.25 ± 0.4 (5)* †	11.6 ± 2 (3)* †
$\alpha\varepsilon$	0.24 ± 0.06 (6)*	2.2 ± 0.6 (6)*
$\alpha\gamma74\varepsilon$	4.2 ± 1.5 (3)*†	6.9 ± 1.6 (3)* †
$\alpha\gamma117\varepsilon$	61 ± 18 (3) †	103 ± 7 (3) †
$\alpha\gamma172\varepsilon$	36.7 ± 8.4 (3) †	132 ± 32 (3) †

*Log Ki significantly different from wild-type γ ($P<0.05$)
†Log Ki significantly different from wild-type ε ($P<0.05$)

Expression of the chimera $\gamma74\varepsilon$ revealed affinities for both ligands significantly lower than wild type ε, suggesting that at least one selectivity determinant lies N-terminal to position 74. Further significant changes in affinity were noted when the γ sequence was extended to form $\gamma117\varepsilon$, suggesting that the region 74-117 may also be involved in cytisine and DHβE selectivity. At present our data do not identify a role for residues C-terminal to position 117 in the selectivity of cytisine and DHβE. However, we are unable to rule out the possibility that although the main determinants of selectivity lie N-terminal to amino acid 117, they may interact with residues in the region 117-172.

Prince, R.J. and Sine, S.M. (1996) J. Biol. Chem. 271: 25770-25777
Taylor, P., Osaka, H., Molles, B.E. et al. (1998) J. Physiol. Paris 92: 79-83

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195P AN INVESTIGATION OF A PUTATIVE BK_{Ca} POTASSIUM CHANNEL ACTIVATOR USING ISOLATED RAT BLADDER AND SINGLE CELL STUDIES

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NS-8 (2-amino-3-cyano-5-(2-fluorophenyl)-4-methylpyrrole), has been reported to be an opener of the high conductance, calcium activated, potassium channel (BK_{Ca}) and to relax rat bladder smooth muscle via this mechanism (Tanaka *et al.*, 1998). The aim of the current studies was to further examine the mode of action of NS-8 in isolated rat bladder and in single cell studies.

For electrophysiology studies the instantaneous current-voltage relationship in the G-292 (human osteosarcoma) cell line was assessed in a conventional way by use of a ramp protocol (Hamill et al., 1981). All recordings were made at room temperature (20-22°C). A voltage ramp was run every 30 seconds for the duration of the experiment. Voltage was stepped from the holding potential of -30mV to +100mV for 50msecs and was then ramped down to -100 mV over the next 200msecs. Control ramps were run until stable ramps were obtained and then the normal buffer was replaced by buffer containing the test compound and this is superfused for 10 ramps. The current amplitude at +60mV from the 10 control ramps were meaned and then the values (current at 60mV) from all of the following ramps were expressed as a percentage of the meaned control values. Concentration effect curves were then constructed and the concentration that caused a 300% increase in the current from control calculated (ED_{300}).

For *in vitro* studies bladders were removed from rats (male, CD, Sprague-Dawley, 250-350g). Bladders were bisected lengthwise and each tissue suspended under 1g tension in aerated (95% O_2/5% CO_2) Krebs-Hensleit solution(2.5mM Ca^{2+}) in a 5ml organ bath.

After a 1hr equilibration the tissues were contracted with either 20mM or 80mM KCl. Cumulative concentration-response curves (CRC) were then constructed with NS-8. The effect of the BK_{Ca} blocker iberiotoxin (IbTX) on the relaxant response to NS-8 was examined after a 1hr incubation. All CRCs were normalised to the maximal relaxation caused by nifedipine (1mM).

NS-8 relaxed both 20 and 80mM KCl-induced contractions of rat bladder with IC_{50} values (mean ± sem, n=4) of 17.4 ± 0.84 and 10.6 ± 3.2μM respectively. Relaxation of an 80mM KCl-induced contraction is not consistent with K^+ channel opening activity. Furthermore, in a separate study, IbTX did not inhibit the relaxant activity of NS-8 against a 20mM KCl-induced contraction (IC_{50} (mean ± sem, n=4) 32.0 ± 2.3 and 40.3 ± 2.7μM with and without IbTX respectively. p>0.05, t-test). In G292 cells NS-8 increased the current (from control) only at the highest concentration tested (100μM) with an ED_{300}>100μM. In contrast, the internal standard NS-1608 (Hu and Kim, 1996) produced a concentration-dependent increase in current (ED_{300} 77.6nM ± 54nM). The increases in current evoked by both NS-8 and NS-1608 were blocked by IbTX (300nM). Increasing NS-1608 concentrations led to an increase in current activation which was blocked by IbTX (300nM).

The results of the electrophysiology studies indicate that NS-8 is a weak activator of BK_{Ca} channels. However, data from the *in vitro* rat bladder studies indicate that this agent acts to relax smooth muscle via a mechanism unrelated to its BK_{Ca} opening activity.

Tanaka, M., Sasaki, Y, Hukui, T. *et al.* (1998), *J. Urol.*, **159**, (5) Suppl., 21
Hamill et al. (1981), Pfleugers Arch. 391, 85-100
Hu, S. and Kim, H.S., (1996), *Eur. J. Pharm.*, **318**, 461-468

196P THE ELECTROPHYSIOLOGICAL EFFECTS OF NEUROPEPTIDE Y (NPY) ON LATERAL HYPOTHALAMUS NEURONES *IN VITRO*

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NPY, thought to mediate certain feeding activities, is an important modulator of neuronal excitability (Laviano *et al.*, 1998). The hypothalamus contains specific subsets of glucose-receptive neurones, which selectively alter their firing rate in response to changes in ambient glucose levels. These comprise glucose-responsive neurones (GRNs), which increase their firing rate as glucose levels rise, and glucose-sensitive neurones (GSNs) which increase their firing rate when glucose levels fall. The lateral hypothalamus (LH) is an important feeding centre (Niijima, 1989) which contains both GSNs and GRNs and is a site of release of NPY. We therefore studied the electrophysiological effects of NPY on glucose-receptive LH neurones *in vitro*, and compared these with glucose-insensitive neurones.

Rats (2-3 weeks old, Wistar, either sex) were anaesthetised with sodium pentobarbital (40 mg/kg.i.p.) and decapitated. Transverse hypothalamic slices (400 μm) were prepared and placed in a recording chamber perfused with gassed modified ACSF at room temperature. Intracellular recordings were made from neurones in LH area, and their glucose responsiveness determined during exposure to normal, low and high glucose levels (respectively 10, 3 and 20 mM in ACSF).

Application of NPY (1.0 μM) induced hyperpolarisation of both glucose-receptive and glucose-insensitive neurones, but to strikingly different degrees. In all glucose-receptive neurones examined, NPY produced prolonged hyperpolarisation averaging 12.5±2.8 mV in peak amplitude, in both GRNs (n=10) and GSNs (n=16). By contrast, NPY-induced hyperpolarisation was significantly less ($P<0.001$, student *t*-test) in glucose-insensitive neurones (3.3±0.6 mV; n=6). In addition, NPY-induced hyperpolarisation in glucose-receptive neurones showed desensitisation when a second application of NPY was given within 1 h after the first one (n=4). In 5 spontaneously active cells, NPY (1.0 μM) substantially reduced the burst firing rate, making them silent. Furthermore, NPY decreased neuronal excitability as evidenced by (a) a significantly reduced input resistance (by 28.2±4.3%, $P< 0.01$; n=12); (b) an increase in the threshold current (depolarising step) required for cell firing (from 0.13±0.01 to 0.24±0.03 nA; $P<0.01$; n=16); and (c) a decreased average numbers of spikes elicited by the depolarising step at a fixed membrane potential (from 2.8±0.5 to 1.2±0.2, n=8; $P<0.01$).

Glucose-receptive neurones are important for long-term regulation of body weight (Bergen *et al.*, 1996). We have shown that glucose-receptive neurones in the LH, a site exquisitely sensitive to the appetite-stimulating effect of NPY, are more sensitive to NPY than glucose-insensitive neurones. Interactions between these major appetite-regulating neuronal systems may play an important role in the overall integration of nutritional signals and in the control of energy homeostasis.

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Bergen, H.T. *et al.*, (1996) *Brain Research* 734, 332-336.
Laviano, A. *et al.*, (1998) *Science*, 280, 503.
Niijima, A. (1989) *Progress in Neurobiology*, 33, 135-147.

197P IMMUNOLOGICAL IDENTIFICATION OF THE H3 HISTAMINE RECEPTOR IN THE ADULT MOUSE STRIATUM

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Histamine has been proposed to modulate dopamine and acetylcholine release in the mouse striatum via presynaptic H_3 receptors, which suggests a role in the control of movement. *In situ* hybridization studies in rat brain revealed mRNA encoding the recently cloned H_3 receptor in all neuronal systems previously associated with H_3 receptor functionalities, including the striatum [Lovenberg *et al.*, 1999]. Due to lack of immunological probes, there is currently no information regarding the anatomical distribution of the cloned H_3 receptor polypeptide in the striatum.

Anti-H_3 (349-358) antibodies were raised in rabbits and used to probe adult rodent (B6/C3Fe mice or Wistar rats) striatum by standard immunoblotting and immunohistochemistry techniques, described previously [Thompson *et al.*, 2000]. Antibody specificity controls included adsorption of the primary antibodies, at their working concentrations, with their respective peptides (100-200 μg/ml) prior to application to the immunoblot or tissue. In all cases, most or all of the staining elicited by the untreated antibody was abolished, demonstrating the specificity of the immunoreaction. Upon immunoblotting, affinity-purified anti-H_3 (349-358) antibodies recognised two prominent protein species with M_r 63,000 $\pm$ 1,000 and M_r 93,000 $\pm$ 4,000 (n = 4, mean $\pm$ S.D.) in adult rodent (rat and mouse) striatum.

The size of the lower molecular weight species is consistent with the predicted molecular size from the deduced amino acid sequence of the H_3 receptor, allowing for the contribution by weight for N-glycosylation [Lovenberg *et al.*, 1999]. There is growing evidence, both pharmacological and biochemical, in favour of H_3 receptor heterogeneity. Therefore, the higher molecular species identified in this present study, may be a further subtype of the H_3 receptor.

Immunohistochemical analysis using affinity-purified anti-H3 (349-358) antibodies yielded a high degree of coincidence with ligand-autoradiographical information, with high levels detected in the CA3 and Dentate gyrus of the hippocampal formation, Laminae V of the cerebral cortex, the olfactory tubercle, Purkinje cell layer of the cerebellum, thalamus and notably the striatum [Pollard *et al.*, 1993].The immunoreactive cells displayed a neuronal morphology with the immunoreactivity associated with both the cell body and proximal processes. In the mouse striatum, high-powered analysis revealed intense bipolar perikaryal staining of many medium-sized neurons (~10 μm), with little evidence of nuclear staining. A few larger (~25 μm) putative cholinergic interneurons were also immunopositive. We are currently attempting to identify the immunopositive neurons by double labelling with selective neurochemical markers.

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198P CHARACTERISATION OF [^{3}H]SHU 9119 BINDING TO HUMAN MC_3 AND MC_5 MELANOCORTIN RECEPTORS

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Molecular cloning has revealed the existence of five melanocortin receptors ($MC_{1\text{-}5}$, Wikberg, 1999). Previously the iodinated ligand, [^{125}I]NDPαMSH, has been used to label MC_1 and $MC_{3\text{-}5}$ receptors. In the present study, binding assays have been developed and validated for human cloned MC_3 and MC_5 receptors using [^{3}H]SHU 9119.

[^{3}H]SHU 9119 (Custom synthesis NEN; 49.8 Ci mmol^{-1}) binding assays were performed in 96 well plates in a final volume of 200µl. Membranes prepared from HEK 293 cells expressing human MC_3 (12µg protein) or MC_5 (4µg protein) receptors (Receptor Biology Inc.) were incubated with [^{3}H]SHU 9119 (saturation binding studies 11 concentrations 0.05-20nM; competition studies 1.2nM) at 25°C for 60 minutes; non-specific binding defined by NDPαMSH (3µM). Binding was terminated by rapid filtration through GF/B filters followed by 4 washes. Bound radioactivity was determined by scintillation counting. Data was analysed using GraphPad Prism software.

[^{3}H]SHU 9119 binding to MC_3 and MC_5 receptors was saturable and of high affinity (K_d=0.68 and 0.81nM; B_{max}=2.13±0.02 and 8.69±0.31pmoles/mg protein; mean±s.e.mean; n=4, respectively). Binding reached equilibrium within 30 minutes for both receptors and was constant for up to 2.5 hours. The pharmacological profile for a range of peptide ligands demonstrated a clear difference between the two receptors (Table 1). NDPαMSH, SHU 9119, MTII and HP228 exhibited similar affinities for MC_3 and MC_5 receptors. In contrast, HS014, β-MSH, α-MSH and ACTH had higher (>10-fold) affinity for MC_3 than MC_5 receptors, with α-MSH and ACTH showing the greatest selectivity. These data are consistent with previous reports (Schioth *et al.*, 1995; 1997; 1998).

In summary, these data show that [^{3}H]SHU 9119 is a suitable ligand for the labelling of MC_3 and MC_5 receptors and represents a useful tool for identifying compounds with affinity for these receptors.

Schioth, H.B. *et al.* (1995) *Eur. J. Pharmacol.* 288, 311-317.
Schioth, H.B. *et al.* (1997) *Neuropeptides* 31, 565-571.
Schioth, H.B. *et al.* (1998) *Br. J. Pharmacol.* 124, 75-82.
Wikberg, J.E.S. (1999) *Eur. J. Pharmacol.* 375, 295-310.

Table 1. Inhibition constants for displacement of [^{3}H]SHU 9119 binding to human MC_3 and MC_5 receptors by a range of peptide ligands

	MC_3			MC_5			Affinity Ratio
Compound	pKi	Ki (nM)	Hill Slope	pKi	Ki (nM)	Hill Slope	MC_5/MC_3
NDPαMSH	9.33±0.06	0.5	0.80±0.05	8.64±0.15	2.3	0.81±0.04	4.6
SHU 9119	9.09±0.11	0.8	1.11±0.06	8.97±0.11	1.1	1.11±0.06	1.4
MTII	7.77±0.09	17	0.93±0.07	7.22±0.04	60	0.94±0.03	3.5
ACTH (1-39, human)	7.51±0.02	31	0.79±0.04	5.73±0.03	1862	0.91±0.02	60.1
HS014	7.18±0.05	66	0.94±0.03	6.10±0.11	794	0.75±0.04	12.0
α-MSH	6.91±0.15	123	0.70±0.05	5.35±0.01	4467	1.16±0.19	36.3
β-MSH	6.69±0.15	204	0.73±0.04	5.34±0.05	4571	0.80±0.02	22.4
HP228	6.59±0.02	257	0.84±0.09	6.28±0.03	525	0.95±0.02	2.0

pKi values and Hill slopes are mean±s.e.mean (n=3). Affinity ratios were calculated from the Ki values. The full names of all compounds tested in this study are given in Schioth *et al.*, 1995; 1997; 1998.

199P CHARACTERISATION OF [^{3}H]MTII AND [^{3}H]SHU 9119 BINDING TO HUMAN MC_4 MELANOCORTIN RECEPTORS

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Molecular cloning has revealed the existence of five melanocortin receptors (MC_{1-5}, Wikberg, 1999). Previously the iodinated ligand, [^{125}I]NDPαMSH, has been used to label MC_4 receptors. In the present study, binding assays have been developed and validated for human cloned MC_4 receptors using [^{3}H]MTII and [^{3}H]SHU 9119.

Membranes (400µl) prepared from HEK 293 cells expressing human MC_4 receptors (Batches 1545/1614; Receptor Biology Inc.) were incubated with 50µl [^{3}H]MTII (Amersham, 71-115Ci mmol^{-1}) or [^{3}H]SHU 9119 (NEN; 49.8 Ci mmol^{-1}) (saturation binding studies, 8 concentrations 0.0625-8nM or 0.0125-1.6nM; competition studies, 0.6nM or 0.15nM; respectively) and 50µl incubation buffer (total binding) or HS014 (1µM non-specific binding) at 25°C for 60 min. Binding was terminated by rapid filtration through GF/B filters using a Skatron cell harvester and radioactivity determined by scintillation counting. Data was analysed using a programme based on Ligand.

In preliminary experiments, binding of [^{3}H]MTII (0.06, 0.54 and 4.6nM) and [^{3}H]SHU 9119 (0.013, 0.19 and 1.65nM) to MC_4 receptors increased linearly with protein concentration ([^{3}H]MTII; 2.2, 4.2 and 8.4µg/tube; [^{3}H]SHU 9119; 0.88, 1.8 and 3.5 µg/tube). Protein concentrations of 4.2 and 3.5µg/tube for [^{3}H]MTII and [^{3}H]SHU 9119, respectively, were chosen for further experiments. Binding reached equilibrium within 60 min for both radioligands and remained constant for up to 3 h. Therefore, an incubation time of 60 min was used. Full saturation analysis revealed that [^{3}H]MTII and [^{3}H]SHU 9119 binding was saturable and of high affinity (K_d = 0.61 ± 0.04 and 0.12 ± 0.01nM and B_{max} = 4.85 ± 0.62 and 4.58 ± 0.06pmol mg protein^{-1}; mean±s.e.mean; n=3, respectively). Inhibition constants for a range of peptide ligands compared well with values reported in the literature (Table 1; Schioth *et al.*, 1995; 1997; 1998). These data show that [^{3}H]MTII and [^{3}H]SHU 9119 are suitable ligands for the labelling of MC_4 receptors and represent useful tools for identifying compounds with affinity for this receptor.

Schioth, H.B. *et al.* (1995) *Eur. J. Pharmacol.*, **288**, 311-317.
Schioth, H.B. *et al.* (1997) *Neuropeptides*, **31**, 565-571.
Schioth, H.B. *et al.* (1998) *Br. J. Pharmacol.*, **124**, 75-82.
Wikberg, J.E.S. (1999) *Eur. J. Pharmacol.*, **375**, 295-310.

Table 1. Inhibition constants for displacement of [^{3}H]MTII and [^{3}H]SHU 9119 binding to human MC_4 receptors by a range of peptide ligands

	[^{3}H]MTII			[^{3}H]SHU 9119		
Compound	Ki	pKi	Hill Slope	Ki	pKi	Hill Slope
SHU 9119	0.21	9.68 ± 1.01	0.90 ± 0.04	0.18	9.76 ± 0.04	1.02 ± 0.05
HS024	0.23	9.63 ± 0.15	0.95 ± 0.13	0.46	9.34 ± 0.05	0.97 ± 0.08
JKC 366	0.49	9.31 ± 0.08	0.85 ± 0.03	1.03	8.99 ± 0.06	1.08 ± 0.08
MTII	0.59	9.23 ± 0.03	0.79 ± 0.02	1.13	8.95 ± 0.04	0.89 ± 0.13
HS014	1.05	8.98 ± 0.07	0.92 ± 0.02	1.35	8.87 ± 0.09	0.94 ± 0.06
NDP-MSH	2.04	8.69 ± 0.02	0.92 ± 0.07	2.92	8.53 ± 0.02	0.92 ± 0.05
HP228	9.25	8.03 ± 0.09	0.91 ± 0.09	18	7.74 ± 0.06	0.81 ± 0.03

pKi values (negative logarithm of the Ki) and Hill slopes are mean±s.e.mean, n=3-4. Ki values (nM) are back-transformed from the pKi.
Full names of peptides are given in Schioth *et al.*, 1995; 1997; 1998. JKC-366 = (Cyclic-[Mpr^3,Nal^4,Arg^5,D-Nal^7,Cys^{11}-NH_2]-α-MSH3-11.

200P A PATHOLOGICAL MUTATION IN PRESENILIN 1 MODULATES Na^+ CHANNEL FUNCTION IN SH-SY5Y CELLS

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Early onset Alzheimer's Disease (AD) is a hereditary condition, characterised chiefly by the over-production and aggregation of amyloid β protein (Aβ). Approximately 40% of early onset AD can be associated with mis-sense point mutations in the presenilin 1 (PS1) gene on chromosome 14. PS1 is a transmembrane protease and has recently been shown to demonstrate γ-secretase activity, providing the final step in Aβ production. PS1 is also implicated in the trafficking of ion channel subunits to the cell membrane (Esler *et al*, 2000).

Here we use the whole cell configuration of the patch clamp technique to study the effect of a pathological PS1 mutation on K^+ channel current in human neuroblastoma SH-SY5Y cells. Wildtype SH-SY5Y (wt) cells were stably transfected to over-express human wildtype PS1 (PS1 wt) or the point mutant leucine to serine substitution at codon 250 PS1 (PS1 L250S) (Tanii *et al*, 2000).

From a holding potential of –70mV cells underwent a 200ms prepulse to either –140 or –50mV before stepping to test potentials between –60 and +70mV for 85ms. The maximum K^+ current density did not differ between test groups when a -140mV prepulse was used. At the +50mV test potential the following currents were measured: wt=65±12 s.e.m. pA/pF, n=26; PS1wt = 65±13 pA/pF, n=16; L250S = 43±8.7 pA/pF, n=18. Similar results were observed when a –50mV prepulse was employed (wt=63.4±13 pA/pF, n=24; PS1wt = 61.8±13.5 pA/pF, n=16; L250S = 57±20 pA/pF, n=18) indicating that the K^+ current in these cells was resistant to inactivation. Sodium currents recorded in the same cells were significantly reduced in the L250S PS1 mutant as compared to the PS1wt. This was true between the -20 and +10mV test potentials ($p<0.05$). Depolarisation to a 0mV test potential from a –140mV prepulse gave the following peak Na^+ current: wt= -56±11 pA/pF, n=25; PS1wt = -40.7±9.5 pA/pF, n=16; L250S = -23±5.1 pA/pF, n=18. No clear effects on channel kinetics were observed.

These data do not support a clear role for the modulation of K^+ channel currents by wildtype or L250S mutant PS1 in SH-SY5Y cells. This contrasts with similar studies in the HEK 293 cell lines where an increase in K^+ channel current with transient PS1wt over-expression was demonstrated (Malin et al 1998). Expression of L250S mutant PS1 reduces Na^+ channel current in SH-SY5Y. This is independent of the non-significant decreases that were observed in the K^+ channel current, which would be expected to increase Na^+ current.

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201P CHANGES IN $GABA_B1A$ AND $GABA_B2$ RECEPTOR GENE EXPRESSION IN THE BASAL GANGLIA AND THALAMUS OF RATS WITH A NIGROSTRIATAL TRACT LESION

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Nigrostriatal tract lesion in Parkinson's disease (PD) produces a multitude of changes in downstream neurotransmitter systems within the basal ganglia. Ligand binding studies in the MPTP treated primate model of PD have indicated that plasticity of $GABA_B$ receptors occurs in the internal globus pallidus (GPi) following lesioning of the nigrostriatal tract (Calon *et al.*, 2000). However, ligand binding cannot distinguish between $GABA_B$ receptor subtypes. Therefore, the aim of this study was to examine, using in-situ hybridisation (ISH), whether changes in two of the known $GABA_B$ receptor subtypes occurs following nigrostriatal tract lesion.

Male Sprague Dawley rats (250-270g) were killed by halothane overdose, 3 weeks after a unilateral 6-OHDA (12.5 µg in 2.5 µl), or sham (2.5 µl 0.1% ascorbate in H_2O), lesion of the median forebrain bundle. Brains were removed, snap frozen and sectioned coronally (15 µm). For ISH, sections were fixed, dehydrated and defatted. ^{35}S-labelled oligonucleotide probes complementary to mRNA encoding the $GABA_B1A$ and $GABA_B2$ receptor were diluted in hybridisation buffer to a specific activity of $3x10^6$ cpm ml^{-1}. Sections were hybridised overnight at 37°C then washed in a series of standard saline citrate solutions (SSC) to a maximum stringency of 60°C and 0.1x SSC. Sections, together with ^{14}C standards, were exposed to film for 19 days. Levels of $GABA_B$ receptor mRNA were semi-quantified by densitometric image analysis. Differences in mRNA levels between lesioned and intact sides were analysed using a paired t-test ($P < 0.05$).

Following a 6-OHDA lesion, $GABA_B2$ gene expression was only significantly reduced in the substantia nigra pars compacta (SNc, ~ 80%). In contrast, $GABA_B1A$ gene expression was significantly increased in the SN pars reticulata (SNr; ~32%), entopeduncular nucleus (EP; ~26%) and subthalamic nucleus (STN; ~16%) and reduced (~16%) in the SNc (Table 1). No significant changes in mRNA levels were noted in the sham animals.

Region	$GABA_B$ Receptor mRNA levels (OD – nCi/g)			
	1A		2	
	Lesioned	Intact	Lesioned	Intact
SNr	24.1 ± 1.6*	18.2 ± 1.0	4.9 ± 0.5	7.4 ± 0.8
EP	23.5± 2.3*	19.6 ± 1.5	7.6 ± 1.7	8.9 ± 3.1
STN	90.3 ± 2.5*	77.9 ± 4.0	39.6 ± 1.3	41.6 ± 2.5
SNc	20.8 ± 3.4*	86.7 ± 6.4	7.3 ± 1.8*	37.4 ± 1.7

Table 1. $GABA_B1A$ and $GABA_B2$ receptor mRNA levels in rats with a 6-OHDA lesion. Values are mean ± s.e.mean. (n=6). * $P<0.05$; lesioned vs. intact. OD = optical density.

The loss of $GABA_B1A$ and $GABA_B2$ mRNA in the SNc suggests that these receptors are present on nigrostriatal tract neurones. The increased $GABA_B1A$ gene expression in the SNr, EP and STN following a 6-OHDA lesion may reflect a compensatory response to the marked overactivity of these nuclei in PD. However, the implications of a lack of change in $GABA_B2$ gene expression in these regions remain to be determined. Analyses of $GABA_B1B$, 1C and 1D splice variants under these conditions are nearing completion.

TJ is supported by an AJ Clark Studentship.

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202P NOVEL PHENYLGLYCINE ANALOGUES AS POTENT AND SELECTIVE ANTAGONISTS OF GROUP III mGLU RECEPTORS ON NEONATAL RAT PRIMARY AFFERENT TERMINALS.

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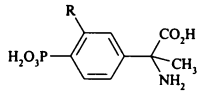
Activation of group II or III metabotropic glutamate (mGlu) receptors on primary afferent terminals in the neonatal rat spinal cord reduces the fast component of the dorsal root evoked ventral root potential (fDR-VRP) likely via attenuation of glutamate release (Watkins and Collingridge, 1994). We have previously synthesised a range of phenylglycine (PG) analogues amongst which (RS)-α-methyl-4-phosphono-PG (MPPG) and (RS)-α-cylclopropyl-4-phosphono-PG (CPPG) were 12- and 30-fold selective, respectively, as antagonists of group III over group II mGluRs (see Table 1) on neonatal rat primary afferent terminals (for a review see Schoepp et al., 1999). In order to probe the structural features required for selective antagonist action at group III mGluRs a range of novel phenylglycine analogues have been synthesised.

Here we describe the actions of 3 novel phenylglycine (PG) analogues as selective group III mGlu receptor antagonists: (RS)-α-methyl-3-chloro-4-phosphono-PG (UBP1110), (RS)-α-methyl-3-methoxy-4-phosphono-PG (UBP1111) and (RS)-α-methyl-3-methyl-4-phosphono-PG (UBP1112) (for structures see Table 1).

All experiments were performed on isolated hemisected spinal cords of 1-4 day Wistar rats of either sex (Evans et al., 1982). Recordings were taken from the ventral root following stimulation of the corresponding dorsal root (30V, 2 pulses min^{-1}). To isolate the non-N-methyl-D-aspartate component of the response (i.e. the fDR-VRP), DR-VRPs were recorded in the presence of 2 mM $MgSO_4$ and 50 µM (R)-2-amino-5-phophonopentanoate (D-AP5). The novel phenylglycines (15 min pre-incubation) were tested for their ability to antagonise either group II ((1S,3S)-1-aminocyclopentane-1,3-dicarboxylate ((1S,3S)-ACPD) or (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R,4R)-APDC)) or group III ((S)-2-amino-4-phosphonobutanoate (L-AP4)) mGlu receptor agonist (agonists applied for 5 min) induced depressions of the fDR-VRP (see Table 1).

Table 1. K_D (µM) for antagonism of agonist-induced effects on mGlu receptors on primary afferent terminals in the rat spinal cord.



Compound	R	Group II mGluRs	Group III mGluRs
UBP1110	Cl	672 ± 75.6	7.4 ± 2.3
UBP1111	CH_3O	52 ± 0.7 % antag at 1 mM	4.7 ± 0.8
UBP1112	CH_3	Not determined	5.1 ± 0.3
MPPG	H	113 ± 13	9.2 ± 0.3
CPPG	-	53	1.7

Results are represented as apparent K_Ds ± s.e.m. (n=3) unless otherwise stated. K_Ds for CPPG are from a Schild plot.

As UBP1110 and UBP1111 are at least 90-fold selective for group III over group II mGlu receptors they are the most potent selective group III mGlu receptor antagonists yet reported. As such they are likely to be useful tools to elucidate the physiological roles of group III mGlu receptors.

The authors would like to thank the BBSRC, Eli Lilly Research centre and the MRC for funding this work.

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203P THE SEDATIVE AND ANTICONVULSANT PROPERTIES OF THE ENANTIOMERS OF AR-A008055, GABAmimetic COMPOUNDS WITH NEUROPROTECTIVE ACTIVITY

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In another presentation to this meeting (Nelson *et al.*, 2000) we reported that both enantiomers of AR-A008055 [1-(4-methyl-5-thiazolyl)-1-phenylmethyl-amine] a GABAmimetic agent and clomethiazole (CMZ; Zendra®) analogue possessed similar neuroprotective potency to CMZ in a model of global ischaemia. In this communication we present some data on the behavioural pharmacology of AR-A008055.

Male Lister Hooded rats (270-320 g) were used in all experiments except in the seizure threshold study when Wistar rats were used to allow comparison with earlier data. Groups of 4 rats were injected i.p. with CMZ, (R)-(+)- or (S)-(-)- AR-A008055 and 30 min later locomotor activity measured for 10 min using an automated open field system with infrared beam break measurement. This was followed by measurement of muscle relaxation using the "pull up" test (Deacon & Gardner, 1984) and the assessment of the righting reflex by placing the animal on its back (response absent or present). Finally, 45 min after injection, the animals were killed and blood and brain samples collected for measurement of drug concentration by h.p.l.c. Anticonvulsant activity was measured by administration of the test compound and measurement of the dose of i.v. infused pentylenetetrazole required to elicit the first myoclonic jerk, using the method described by Green & Murray (1989).

Both enantiomers of AR-A008055 were less sedative than clomethiazole (Fig 1a) when measuring locomotor activity, but results indicated no difference in activity between the 2 enantiomers. In contrast, while both enantiomers were less effective in producing muscle relaxation than clomethiazole (Fig 1b), the (S)-(-)- enantiomer was more potent as a myorelaxant than (R)-(+)-AR-A008055. Similarly, no rats injected with (S)-(-)-AR-A008055 (1000 μmol kg^{-1} i.p.) showed a righting reflex while this reflex was still present in all rats injected with (R)-(+)-AR-A008055 (1000 μmol kg^{-1} i.p.). At the same (210 μmol kg^{-1}) dose which raised seizure threshold by 75 % following CMZ (Green & Murray, 1989), (R)-(+)-AR-A008055 failed to produce a statistically significant increase in threshold (+19±21%) while (S)-(-)-AR-A008055 increased it by +119±21% ($p<0.05$; unpaired 't' test).

These data emphasize the separation between neuroprotection and the sedative and anticonvulsant activity of GABAmimetic compounds as previously indicated in the study on clomethiazole (Cross *et al.*, 1995).

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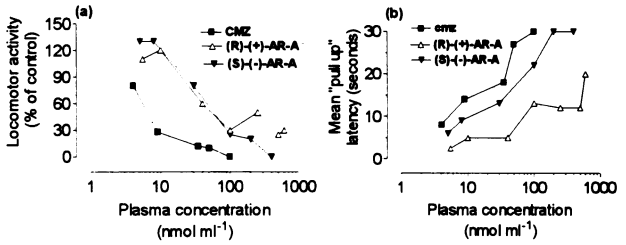

Figure 1. (a) Plasma conc. versus mean inhibition of locomotor response and (b) plasma conc versus mean "pull up" time in groups of rats (n=4) injected with CMZ and the 2 enantiomers of AR-A008055.

204P AR-A008055, A CLOMETHIAZOLE ANALOGUE, IS NEUROPROTECTIVE IN A GLOBAL ISCHAEMIA MODEL *IN VIVO* AND INHIBITS ISCHAEMIA-INDUCED GLUTAMATE RELEASE *IN VITRO*

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Clomethiazole (CMZ, Zendra®) is an effective neuroprotective agent in several *in vivo* models of acute ischaemic stroke (Green *et al.*, 2000). Recently we showed that CMZ inhibited ischaemia-induced glutamate efflux *in vitro* by a GABAergic mechanism (Nelson *et al.*, 2000). We have now examined a CMZ analogue, AR-A008055 [1-(4-methyl-5-thiazolyl)-1-phenylmethylamine], on ischaemia-induced glutamate efflux *in vitro* and in a model of global ischaemia. Both enantiomers of this compound, (S)-(-)- and (R)-(+)-AR-A008055, were examined.

Transient forebrain ischaemia was induced in male gerbils (60-80 g) by bilateral carotid artery occlusion for 5 min (Cross *et al.*, 1995). AR-A008055 (600 μmol kg^{-1} i.p.) was injected 60 min later. The % of neurodegenerated CA1 region of the hippocampus was assessed 4 days later (Cross *et al.*, 1995). Glutamate efflux from rat brain tissue exposed to ischaemic conditions was examined by the method of Nelson *et al.* (2000).

Both (S)-(-)- and (R)-(+)-AR-A008055 (600 μmol kg^{-1}) produced substantial neuroprotection against a global ischaemic insult [Control group: 90±7, n=10: (R)-(+)-AR-A008055: 22±8, n=10; (S)-(-)-AR-A008055: 11±8, n=8, results shown as % CA1 damage; both AR-A008055 groups differed from control: $p<0.01$, ANOVA followed by Dunnett's 't' test]. Protection was similar to that seen following the same dose of CMZ (Cross *et al.*, 1995). *In vitro* ischaemic conditions (30 min) enhanced glutamate efflux from cerebral tissue and (S)-(-)- and (R)-(+)-AR-A008055 and CMZ (all at 100 μM) inhibited glutamate efflux to a comparable degree (Fig. 1). The inhibitory effect of all 3 drugs was antagonized by picrotoxin (100 μM).

These data demonstrate the neuroprotective efficacy of both enantiomers of AR-A008055 and further show that both antagonize ischaemia-induced glutamate efflux, a probable initiator of the neurodegenerative process (Kristian & Siesjö, 1998). The picrotoxin data further demonstrate that the effect of AR-A008055 on glutamate efflux is probably through enhancement of GABA function, in a manner similar to CMZ (Nelson *et al.*, 2000; Green *et al.*, 2000). This may contribute to the neuroprotective effect of AR-A008055

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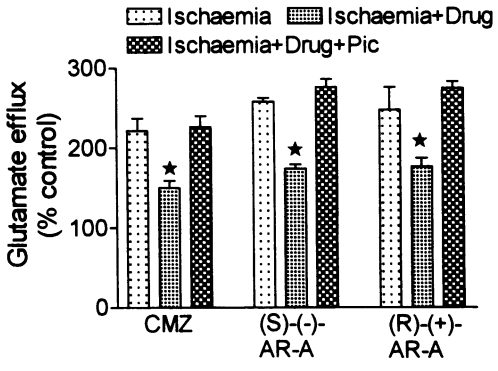

Fig. 1. Efflux of glutamate (% of control) in ischaemic medium in absence or presence of CMZ, (S)-(-)- or (R)-(+)-AR-A008055 (each at 100 μM) or drug + picrotoxin (100 μM). ★: Different from the 2 other conditions: $p< 0.01$ ($n \geq 6$). CMZ data from Nelson *et al.* (2000).

205P INDUCTION OF Arc mRNA EXPRESSION BY 3,4-METHYLENE-DIOXYMETHAMPHETAMINE (MDMA) IN THE BRAIN OF THE DARK AGOUTI RAT

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3,4-Methylenedioxymethamphetamine (MDMA, 'Ecstasy') is an amphetamine analogue which induces the release of monoamines, principally 5-HT and dopamine, in brain tissue following administration to the rat (Shulgin, 1986; White et al., 1996). Arc (activity regulated cytoskeleton associated protein) is an effector immediate early gene whose mRNA expression is localised in neuronal dendrites. Its expression has been shown to be induced by agonist stimulation of 5-HT_{2A} receptors (Pei et al., 2000) or D1 dopamine receptors (Fosnaugh et al., 1995). In the present study we have investigated the effects of MDMA on Arc mRNA expression in rat brain using the Dark Agouti strain, as part of an ongoing investigation into the long-term neurotoxic effects of MDMA.

Male Dark Agouti rats (190 - 210g) were administered (i.p.) either saline (1ml/kg) or MDMA (12.5 mg/kg), which induced a clear hyperthermic response when measured 2h later. The rats were killed 2h after drug/saline administration and the brains isolated and frozen in isopentane then stored at -70°C prior to use. Arc mRNA expression was analysed by in situ hybridisation histochemistry using [^{35}S]-dATP labelled oligonucleotide probe as described previously (Pei et al., 2000). The relative abundance of mRNA in selected areas was determined by densitometric quantification of autoradiograms using the Scion-Image system. Statistical analysis of the effects of MDMA were made using Student's unpaired t-test.

Acute administration of MDMA induced a significant increase in Arc mRNA expression in several brain areas (Table 1), particularly in the orbital, cingulate and parietal cortex. Within these cortical areas Arc mRNA expression was particularly dense within layers 2 and 5, corresponding with the distribution of 5-HT_{2A} receptors and similar to the distribution of Arc mRNA induced by DOI (Pei et al., 2000). MDMA also induced a five-fold increase in the striatum. This was similar to the effect induced by cocaine (Fosnaugh et al., 1995) and was substantially greater than that previously observed in response to DOI in this area.

Table 1 : Effect of MDMA on Arc mRNA expression.

Treatment	Orbital Cortex	Cingulate Cortex	Parietal Cortex	Striatum
Saline	96 ± 15	83 ± 8	210 ± 14	35 ± 3
MDMA	576* ± 115	352* ± 65	1085* ± 132	179* ± 44
% Increase	497	323	417	419

Values are expressed in nCi/g tissue as mean ± SEM for n=6 per group. * P<0.01 v Saline, Student's unpaired t-test.

We conclude that acute administration of MDMA in the rat induces significant expression of Arc mRNA in selected brain regions. Comparison to previous studies suggests that these effects may be mediated through serotonergic mechanisms in the cortex and predominantly dopaminergic stimulation in the striatum. The use of selective antagonists will be essential in the identification of the receptors mediating these acute effects of MDMA.

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206P DEPRESSION OF SYNAPTIC TRANSMISSION IN THE RAT SPINAL CORD MEDIATED VIA GROUP III METABOTROPIC GLUTAMATE RECEPTORS IS SENSITIVE TO NMDA RECEPTOR ANTAGONISTS.

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It is known that group III metabotropic glutamate receptors (mGluRs) are present on primary afferents in the neonatal rat spinal cord. Activation of these receptors by the selective agonist (S)-2-amino-4-phosphonobutyrate (L-AP4) has previously been shown to mediate a reduction in the amplitude of the fast component of the dorsal root evoked-ventral root potential (fDR-VRP) likely via attenuation of glutamate release (Jane *et al.*, 1994). Previous work has examined the affects of L-AP4 in the presence of 2 mM $MgSO_4$ and 50 μM (R)-2-amino-5-phosphopentanoate (D-AP5) in order to block the *N*-methyl-*D*-aspartate (NMDA) receptor-mediated contribution to the fDR-VRP (Jane *et al.*, 1994). Here we report evidence that the ability of L-AP4 to reduce the amplitude of the fDR-VRP is diminished when the NMDA receptors are activated.

All experiments were performed on isolated hemisected spinal cords from neonatal rats (2-3 days old) bathed in a physiological saline (Evans *et al.*, 1982). Recordings were taken from the ventral root following stimulation of the corresponding dorsal root (30 V, 2 pulses min^{-1}). Concentration-response curves (CRCs) were then constructed to L-AP4. The perfusate was then changed one containing either 2 mM $MgSO_4$ + 50 μM D-AP5, 2 mM $MgSO_4$ or 50 μM D-AP5 and a second CRC to L-AP4 constructed.

CRCs constructed to L-AP4 in the absence of $MgSO_4$ and D-AP5 were biphasic. Therefore, under these conditions L-AP4 appears to be a partial agonist on the receptor that is mediating the high affinity portion of the curve as this component was unable to produce a 100% depression of the fDR-VRP. An estimate of the EC_{50} for the high-affinity portion of the curve was calculated and was 1.80±0.71 μM (mean ± S.E.M, n=10). An estimate of the EC_{50} for the low-affinity portion of the curve could not be calculated due to L-AP4 producing depolarisations of motorneurones at high concentrations via activation of NMDA receptors (Evans *et al.*, 1982).

Next, either 2 mM $MgSO_4$, 50 μM D-AP5 or 2 mM $MgSO_4$ + 50 μM D-AP5 was added to the perfusate for 30 minutes and a second CRC constructed to L-AP4. The presence of 2 mM $MgSO_4$, 50 μM D-AP5 or a combination of both all produced similar responses; namely the CRC to L-AP4 was no longer biphasic, and so in these experiments L-AP4 appears to be acting as a full agonist. In addition the EC_{50}s to L-AP4 in the presence of $MgSO_4$, D-AP5 or $MgSO_4$ + D-AP5 were 2.77 ± 1.23, 3.15 ± 1.67 and 2.11 ± 0.92 μM, respectively, (mean ± S.E.M, n=3). There was no significant difference between any of these three EC_{50}s and the EC_{50} of the high affinity component in the absence of $MgSO_4$ and/or D-AP5 (P=0.791, one-way ANOVA).

As the effects of adding $MgSO_4$, D-AP5 or both $MgSO_4$ + D-AP5 were similar it is likely that the potentiation of the effect of L-AP4 on the fDR-VRP caused by these treatments is due to removing the influence of the NMDA receptors.

It is difficult to envisage a hypothesis whereby antagonism of post-synaptic NMDA receptors could potentiate the presynaptically mediated effects of L-AP4. However, it is known that there are presynaptic NMDA receptors present in the spinal dorsal horn (Liu *et al.*, 1994). It is possible that blocking these pre-synaptic NMDA receptors causes a decrease in glutamate release, thereby removing a mechanism that would normally oppose the effects of group III mGluR activation, thus leading to an increase in efficacy of L-AP4 for depressing the fDR-VRP.

This work was supported by the MRC.

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207P INHIBITORY INFLUENCE OF ELECTRICAL FIELD STIMULATION ON N-METHYL-D-ASPARTATE RECEPTOR-INDUCED ILEAL CONTRACTIONS

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Contractile responses of ileal tissues to $GABA_A$ receptor agonists were found to be significantly inhibited by prior periods of electrical field stimulation (EFS), whilst responses to other agonists, including acetylcholine, 5-HT and histamine, were not affected by EFS (Alyami *et al*, 1990). $GABA_A$- and NMDA-receptor agonists produce similar effects in gut tissue, via activation of cholinergic nerves (Campbell *et al*, 1991) and it was of interest to examine whether responses to NMDA receptor agonists were also affected by periods of EFS.

Longitudinal muscle myenteric plexus (LMMP) preparations taken from guinea-pig ileum were mounted under 0.5g tension in Mg^{2+}-free Krebs' solution containing 10μM glycine (37°C, 95 % O_2/5% CO_2). Changes in tension due to the application of drugs and electrical field stimulation (0.1 Hz, 40 volts, 0.2ms duration) were expressed as the mean ± s.e.m (n = 4); statistical differences were sought using analysis of variance (ANOVA) followed by Dunnett's t-test.

In EFS-free preparations, glutamate (2-200μM), N-methyl-D-aspartate (NMDA, 20-100μM) or L-aspartate (20-200μM) induced concentration-dependent contractions. Glutamate (EC_{50} 28 ± 5μM) was significantly ($p<0.01$) more potent than either aspartate (EC_{50} 210 ± 30μM) or NMDA (EC_{50} 290 ± 50μM). Sub-maximal contractions to these agonists were antagonised by atropine (1μM) and by the NMDA receptor antagonist AP-5 (20μM).

Sub-maximal contractile responses to aspartate (800μM) were elicited immediately before EFS (control) and 20-420s after ceasing EFS (test). The application of EFS (20 min) significantly ($p<0.05$) reduced the contractile response to aspartate applied 20s after cessation of EFS, to 6.5 ± 4.2% of control. The extent of inhibition was dependent on the duration of EFS; at durations of 5, 10, 15 and 20 min of EFS, the contractile response to aspartate (administered 1 min after termination of EFS) was reduced significantly ($p<0.01$) to 68.7 ± 8.5%, 51.7 ± 3.2%, 45.3 ± 3.7% and 32.8% ± 5.5% of control respectively.

The test response to aspartate recovered with time, to 97.3 ± 2.7% of control when aspartate was applied 420s after ceasing EFS. Whilst significant inhibition of responses to aspartate by EFS occurred at 20, 60 and 180s after cessation of EFS, in the presence of the $GABA_B$ antagonist, CGP35348 (30μM) the responses to aspartate were significantly inhibited (52 ± 9.9% of control, $p<0.001$) only when applied 20s after cessation of EFS but not after 60 or 180s. The same profile of response was observed when glutamate (80μM) and NMDA (800μM) were employed as agonists.

These results show that periods of EFS can inhibit contractile responses to NMDA receptor agonists, as previously reported for $GABA_A$ receptor agonists (Alyami *et al*, 1990). Furthermore, the inhibition was found to be attenuated by the $GABA_B$ receptor antagonist CGP35348, suggesting that activation of these receptors may be central to the inhibitory effect of EFS.

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208P ISOLATION REARING REDUCES THE ABILITY OF 7-OH-DPAT TO ATTENUATE RESPONDING FOR A FOOD-PAIRED CONDITIONED STIMULUS IN THE RAT

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Isolation rearing enhances the effects of the dopamine (DA) releasing agent d-amphetamine to increase responding for a food paired conditioned stimulus (CS) (Jones *et al*., 1990; Smith et al., 1997a). It has been suggested that the DA D_3 receptor is involved in the mediation of reward (Chaperon and Thiebot, 1995). Previous studies in our laboratory demonstrated that the D_2/D_3 receptor agonist 7-OH-DPAT attenuated responding for food paired CS in group housed rats (Smith *et al*., 1997b). The aim of the present study was to investigate the influence of isolation rearing on sensitivity of rats to the effect of 7-OH-DPAT on responding for a food paired CS. Female Hooded Lister rats (n=40) were obtained at weaning (21-23 days) and randomly allocated to either isolation housing in individual cages or social groups of 5 per cage. Rats were food deprived to 85% of their free feeding weight (250-300g) and trained to associate the delivery of food with presentation of a CS over 12 days following the protocol of Smith et al., (1997a). Briefly, in the test phase two levers were introduced into the chamber. Drugs, d-amphetamine, 7-OH-DPAT (D_3 preferring agonist), quinpirole (D_2 preferring agonist) or vehicle (0.9% saline), were administered i.p. 30min prior to testing with test sessions of 30min duration. All data were subject to square root transformation before subsequent analysis by 2-way ANOVA followed by post-hoc Bonferroni corrected t-test. None of the compounds had a significant effect on responding on the NCR (inactive) lever in either group of rats.

d-Amphetamine (0.125-0.5 mg/kg) produced a significant effect of drug ($F(3,51)= 44.9$ $p<0.001$) and housing ($F(1, 51)= 14.4$ $p<0.05$). Post-hoc analysis revealed that a dose of 0.5mg/kg significantly increased responding on the CR lever in both social and isolated rats ($p<0.05$). A dose of 0.25mg/kg d-amphetamine significantly increased responding on the CR lever (active) in isolated rats only ($p<0.05$). At this dose CR responding in isolated rats was significantly increased compared with the social group ($p<0.05$) (Table 1). Following the administration of quinpirole (0.01-1mg/kg) a significant effect of drug was observed ($F(3,54)= 4.82$ $p<0.05$), and no significant effect of housing ($F(1,54)= 1.8$ N.S.). A dose of 1mg/kg significantly reduced responding for the CS in isolated rats only ($p<0.05$). 7-OH-DPAT (0.01-1mg/kg) revealed a highly significant effect on housing ($F(1,90)= 24.8$ $p<0.001$). Further analysis revealed that doses of 0.1 and 0.5mg/kg significantly decreased responding for the CS in social animals only (Table 1).

In agreement with previous studies (Jones *et al*., 1990; Smith *et al*., 1997a) isolates were more sensitive to the increase in CR responding induced by d-amphetamine compared with socially housed rats. Furthermore, the present results support our previous work, showing that 7-OH-DPAT reduces CR responding in social animals and extends it to show a lack of effect in isolation reared rats. Further studies using selective D_3 receptor antagonists are required to investigate the role of D_3 receptors in these effects.

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Smith J.K. *et al* (1997a) *Psychopharmacology.* 131, 23-33.

Smith J.K. *et al* (1997b) *Br.J.Pharmacol.* 120, 249P

Table1: The effect of housing conditions on responding for a conditioned reinforcer following treatment with DA agonists

d-Amphetamine			7-OH-DPAT			Quinpirole		
Dose mg/kg	Social	Isolated	Dose mg/kg	Social	Isolated	Dose mg/kg	Social	Isolated
0	5.4 ± 0.7	7.2 ± 0.6	0	4.9 ± 0.3	6.8 ± 0.9	0	4.0 ± 0.7	6.0 ± 0.6
0.125	7.5 ± 0.6	9.8 ± 1.1	0.01	3.9 ± 0.4	5.6 ± 0.9	0.01	3.2 ± 0.6	4.4 ± 0.5
0.250	9.8 ± 1.3	14.9 ± 0.6 *♣	0.05	4.2 ± 0.4	4.7 ± 0.8	0.10	4.7 ± 0.6	4.9 ± 0.9
0.500	15.7 ± 0.5 *	16.2 ± 1.2 *	0.10	3.1 ± 0.3 †	4.8 ± 0.5	1.00	2.7 ± 0.8	2.2 ± 1.0 †
			0.50	2.8 ± 0.4 †	6.1 ± 1.3			
			1.00	3.0 ± 0.7	5.4 ± 1.8			

Data are expressed as mean ± s.e.m. square root of lever responses on CR lever (n=9-10 per group). Effect of drug compared to vehicle treatment; significant increase: *$p<0.05$, significant attenuation: †$p<0.05$. Effect of housing condition; ♣$p<0.05$ (Bonferroni corrected t-test).

209P EFFECT OF PREGABALIN ON MAINTENANCE OF CONDITIONED PLACE PREFERENCE (CPP) TO MORPHINE IN THE RAT

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Pregabalin, has been shown to block the development of CPP to morphine (Andrews et al., this meeting). While this may demonstrate a possible beneficial interaction between the action of gabapentinoids and opioids it does not directly address the clinical situation where patients present with an already established propensity to take opioids. Thus the aim of the study was to determine the ability of pregabalin to reverse an established CPP.

Male Hooded Lister rats (250-350g) were used for all studies. The place preference chambers were balanced for tactile and visual cues (unbiased) and initial training took place over 4 days. Briefly, each rat was injected and placed immediately into one side or other of the box for 45 min (with no access to the other side). On Day 5 each rat was placed (uninjected) into the box and allowed free access to both sides of the box for 15 min. Time spent in each side was recorded and preference expressed as time in drug side minus time in saline side (Students paired t-tests were used to analyse for differences between time spent in the drug (D) and saline-paired (S) sides). Graphical representations are of the difference (D minus S) between the times spent in the two compartments.

In Experiment 1, rats (n=6 or 7/group) were trained as outlined above. Individual groups were then tested over several days (without further drug treatment or training) to determine the time course over which the CPP dissipated. CPP was found to be present on each of four days following the initial test for preference (see Figure 1A). In Experiment 2 CPP to morphine was established in rats over four days and the presence of CPP assessed the following day (initial test; fig 1B). Rats showing CPP were then assigned to one of two groups (n=10/group) and the following day administered either pregabalin (10mg/kg p.o.) or saline one hour prior to a second preference test. The saline group maintained CPP but the group given pregabalin showed no CPP, demonstrating a blockade by pregabalin of the maintenance of morphine-induced CPP (Fig 1B). On re-testing the two groups over two subsequent days (without further treatment with saline or pregabalin) significant CPP was maintained in the saline group and also returned in the pregabalin-treated group indicating one dose of pregabalin was insufficient to permanently reverse the place preference to morphine (Fig 1B).

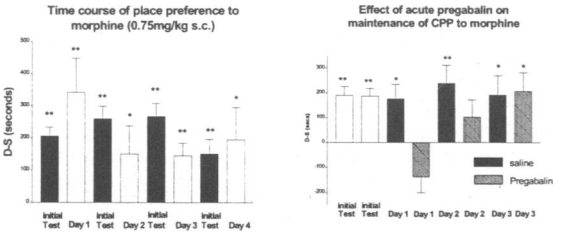

Figure 1A

*p<0.05, **p<0.01 paired t-test D vs S

Figure 1B

In conclusion, these experiments show that it may be possible to inhibit an established place preference to morphine by administration of pregabalin, however this is reversible. Further work is required to determine the effect of chronic pregabalin. Overall, these data provide early evidence for a role of gabapentinoids in the treatment of opioid dependence.

References

Andrews, N., Loomis, S., Oles, R.S., et al., (this meeting)

210P THE EFFECT OF NALOXONE ON ADAPTATION TO MOTION SICKNESS IN *SUNCUS MURINUS*

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It has been suggested that endogenous opioids may be involved in the genesis of motion sickness (Yasnetsov *et al.*, 1986). The aim of the present study was to investigate the effect of the opioid receptor antagonist naloxone on adaptation to motion sickness in the Japanese House Musk shrew, *Suncus murinus* .

Three groups of adult male *Suncus murinus* (60-65 g) were used. Each animal was placed individually in a transparent cage (100Wx150Lx150H mm) of 6 linked units. After 30 min, a horizontal motion stimulus was commenced with an amplitude (peak to peak displacement) of 40 mm and frequency of 1 Hz; this was applied every second day for a total number of 11 trials. All groups received vehicle (saline) on the first trial and in subsequent trials they were treated with either vehicle or naloxone (1.0 or 10.0 mg/kg, i. p.) 30 min prior to the motion stimulus. The number of emetic episodes (vomiting/retching) and the latency of onset (s, the time from the start of the shaking to the first vomit) were recorded over a 10 min period. Data were expressed as the mean±s.e.mean of n=6 and analysed using one-way ANOVA which was followed by Bonferronni-Dunnett's t-test.

Animals that received vehicle only responded on the first trial with 11.5 ± 2.1 emetic episodes which was reduced to 3.7 ± 1.3 ($p < 0.05$) emetic episodes on the second trial. During the subsequent 11 trials the emetic episodes were observed in all animals but the intensity of emesis remained at a level of approximately 20 to 25% of the original value and this was also recorded in the naloxone (1.0 mg/kg) treatment group (Figure 1). However, the animals receiving naloxone at 10.0 mg/kg prior to the second and subsequent challenges, showed no significant reduction in the intensity of emesis compared to the first trial (Figure 1).

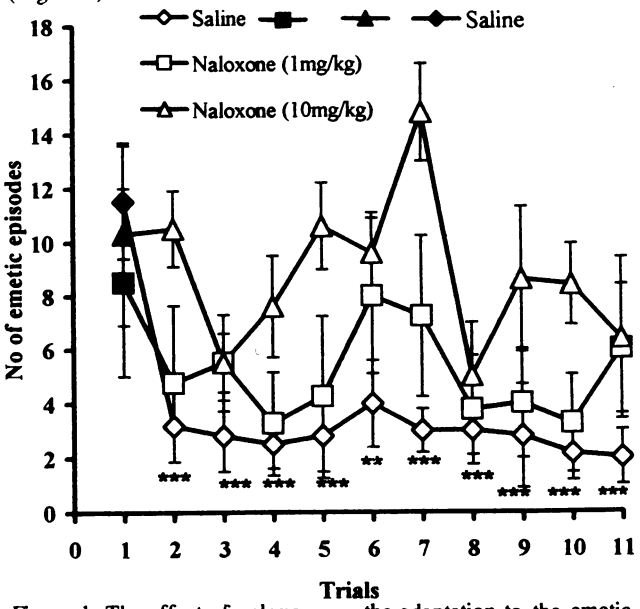

Figure 1. The effect of naloxone on the adaptation to the emetic stimulus of motion sickness in *Suncus murinus*. **p<0.01 and ***p<0.001 compared to the vehicle treated animals in the first trial.

The data suggest that a repeated challenge with motion stimulus may induce the release of an opioid-mediated anti-emetic response that is blocked by naloxone.

Yasnetsov V. V., Vakulina O. P., Sabaev V. V. et al. (1986) *Bull. Exp. Biol. Med.* 100, 164-167.

211P NO INHIBITS CARBACHOL- AND THAPSIGARGIN-INDUCED CONTRACTION OF β-ESCIN-PERMEABILISED SMOOTH MUSCLE

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We have recently reported that the NO/guanylyl cyclase/cyclic GMP pathway can relax the mouse anococcygeus muscle without reducing the cytoplasmic Ca^{2+} concentration (Wallace *et al.*, 1999). To help clarify the nature of this Ca^{2+}-independent relaxation, we have now investigated the effects of NO on excitation-contraction coupling in muscles permeabilised with β-escin, which allows free access of Ca^{2+} through the plasma membrane whilst preserving receptor-effector function

Anococcygeus muscles were dissected from male mice (LACA strain; 25 – 35 g) and set up initially in Krebs solution containing phentolamine (1 μM), L-N^G nitro arginine (50 μM) and verapamil (10 μM). The tissues were pre-incubated with 30 μM guanethidine (10 min) before contractile viability was assessed with carbachol (50 μM; 407 ± 39 mg tension). Muscles were then transferred to a relaxing solution containing PIPES 20 mM, $MgCl_2$ 7.1 mM, KCl 108 mM, EGTA 2 mM, Na_2ATP 5.9 mM, creatine phosphate 2 mM, creatine phosphokinase 4 u ml^{-1}, E-64 1 μg ml^{-1} and FCCP 1 μM) and permeabilised by a 10 min exposure to 50 μM β-escin. All experiments were conducted at 25°C; results are expressed as mean ± sem (n > 5).

Addition of Ca^{2+} (0.1 – 50 μM; buffered with 2 mM EGTA) to permeabilised tissues caused a concentration-dependent contraction. 10 μM Ca^{2+} raised tone by 35 ± 6 mg and was used in all subsequent experiments; NO (96 μM) had no effect on the contraction produced by Ca^{2+} alone. When the Ca^{2+}-induced response had reached a peak (after 100 s), subsequent addition of the substrate for guanylyl cyclase, GTP (100 μM), produced a further increase in tension (to 169 ± 26% of the peak Ca^{2+} response), but again this combined Ca^{2+}/GTP-induced tone was unaffected by NO (96 μM). Carbachol (50 μM) enhanced the Ca^{2+}/GTP contraction (to 343 ± 42% of the peak Ca^{2+} response) and in this case the carbachol component was greatly relaxed by NO (96 μM). This relaxant effect of NO was concentration-related; 1.5, 6, 24 and 96 μM NO relaxed carbachol-induced tone by 40 ± 3%, 65 ± 12%, 86 ± 19% and 88 ± 19% respectively. Addition of 100 nM thapsigargin (Tg), rather than carbachol, also enhanced the Ca^{2+}/GTP contraction of permeabilised tissues and this Tg-induced component was again relaxed by NO (96 μM). NO-mediated relaxations of both carbachol and Tg tone were abolished by the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ; 5 μM).

The results suggest that NO can relax the mouse anococcygeus by inhibiting Ca^{2+}-sensitisation mechanisms activated by receptor agonists; these mechanisms may be related to store depletion since they were also activated by Tg.

The authors thank the Wellcome Trust. S.A. is a BBSRC CASE student with Pfizer, UK.

Wallace, P., McFadzean, I. & Gibson, A. (1999) *Br. J. Pharmacol.* 128, 299P

212P BARIUM CAN DISTINGUISH BETWEEN CONTRACTIONS OF SMOOTH MUSCLE MEDIATED VIA STORE-OPERATED AND VOLTAGE-OPERATED CALCIUM ENTRY

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Store-operated (capacitative) calcium entry plays an important role in excitation-contraction coupling in several smooth muscles (Gibson et al., 1998), yet there are relatively few chemical entities which have been found to modify selectively store-operated channels (SOCs) over other calcium entry processes. We have therefore begun a series of studies to try and identify such entities and, here, we report that barium ions (Ba^{2+}; as BaCl) can distinguish between contractions of the mouse anococcygeus mediated by SOCs (activated by thapsigargin, Tg) and those mediated by voltage-operated calcium channels (VOCCs; activated by high K^+ Krebs).

Anococcygeus muscles were dissected from male mice (LACA; 25-35 g) and set up for the recording of isometric tension responses as described previously (Wallace et al., 1999). The Krebs solution contained phentolamine (1 μM), L-N^G-nitro arginine (50 μM) and either verapamil (10 μM) or nifedipine (1 μM); in addition, muscles were preincubated with guanethidine (30 μM; 10 min). When responses to high K^+ Krebs (60 mM KCl; NaCl reduced appropriately) were to be studied, verapamil/nifedipine was omitted. Results are given as mean ± sem (n > 4).

Both Tg (100 nM) and high K^+ (60 mM) produced sustained contractions of the mouse anococcygeus. The response to Tg was relatively slow, reaching peak tension (550 ± 50 mg) 10 min after drug addition; the contraction to high K^+ was faster, and consisted of a rapid initial phase which then fell within 5 min to the sustained level (440 ± 40 mg). Ba^{2+} (0.1 – 13 mM) produced concentration-related relaxations of Tg-induced tone (IC_{50} = 0.5 mM) with complete relaxation at 13 mM. No such relaxations were observed against high K^+ tone; indeed, at concentrations above 1 mM, Ba^{2+} enhanced high K^+-induced contraction (by 162 ± 28% at 13 mM Ba^{2+}). In contrast to Ba^{2+}, the general smooth muscle relaxant papaverine (1 – 33 μM) was equipotent as a relaxant of tone induced by either Tg or high K^+ (IC_{50} = 2 μM in each case). In calcium-free medium (with 0.5 mM EGTA and 1 μM nifedipine), carbachol produced a small transient contraction (25 ± 5 mg), which could not be repeated unless the internal stores were allowed to refill by exposure to normal Krebs for 15 min. Inclusion of 30 mM Ba^{2+} during the calcium-reloading period greatly reduced the subsequent response to carbachol in calcium-free medium (8 ± 4 mg); inclusion of Ba^{2+} in the calcium-free medium, after the reloading, had no effect on the carbachol response.

The results provide evidence that Ba^{2+} produces selective inhibition of contractions mediated via SOCs, and inhibits store re-filling through capacitative calcium entry, in the mouse anococcygeus. Attempts to identify organic molecules with similar pharmacological properties are continuing.

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Wallace, P., Ayman, S., McFadzean, I. *et al.* (1999) *N.S. Arch. Pharmacol.* 360, 368-375.

213P EVIDENCE FOR 5-HT_3 RECEPTOR INVOLVEMENT IN THE 5-HT-INDUCED CONTRACTION IN THE MOUSE ISOLATED ILEUM

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The 5-hydroxytryptamine (5-HT) receptor mediating tension changes in the intestine has been studied mainly using tissues from the guinea-pig (Costall & Naylor, 1990). The aim of the present study was to study the contractile response to 5-HT in the mouse ileum and characterize the 5-HT receptor involved in the response.

Ileal segments (up to four segments taken 2-15 cm from the ileo-caecal junction) were obtained from BKW mice of either sex (24-35g) and mounted in 10 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit solution (37°C) containing methysergide (1 μM) under an initial tension of 0.25 g. The tissues were allowed to equilibrate for 1 hour and concentration response curves to 5-HT and other agonists were obtained in a non-cumulative manner using a 10 min cycle and a 30 s contact time. A comparison of the effects of agonists and antagonists was carried out using paired preparations. Antagonists were added to the buffer reservoir and allowed to equilibrate for 1 hr before application of the agonists.

In the presence of methysergide, the non-cumulative addition of 5-HT (0.3 - 100 μM) produced a concentration-related contraction with a pEC_{50} ± s.e.mean value of 5.47 ± 0.09 (n=12). The 5-HT_3 receptor selective agonist 2-methyl-5-HT (3 - 100 μM) mimicked the 5-HT response with a pEC_{50} value of 5.00 ± 0.07 (n=10) and produced a maximum response not significantly different from that of 5-HT. The individual contractile response to 5-HT and 2-methyl 5-HT consisted of a spike-like phasic response with little or no tonic contraction. Unlike 2-methyl-5-HT, the 5-HT_4 receptor agonists 5-methoxytryptamine and RS 67506 (Eglen *et al.*, 1995) and the 5-HT_2 receptor agonist α-methyl-5-HT (0.1 -100 μM) failed to mimic the contractile response of 5-HT, with only a few tissues exhibiting contraction which did not exceed 25% of the maximum response to 5-HT.

The 5-HT_2 receptor antagonists ritanserin (0.1 μM) and ketanserin (1 μM) or the 5-HT_4 receptor selective antagonist SB 204070 (0.1 μM) (Wardle *et al.*, 1994) failed to alter the concentration response curve to 5-HT (n=5-10). But the 5-HT_3 selective antagonists granisetron (1 nM) and tropisetron (2 nM) caused a dextral shift of the concentration response curve to 5-HT. The antagonism with both granisetron and tropisetron was unsurmountable with the maximum response to 5-HT being significantly reduced in the presence of either granisetron or tropisetron.

The inclusion of methysergide in the bathing medium and inability of ritanserin, ketanserin or SB 204070 to antagonise the response to 5-HT suggest that 5-HT_1, 5-HT_2 and 5-HT_4 receptors are not involved in the contractile response to 5-HT in the mouse ileum. The failure of 5-methoxytryptamine, RS 67506 and α-methyl-5-HT to mimic the 5-HT response further supports this conclusion. The ability of 2-methyl-5-HT to mimic the 5-HT response and antagonism of the 5-HT response with nanomolar concentrations of granisetron and tropisetron indicates that the response is mediated by a 5-HT_3 receptor. The unsurmountable nature of the antagonism with 5-HT_3 receptor antagonists is not fully understood but was not unexpected (Hoyer *et al.*, 1994).

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Eglen, R.M. *et al.* (1995) *Br. J. Pharmacol.*, **115**, 1387- 1392.
Hoyer, D. *et al.* (1994) *Pharmacol. Rev.*, **46**, 157-203.
Wardle, K.A. *et al.* (1994) *Br. J. Pharmacol.*, **112**, 789-794.

214P ENDOTHELIN-1-INDUCED HYPERPOLARISATION OF SMOOTH MUSCLE CELLS IN THE GUINEA-PIG LOWER OESOPHAGEAL SPHINCTER

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It is well known that endothelin-1 (ET-1) is a potent vasoconstrictor. Recently, it has been reported that ET-1 produces an initial, transient relaxation of gastrointestinal sphincter smooth muscle followed by a sustained secondary contraction (Chakder & Rattan, 1999). In the present study we investigated whether the effects of ET-1 were associated with changes in membrane potential. Conventional intracellular recording techniques were used to record the membrane potential of individual smooth muscle cells in the lower oesophageal sphincter of the guinea-pig. The average resting membrane potential was -44.1 ± 0.3 mV (mean ± s.e.mean; n = 52). In separate experiments, isometric force transducers were used to measure mechanical responses. It was confirmed that ET-1 elicited an initial transient small relaxation followed by a sustained contraction. Surprisingly, ET-1 (1 - 30 nM, n = 3 - 9) elicited a concentration-dependent, slow onset, sustained membrane hyperpolarisation that was insensitive to nifedipine (1 μM) (ET-1 at 10 nM, control, 12.3 ± 1.1 mV, n = 6; nifedipine, 12.7 ± 0.7 mV, n = 6). The ET-1-induced hyperpolarisation was abolished by the small conductance Ca^{2+}- activated K^+ channel blocker apamin (0.1 μM). The ET_A receptor antagonist BQ-123 (1 μM) abolished, and the ET_B receptor antagonist BQ-788 (1 μM) partially inhibited the ET-1-induced hyperpolarisation. The selective ET_B receptor agonist sarafotoxin 6c (10 nM) did not alter membrane potential significantly. In Ca^{2+}- free Krebs solution, the ET-1 induced hyperpolarisation was reduced to 2.6 ± 0.4 mV (n = 5) and following re-perfusion with physiological Krebs solution recovered to 13.4 ± 1.1 mV (n = 5). SKF 96365 (50 μM), a receptor-operated Ca^{2+} channel blocker (Merritt *et al.*, 1990), partially inhibited the ET-1-induced hyperpolarisation (control, 12.2 ± 1.0 mV, n = 6; SKF 96365, 4.3 ± 0.7 mV, n = 6) and the residual component was blocked by pretreatment with the sarcoplasmic reticulum Ca^{2+}- ATPase inhibitor thapsigargin (1 μM) (1.2 ± 0.4 mV, n = 5). To investigate the relationship between hyperpolarisation and mechanical activity, the effects of apamin on ET-1-induced biphasic mechanical responses were studied. Apamin (0.1 μM) abolished the initial transient relaxation and potentiated the subsequent maintained contraction (ET-1, 13.1 ± 1.1 mN, n = 4; ET-1 in apamin, 23.5 ± 1.3 mN, n = 4).

These data suggest that ET-1 acts mainly through ET_A receptors, producing a sustained hyperpolarisation by activating apamin-sensitive K^+ channels. K^+ channel activity is triggered mainly by extracellular Ca^{2+}, acting on receptor-operated Ca^{2+} channels, and partly through Ca^{2+} release from intracellular stores. Presumably the ET-1-induced hyperpolarisation triggered the initial transient relaxation, and acts to oppose the subsequent maintained contraction.

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215P A PRELIMINARY STUDY OF THE ROLE OF CGMP IN ISOLATED HUMAN MYOMETRIUM FROM PREGNANT DONORS

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It has been suggested that in pregnant guinea pigs cGMP plays a role in maintaining uterine quiescence during pregnancy (Weiner *et al.*, 1994). However, little is known regarding the role of cGMP in human uterus. The aim of this study was to investigate cGMP involvement in isolated human myometrium using bradykinin (BK) to inhibit myogenic activity (Abbas *et al.*, 1996), methylene blue (a guanylyl cyclase inhibitor), sodium nitroprusside (SNP) (a nitric oxide (NO) donor) and zaprinast (a phosphodiesterase type 5 (PDE-5) inhibitor).

Samples of human myometrium were obtained from pregnant donors (who were at term but had not gone into labour) during Caesarean section (all patients gave written consent). The myometrial strips were set up for superfusion (2g tension) in Krebs solution (37 C, 95% $O_2$5% CO_2) at 2ml min^{-1} as previously described by Senior *et al.* (1991). After equilibration of the tissues bolus doses (10µl volume) of zaprinast (10^{-15} - 10^{-9} mol) were injected directly into the flow of the superfusate. Inhibitory responses were measured in minutes (see Senior *et al.*, 1991). When used methylene blue was added to the superfusate (10µM) 20 min before bolus administration of BK (0.1 & 1 nmol). SNP (10µM) was added as a bolus dose. In all cases n=5. Statistical comparisons were made using ANOVA with a *post-hoc* Dunnett's test.

SNP inhibited myometrial activity for 15±3min. Methylene blue alone had no effect on the myogenicity of the tissue, however, the inhibitory responses induced by both doses of BK were significantly reduced ($P<0.001$) in the presence of methylene blue.

Zaprinast evoked a dose-related inhibition of myogenic activity (the interval between spontaneous contractions was increased by 0.5±0.04, 1±0.09 , 2±0.19 & 4.5±0.2min at 10^{-14}, 10^{-13}, 10^{-11} & 10^{-9} respectively).

The results of the study using SNP and methylene blue suggest that cGMP is involved in the inhibition of myometrial activity. The results obtained with zaprinast may indicate that there is PDE-5 activity in human myometrium from pregnant donors (zaprinast had no effect on tissue from non-pregnant donors, data not shown). PDE-5 is cGMP specific, therefore, this finding also suggests a role for cGMP in maintaining myometrial quiescence during pregnancy. It has been demonstrated that PDE-5 activity can be inhibited via a G-protein dependent mechanism (Lochhead *et al,* 1997) and thereby it is possible that stimulation of, for example, B_2 receptors could inhibit PDE-5 via this mechanism resulting in an increase in cGMP levels which in turn would evoke inhibition of myometrial activity.

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216P THE EFFECT OF SMOKING ON THE RESPONSE TO A TP-MIMETIC ON PERFUSED SEGMENTS OF THE HUMAN ISOLATED UMBILICAL ARTERY.

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Previous studies in our laboratory have shown that rings of isolated human umbilical artery respond to thromboxane analogues and that this response can be antagonised by TP-receptor antagonists (Duckworth *et al.*, 1998). The effect of smoking throughout pregnancy is associated with a decrease in the prostacyclin (PGI_2)/thromboxane (TXA_2) ratio (Dadak *et al.*, 1981). In this study perfused segments of human umbilical artery were used to evaluate the response to the stable thromboxane mimetic, U46619. Tissues were used from the placental and foetal portions of the umbilical artery from both smoking (5 - 20 cigarettes a day) and non-smoking mothers.

Human umbilical cords were obtained from full-term pregnancies (all women gave written consent), at either vaginal delivery or Caesarean section, and were placed immediately in Krebs solution at room temperature. The cords were then transported to the laboratory and set up within 60 minutes. The placental end of the cord was identified by a cotton ligature whilst the foetal end was left free. The artery was carefully dissected from the surrounding Wharton's jelly and cut into 1.5 - 2 cm lengths. Each length was attached to a cannula. This in turn was connected to a pressure transducer and responses were measured as changes in pressure (mmHg). Krebs physiological solution was aerated (2.5 % O_2, 8 % CO_2 & balanced with N_2) and was pumped through heating coils (37°C) at a flow rate of 2 ml min^{-1}. Indomethacin (1µM) was included in the Krebs solution at all times.

The artery lengths were left to equilibrate for at least 60 minutes before constructing a dose response curve. Agonists were added as bolus doses to the perfusion fluid and results are expressed as a mean (± sem) change in pressure (mmHg) of 5 determinations. When the TP-antagonist, Bay u3405 was used (McKenniff *et al.*, 1991) it was added to the superfusate (10^{-6}M) and left to equilibrate for at least 30 minutes. Statistical comparisons were made using ANOVA with a *post-hoc* Dunnett's test.

In samples taken from both ends of cord from both patient groups (smokers and non-smokers), U46619 (0.01-10 nmols) evoked a dose related vasoconstrictor response. However, the response to 10 nmols of U46619 at the foetal end of the cord declined in both smokers (S) and non-smokers (NS). Maximum responses, ± s.e.mean, (mmHg) for placental-end artery were 170 (± 40) and 52 (± 11) for S and NS respectively. At the foetal end, maximum responses were 180 (± 35) and 127 (± 28) for S and NS respectively. Tissue taken from the placental end of the cord from smoking mothers was found to be more sensitive to U46619 and this difference was statistically significant ($P<0.05$ - 0.001) when compared tissue from non-smokers. Responses were significantly attenuated by the TP-antagonist, Bay u3405 ($P<0.05$ - 0.001).

In conclusion, TP-receptor stimulation produces a more marked vasoconstriction in umbilical artery taken mothers who smoke. Such a vasoconstriction *in vivo* would result in restricted blood flow to the placenta.

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217P INVESTIGATIONS INTO THE USE OF MINIATURISED FIELD EFFECT TRANSISTORS (FETs) AS A NOVEL ELECTROPHYSIOLOGICAL TOOL IN PHARMACOLOGICAL RESEARCH

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Short-term measurements of the effects of drugs on the heart are commonly performed using isolated whole organ (eg. Langendorff preparation) or electro-physiologically (eg. patch clamp) on cultured cardiac myocytes (CM). Recently, FETs array (currently with 16 gates) have been used as a tool for monitoring electro-chemical signals from neuronal cultures (Offenhäusser *et al.*, 1997) and CM (Sprössler *et al.*, 1998, 1999). However, a quantitative use of the FET array as a tool for pharmacological measurements has not been fully validated. In the present study, FETs are used to detect the changes of myocyte responses in the presence of a number of well-known cardioactive agents: Isoproterenol (ISO, a ß-adrenoceptor agonist, stimulates G-proteins and enhances L-type calcium channel activity); verapamil (VP, L-type calcium channel antagonist) and carbamylcholine (CARB, receptor-coupled, reduces cAMP, inhibits Ca^{2+} current and opens K^+channels). We aim to demonstrate that FET array maybe useful for future long-term pharmacological bioassays.

Dissociated CM were cultured from 8-10 embryonic Sprague Dawley rats (gestation day 15 to 18) using the method previously described (Denyer *et al.*, 1998). The resulting cell suspension was counted and approx. $1\text{-}2\times10^4$ cells were plated onto each encapsulated FET array. The cells were allowed to adhere to the surface of the FETs for 4 –5 hours and then the encapsulated devices (volume=300µl) were flooded with fresh 5% medium. A simple drug-cycle regime was employed. Basal beat frequency, beats/min (bpm), of the cells on each device was recorded for 60s prior to drug administration (Control, C). ISO (0.1µM) or VP (10µM) or CARB (10µM) was added and remained in contact with the cell layer for 60s (Test, T). The device was then washed with pre-warmed fresh external solution 5 times at 1min intervals, re-equilibrated for a further 5min and then another 60s control recording was taken to ensure complete recovery. Vehicle has no effect.

All readings were taken at 33-35°C in the dark (FET is light sensitive). The stock solutions of ISO was made up in 1/100N HCl, VP and CARB in distilled water. All subsequent dilutions were made in external recording solution containing (in mM): KCl, 5; NaCl, 150; $MgCl_2$, 1; HEPES, 10; $CaCl_2$, 2.5; glucose, 10. The results are expressed as beat frequency (C *vs* T), bpm ± s.e.m. The differences were analysed using Student's unpaired t-test.

The beat frequency of CM appeared to stabilise after 1-2min on the heated platform with beat frequency 50.5±7.8 bpm (n=61). ISO caused significant increased in beat frequency (29±3 bpm, n=24, *vs* 215±6 bpm, n=13, P<0.001) with no signifcant change in membrane Na^+ current. Although VP did not cause a reduction in beat frequency, it completely diminished the Ca^{2+} current and reduced the Na^+ current by 25% (n=4). CARB caused 95% reduction of beat frequency (214±12 bpm, n=7, *vs* 11±7 bpm, n=7, P<0.001).

The results in the present study demonstrate the detection of responses of some well-known agents by the FET array for a long period of time. One single culture could yield enough cells to prepare between 50 to 100 encapsulated devices with potentials to perform multi-channels and multi-FETs recordings simultaneously. The experiment itself is straightforward and will be particularly advantageous when only a small quantity of drug is available or tissue availability is limited. More refinements are currently being considered in terms of the operating systems, FET fabrication and drug delivery. Nonetheless, the current FET-based bioassay system shows definite promise for drug testing on variety of cells in the near future.

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218P MONOCLONAL ANTIBODY INJECTION WITHOUT A NEEDLE

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Monoclonal antibodies as a class of drugs are gaining in commercial significance. The requirement to deliver antibodies using a needle is often perceived as limiting their applicability. Intraject is a novel needlefree injection device, developed by Weston Medical, which is prefilled and disposable, utilising a compact, high-pressure gas source to deliver liquid drug subcutaneously. Administration is simple and painfree. We set out to demonstrate that it is possible to successfully deliver a monoclonal antibody, CAT-192 with Intraject. CAT–192 is a fully human monoclonal antibody, which neutralises transforming growth factor-beta-1 ($TGF\beta_1$) and is in clinical development as an inhibitor of fibrotic disease.

Delivery *in vitro* was investigated by passing CAT-192 through a needle or Intraject, and analysing the product by SDS-PAGE, SE-HPLC, IEF, absorbance and $TGF\beta_1$ binding. Intraject injected CAT-192 was indistinguishable from material that had been passed through a needle.

Delivery *in vivo* was investigated by administration of CAT-192 in the pig (*Sus scrofa*). The animals used were male, 8-10 weeks old and weighed 23-28 kg at the start of the study. 3 animals were dosed with 0.5 ml of 63 mg/ml CAT-192 (in PBS) via Intraject and 3 animals subcutaneously using a conventional 21G 1.5" needle and syringe.

In all cases the dose was administered at a site on the inner aspect of the hind leg. Plasma samples were taken over 15 days. There was no reaction at the site of injection in any animal. The CAT-192 plasma concentrations were determined by the ability to bind to $TGF\beta_1$ in an ELISA. The results of these assays are summarised in Fig 1.

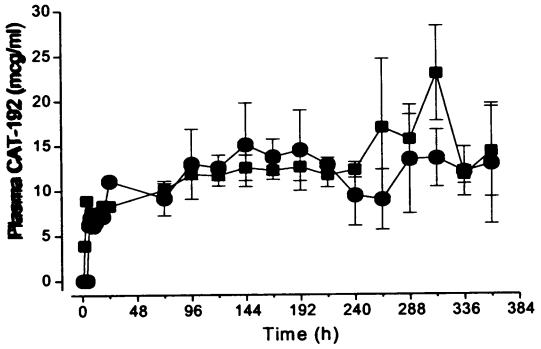

Figure 1. Determination of plasma concentrations of CAT-192 with Intraject (●) and needle and syringe (■). Values are mean and s.e.mean.

The CAT-192 delivered by Intraject produced a similar pharmacokinetic profile to subcutaneous injection with a needle. Importantly the assays indicate that the Intraject delivered product was intact and functional.
This work demonstrates that monoclonal antibodies can be delivered by the Intraject needlefree injection device without compromising their integrity or activity.

219P CONTINUOUS MONITORING OF CHANGES IN BRAIN EXTRACELLULAR LACTATE USING MICRODIALYSIS COUPLED TO ENZYME-AMPEROMETRIC ANALYSIS

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Lactic acid accumulation in the brain parenchyma is a reliable index of increased neuronal activity, because energy demand is tightly coupled to neuronal activity, and oxygen is the limiting substrate for aerobic metabolism under normal glycaemia. We have developed and assessed a novel method for the continuous monitoring of changes in extracellular lactate, in individual brain structures of laboratory animals.

Our strategy was to combine microdialysis with flow analysis of dialysate lactate by enzyme-amperometry (biosensor). The flow-cell previously described for glutamate detection (Zilkha *et al.*, 1994) was adapted to measure much larger concentrations of substrate, as extracellular levels of lactate are around 1000-fold those of glutamate. Substrate selectivity was provided by lactate oxidase, immobilised with glutaraldehyde on surfaces adjacent to the electrode system. A film of 1,2-diaminobenzene on the working electrode eliminated interference from endogenous electroactive compounds (e.g. ascorbic acid).

In vitro assessment of the lactate biosensor showed appropriate sensitivity (1.3 ± 0.3 nA/μM lactate, mean ± s.d., n =10) and fast time response (around 30 s for 90 % of maximum response). At 1 μl/min flow rate, and with solutions equilibrated with air, the maximum concentration of lactate measured without oxygen-limitation was 200 μM (corresponding current, 260 nA). The biosensor showed good stability (usable for up to 14 days), providing solutions were filtered through 0.2 μm micropore filter to reduce the risk of contamination of the measuring system with microorganisms.

In vivo assessment of the method was performed with male Sprague-Dawley rats (280-320 g) anaesthetised with halothane (1.5-2 %) in N_2O:O_2 (2:1). Microdialysis probes (1-mm fiber length), incorporating an electrode for extracellular direct current (d.c.) potential recording, were implanted in the frontoparietal cortex, and perfused for 2 min with increasing concentrations of *N*-methyl-D-aspartate (NMDA, 50-400 μM) or quinolinic acid (QUIN, 1-3 mM). NMDA and QUIN produced dose-dependent, biphasic increases in dialysate lactate, with most of the increase occurring subsequent to repolarisation (Fig. 1), as previously observed with K^+-induced depolarisation (Taylor *et al.*, 1994).

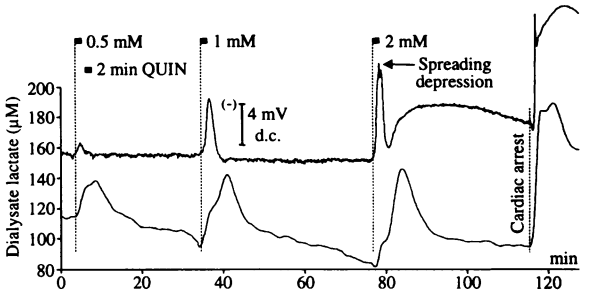

Figure 1. Changes in d.c. potential and dialysate lactate associated with perfusion of QUIN and terminal ischaemia.

Suitable sensitivity, high time resolution, and immediate availability of the results, make this novel method especially appropriate for the study of metabolic changes associated with increased neuronal activation (e.g. drug-induced seizures).

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220P SYSTEMIC CORTISOL RESPONSE TO *HELICOBACTER PYLORI* VACUOLATING TOXIN IN IDIOPATHIC PARKINSONISM AND CONTROLS

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Idiopathic parkinsonism is associated with evidence of a local, and systemic, immune/inflammatory process (Dobbs *et al.*, 2000a), prodromal peptic ulcer and seropositivity for IgG antibodies against *Helicobacter pylori* urease, and the virulence markers, vac A (vacuolating toxin) and cagA (product of the cytotoxicity-associated gene) (Dobbs *et al.*, 2000b, Oxlade *et al.*, 2000). These antigens may drive the elevated serum cortisol (Charlett *et al.*, 1998), in parkinsonism. We contrast the relationship of serum cortisol concentration to *H. pylori* antibodies, between 115 subjects with idiopathic parkinsonism (up to 12 men, 12 women in each decade from age 40-89 years) and 184 without (aged 30-89 years), in an analysis of covariance. Unfitness, chronic or intercurrent illness, or past treatment for *H. pylori* led to exclusion.

Serum samples were stratified for age, gender and subject group, the same proportion from each stratum being included in every assay. Within assay, they were randomly ordered. Cortisol was measured by solid-phase radioimmunoassay, using ^{125}I-labelled cortisol (Cort-A-Count, Diagnostic Products Corporation, Los Angeles). The between assay coefficient of variation, for samples assayed in duplicate, was 5.9, 4.8 & 2.9%, at concentrations of 77, 339 & 1086 nmol.l^{-1}. Enzyme-linked immunosorbent assay measured IgG antibody against high molecular weight, cell bound *H pylori* urease (SIA *Helicobacter pylori* (HM-CAP), Sigma-Aldrich Ltd, Poole). A calibration curve converts absorbence to an "ELISA value" (EV). The recommended cut-point for seropositivity is EV >2.2: the proportion (95% C.I.) of true positive external quality control sera correctly identified (sensitivity) was 0.95 (0.75, 0.99), that of true negatives (specificity) 1.00 (0.83, 1.00). Antibodies against vacA and cagA were detected by Western Blotting (RIDA Blot *Helicobacter* IgG, Quadratech Diagnostics Ltd, Epsom). Antigens of strain ATCC 60 190 had been separated, electrophoretically, on a nitrocellulose membrane, by molecular weight. Specific antibodies bind to corresponding protein bands. Membrane strips, exposed to test and control sera, are compared with a control strip. For analysis of covariance, cortisol values were $\log_e$ transformed, to ensure normality and equality of residual variance, and adjusted for gender and sampling time (Charlett *et. al.*, 1998).

Any effect of urease, vacA and cagA seropositivity on cortisol concentration was independent of the presence of parkinsonism ($P \geq$ 0.5 for disease status. antibody status interactions). Overall, vacA seropositivity (25% of subjects) was associated with a 15.5 (95% C.I.: 2.1, 30.6)% elevation of cortisol (P=0.02), parkinsonism with an additional 18.3 (8.3, 29.2)% (P<0.001). Neither urease (44%) nor cagA (31%) seropositivity added to the variance explained.

Parkinsonism is an insidious disease, which, like *H. pylori* infection, may originate in childhood. A causal insult may not, invariably, operate throughout its course: hence the importance of potential markers of activity. Relationships of elevated cortisol to vacA seropositivity and parkinsonism could be independent. Alternatively, they may depend on there being, in parkinsonism:- (1) host predisposition to a systemic response to vacA, (2) association with particular vacA allele(s), (3) a bacterial promoter of the response to vacA.

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221P ENHANCED PROLIFERATION AND MIGRATION OF PRIMARY HUMAN DERMAL FIBROBLASTS INDUCED BY VARIOUS GROWTH FACTORS: IMPLICATIONS FOR DERMAL REPAIR

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During wound repair, a key process is one of cellular migration both into and over the wound bed. This is a complex process subject to precise control by a number of guidance cues (Britland *et al*, 1996). One such cue is the presence of chemotaxic stimuli such as growth factors. Growth factors are polypeptide molecules who through interaction with specific receptors on the cell surface regulate the recruitment of cells whose functions are believed necessary to ensure a satisfactory wound response (Moulin, 1995; Steed, 1997). This study examines the effect of acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF) on the proliferation and migration of primary human dermal fibroblasts (HDF).

HDF from passage 1-5 were plated out at a density of 1 x 10^4 cells/cm^2 using Hams F10 nutrient medium containing 1.2g/L $NaHCO_3$, 5% foetal bovine serum, 100umits/ml penicillin and 100µg/ml streptomycin. After 24 hours cell counts were performed and media was replaced with growth media containing aFGF (10ng/ml), EGF (10ng/ml), PDGF (5ng/ml) and HGF (5ng.ml). Control was growth medium without presence of additional growth factors other than those present in the serum supplement. Cell counts were made on days 1, 3, 6, 9, 11, 14 and expressed as a percentage of the initial cell count. For analysis of cell motility, cells were viewed using a CCD digital camera attached to a phase contrast microscope. Cultures were filmed for 24-72 hours at a rate of 8 frames per hour, during which time temperature of the medium was maintained at 37°C by a temperature control unit. Time-lapse films were converted into a series of still images and movement of cells was plotted using a macro developed for Scion Image. Co-ordinates of cell movement were then entered into a macro in Microsoft Excel which calculated arithmetically various aspects of cell behaviour such as velocity, persistence and total distance travelled.

Proliferation of HDF was found to be accelerated by aFGF, EGF and PDGF, this effect was marked with aFGF which induced more than a 10-fold increase [1200.8 ± 9.7 (mean ± s.e.mean); n=6) in cell numbers over 14 days, compared to only a 5 -fold increase (530.3 ± 9.4; n=6) observed in control cultures. Rate of cell migration was accelerated by aFGF, EGF and PDGF with mean velocities (µm/hour) of 4.6 ± 0.3 (n=15), 4.4 ± 0.4 (n=14) and 4.1 ± 0.6 (n=12) respectively compared to velocity in control cultures of 2.9 ± 0.2 (n=10). HGF did not demonstrate significant effects on either proliferation or growth of cells.

The results in the present study demonstrate the ability of aFGF, EGF and PDGF to enhance the proliferation and migration of HDF. *In vitro* studies investigating the role of growth factors on cell behaviour will provide important information on how proliferation and migratory behaviour may be potentiated. Further studies in our laboratory will investigate the effects of these and other growth factors on behaviour of other cells involved in the wound response such as keratinocytes.

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Moulin, V. (1995). *Eur. J. Cell Biol.* 68: 1-7

Steed, D.L. (1997). *Surg. Clin. North Am.* 77 (3): 575-586

222P GLUTAMATE RECEPTORS AND SYNAPTIC PLASTICITY

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The discovery of multiple glutamate receptor subtypes and the development of selective antagonists for these receptors has enabled major insights into brain function in health and disease. We have been interested in the roles of glutamate receptors in synaptic transmission and plasticity in the hippocampus; in particular in the processes of long-term potentiation (LTP). This model for studying synaptic plasticity is providing insights into plastic changes that occur in the brain during learning and memory and various pathological states, including epilepsy, excitotoxicity and recovery from neuronal injury. Here, I shall summarise the current state of knowledge of LTP mechanisms in the hippocampus.

Most studies have focussed on the CA1 region of the rat or mouse hippocampus. Here, LTP is induced via the transient activation of the NMDA subtype of glutamate receptor and is expressed as a persistent increase in the efficiency of synaptic transmission mediated via both the AMPA and NMDA receptor subtypes. Activation of NMDA receptors results in a highly localised Ca^{2+} transient and this is believed to result in the activation of kinases, in particular CaMKII. With respect to LTP of AMPA receptor-mediated synaptic transmission, there is evidence that CaMKII can directly phosphorylate AMPA receptors (on the GluR1 subunit) and this leads to an increase in the amount of current that permeates AMPA receptors in unit time. In addition, activation of CaMKII may cause the rapid insertion of new AMPA receptors to increase the physical number of receptors at synapses.

In addition to NMDA receptors, activation of metabotropic glutamate (mGlu) receptors is also involved in the induction of LTP at CA1 synapses. However, their role is more complex and not fully understood. One role has been found for mGlu5 receptors that, via activation of CaMKII and PKC, induce a form of metaplasticity, which negates further requirement for the synaptic activation of mGlu receptors – a so-called "molecular switch".

The mossy fibre pathway innervating CA3 neurons is unusual in that NMDA receptors are not involved in the induction of LTP at these synapses. Instead, recent evidence suggests that kainate receptors may act as the trigger. In this pathway PKA seems to be the major signalling molecule involved. Again there is also evidence for a role for mGlu receptors.

Supported by the MRC