71P CHARACTERISATION OF P2 PURINOCEPTORS MEDIATING INCREASES IN INTRACELLULAR FREE CALCIUM LEVELS IN HL60 CELLS USING FLIPR (FLUORESECENCE IMAGING PLATE READER)

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Extracellular nucleotides regulate a broad range of physiological responses through cell surface P2 receptors (P2X and P2Y subtypes). The activation of some P2Y receptor subtypes, such as $P2Y_2$, leads to the elevation of intracellular free calcium ($[Ca^{2+}]_i$) levels. For example, nucleotide-stimulated increases in $[Ca^{2+}]_i$ in the HL60 cell (human promyelocytic leukaemic cell line) is mediated by $P2Y_2$ receptors as inferred by the agonist potency order using traditional spectrofluorimetric methods (Cowen *et al.*, 1989). The aim of this study was to characterise the P2 receptor mediating intracellular Ca^{2+} release and Ca^{2+} influx using a Ca^{2+} re-addition protocol to dissociate these two components of the $[Ca^{2+}]_i$ increase.

Changes in $[Ca^{2+}]_i$ were measured at room temperature (RT) in Fluo-4 (Molecular Probes) loaded HL60 cells (ATCC, $1.5x10^5$ per well) in 96 well plates using FLIPR (Molecular Devices). Hanks buffered salt solution (HBSS) containing 1mM $CaCl_2$ and 1mM $MgCl_2$ was used in preliminary experiments characterising the P2 receptors mediating increases in $[Ca^{2+}]_i$. In Ca^{2+} re-addition experiments cells were stimulated with UTP or thapsigargin (inhibitor of endoplasmic reticulum Ca^{2+}-ATPase) in HBSS containing 0.07mM $CaCl_2$ and 1mM $MgCl_2$. When the $[Ca^{2+}]_i$ had returned to basal levels, 1mM extracellular $CaCl_2$ ($[CaCl_2]_{ex}$) was added to enable quantitation of Ca^{2+} influx. Cells were pre-treated with suramin (P2 receptor antagonist, Sigma) or Ca^{2+} channel blockers (Sigma) for 10 min or 2 min respectively (RT) prior to stimulation.

ATP, UTP, ADP and UDP stimulated increases in $[Ca^{2+}]_i$ (measured as peak increase in $[Ca^{2+}]_i$) in the presence of 1mM $[CaCl_2]_{ex}$. EC_{50} values (mean±s.e.m.) of the non-cumulative concentration response curves (CRC) were 0.09±0.03 μM, 0.06±0.02 μM, 3.80±0.78 μM and 5.30±1.15 μM respectively (n=3 for each). The potency order of ATP = UTP > ADP = UDP is consistent with a $P2Y_2$ receptor.

UTP concentration-dependently stimulated intracellular Ca^{2+} release (peak increase $[Ca^{2+}]_i$) in the presence of 0.07mM $[CaCl_2]_{ex}$ with an EC_{50} value of 0.04±0.01μM (n=4), similar to the EC_{50} value obtained in the presence of 1mM $[CaCl_2]_{ex}$. The EC_{50} value for UTP-stimulated Ca^{2+} influx (measured as area under the curve post re-addition of 1mM $[CaCl_2]_{ex}$) was higher, 0.36±0.09 μM (n=6). Pre-treatment of HL60 cells with suramin caused concentration-related rightward shifts of the UTP CRCs for both intracellular Ca^{2+} release and Ca^{2+} influx consistent with its pA_2 value of 4.3 (Charlton *et al.*, 1996) i.e. a 4.5±1.1 fold (100 μM suramin, n=4) and 15.4±5.2 fold (300 μM suramin, n=4) shift of the UTP CRC for intracellular Ca^{2+} release, and a 5.0±0.4 fold (100 μM suramin, n=5) and 17.1±2.4 fold (300 μM suramin, n=5) shift of the UTP Ca^{2+} influx CRC. Suramin (300 μM) did not inhibit thapsigargin (1μM)-stimulated intracellular Ca^{2+} release or Ca^{2+} influx. In addition, UTP (10μM)-stimulated Ca^{2+} influx was not inhibited the selective L-type voltage-operated Ca^{2+} (L-VOC) channel blocker nifedipine (20.0±5.5%, 30 μM, n=3, $P>0.05$, unpaired Students t-test) but was inhibited by the Ca^{2+} influx blocker SK&F 96365 (82.1±3.1%, 10 μM, n=3, $P<0.0001$) which blocks receptor-operated and voltage-operated Ca^{2+} channels.

These data suggest that higher concentrations of UTP acting at a $P2Y_2$ receptor are required to stimulate Ca^{2+} influx than intracellular release Ca^{2+} in HL60 cells and that the Ca^{2+} influx is mediated via receptor-operated and not L-type VOC channels.

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72P EFFECT OF A_{2A} ADENOSINE RECEPTOR KNOCKOUT ON RELAXANT EFFECTS OF ADENOSINE AND ANALOGUES IN MOUSE ISOLATED AORTA.

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Adenosine A_{2A} receptors are regarded as playing a key role in vasodilation in many tissues and A_{2A} receptor knock-out mice have been produced which are hypertensive, suggesting a tonic role of A_{2A} receptors in the regulation of blood pressure (Ledent *et al.*, 1997). We compared responses to adenosine and its analogues in isolated blood vessels from wild-type (+/+) and A_{2A} receptor knock-out (-/-) mice to investigate the role of peripheral A_{2A} receptors in the control of vascular tone. Data obtained in aortic rings from rat and hamster have shown that both adenosine A_2 receptors and an antagonist-resistant site mediate relaxations to adenosine and its analogues (Prentice & Hourani, 1996; Prentice & Hourani, 2000).

In the present study, we therefore looked at the effects of adenosine and analogues on murine aortic rings from adult male CD1 mice (30-35g) set up in organ baths containing Krebs solution gassed with $95\%O_2/5\%CO_2$ and maintained at 37°C. Tissues were precontracted with 0.1μM phenylephrine prior to construction of cumulative relaxant agonist curves and, where the effects of the non-selective antagonist 8-sulphophenyltheophylline (8-SPT) were investigated, an incubation period of 60 minutes before construction of agonist curves was used. In tissues from both +/+ and -/- mice adenosine, CGS21680 (2-*p*-(2-carboxyethyl)phenyl-ethyl-amino-5'-*N*-ethylcarboxamidoadenosine) a potent and selective A_{2A} receptor agonist and NECA (5'-*N*-ethylcarboxamidoadenosine) a non-selective agonist only elicited relaxations at concentrations of 30μM and above and these were not blocked by 8-SPT (100μM).

These data suggest the absence of A_{2A} receptors in tissues from both -/- and +/+ mice. Since there are apparently no A_{2A} receptor mediated responses in aortic preparations from the +/+ animals we confirmed functional knock-out of A_{2A} receptors in their genetically modified litter mates in experiments using the vas deferens preparation (Hourani *et al.*, 2001). Interestingly, R-PIA, (N^6-*R*-phenylisopropyladenosine) which is normally considered as an A_1 receptor selective agonist, elicited relaxations in both +/+ and -/- ($p[A]_{50}$ +/+ = 4.28 ± 0.12, -/- = 4.35±0.05, n=3&4) but again these were not blocked by 100μM 8-SPT ($p[A]_{50}$ + 8-SPT +/+ = 4.08±0.06, -/- = 4.40 ± 0.08, n=3, $p>0.05$) suggesting activation of an antagonist resistant site distinct from the currently cloned receptors.

These findings are in agreement with data obtained in aortic rings from normal CD1 mice in which relaxant responses to adenosine and analogues are also mediated by an antagonist resistant site (Prentice *et al.*, 2001). There were no significant differences between responses to analogues in +/+ and -/- tissues suggesting no compensatory changes in other adenosine receptor subtypes in the A_{2A} receptor knock-outs, which is perhaps not surprising considering the apparent lack of A_{2A} receptors in the murine aorta.

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73P EFFECT OF A_{2A} ADENOSINE RECEPTOR KNOCK-OUT ON INHIBITION BY NECA AND CGS21680 OF KCl-INDUCED CONTRACTIONS IN MURINE VAS DEFERENS

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Adenosine receptors on the vas deferens from species such as the rat have been characterised and the presence of post-junctional A_1 and A_2 receptors established which enhance and inhibit smooth muscle contraction respectively (Hourani *et al.*, 1993; Hourani & Jones, 1994). However, post-junctional receptor expression in vas deferens is species dependent and in guinea pig and rabbit only excitatory A_1 and inhibitory A_2 receptors respectively have been shown to modulate contractions (Smith *et al.*, 1999). As yet the presence of post-junctional adenosine receptors has not been established in murine vas deferens. Therefore we have used vas deferens from wild-type (+/+) and A_{2A} receptor knockout (-/-) mice to investigate the involvement of A_{2A} receptors in the inhibition of KCl induced contractions.

Vas deferens were removed from adult CD-1 mice (30-35g) and mounted in organ baths containing Krebs solution gassed with 95%O_2/5%CO_2 and maintained at 37°C. Contractions were elicited by 50mM KCl and the effects on these contractions of increasing concentrations of NECA (5'-*N*-ethylcarboxamido-adenosine) a non-selective agonist and CGS21680 (2-*p*-(2-carboxyethyl)phenylethylamino-5'-*N*-ethylcarboxamidoadenosine) an A_{2A}-selective agonist were investigated by administering these compounds 1 min prior to KCl addition. Curves were constructed non-cumulatively and 15 minutes allowed between each dose.

In antagonist studies, 8-SPT (8-sulphophenyltheophylline) was incubated for 30 minutes prior to administration of each agonist dose.

In tissues from +/+ animals both NECA and CGS21680 caused dose-dependent inhibition of KCl-induced contraction. In the tissues from -/- mice the responses to NECA were markedly reduced and those to CGS21680 completely abolished except for those elicited at 100μM. This residual response to both NECA and CGS21680 was completely abolished by 100μM 8-SPT (% reduction in KCl contraction at 100μM NECA + & - 8-SPT = 36.0±6.9 & 3.8±11.8 and 100μM CGS21680 + & - 8-SPT= 30.4±6.0 & -0.6±8.5; $p<0.05$).

The results suggest that inhibitory responses to both NECA and CGS21680 are almost entirely mediated by post-junctional A_{2A} receptors since these responses are largely absent in tissues from -/- mice. However, at the highest concentration used (100μM), both agonists appeared to activate a receptor which was susceptible to blockade by 8-SPT and is therefore likely to be an A_{2B} receptor. The incomplete abolition of responses to NECA in tissues from -/- mice at concentrations below 100μM may suggest that a component of the inhibitory effect is also mediated by A_{2B} receptors in the mouse vas deferens.

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74P A COMPARATIVE STUDY OF P2 RECEPTOR EFFECTS ON SMOOTH MUSCLE TONE IN THE PORCINE ISOLATED COMMON DIGITAL ARTERY AND PALMAR LATERAL VEIN

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Receptors for purine and pyrimidine nucleotides are expressed in most blood vessels and are involved in the modulation of vascular tone (Ralevic & Burnstock, 1988), but it is unclear whether there is a specific relationship in expression of P2 receptors in arteries and veins in the same vascular bed. In the present study, therefore, we have examined responses to purine and pyrimidine nucleotides in the porcine isolated common digital artery (PCDA) and palmar lateral vein (PPLV).

Segments of PCDA and PPLV (dissected from trotters transported on ice from the abatoir) from pigs (either sex) were mounted for isometric tension recordings, as described previously (Alexander *et al.*, 2000). Tissue viability was assessed by eliciting contractions in the presence of 60 mM KCl. Non-cumulative concentration-response curves were generated to agonists (40 min intervals), each in a separate tissue. The thromboxane A_2 analogue U46619 (11α,9α-epoxymethano-PGH_2) was used (up to 100 nM) to elicit a contraction of about 60% of that to KCl. Thereafter, concentration-relaxation curves were constructed in the presence of increasing cumulative concentrations of agonists. Data reported are means ± s.e.m. of results from at least five experiments. All data were compared by ANOVA.

Nucleotides elicited concentration-dependent contraction of the PCDA, but failed to reach an asymptote over the concentration ranges employed. By comparing concentrations which evoked a contraction to 50 % of the KCl-induced response (pC_{50}), a rank order of potency could be established of α,β-methylene ATP (α,β-meATP, 5.87 ± 0.10) > ATP (3.13 ± 0.09). UTP and UDP failed to evoke a significant contraction at concentrations up to 3 mM. Nucleotides evoked contractions in the PPLV also, in which tissue responses to α,β-ATP and ATP achieved saturation with maximal responses not significantly different from the KCl response (allowing calculations of pEC_{50} values) and UTP evoked a signify-cant response. A rank order of potency was, therefore: α,β-meATP (6.33 ± 0.14) > ATP (3.91 ± 0.22) > UTP (pC_{50} 3.61 ± 0.33) > UDP (no significant response). In U46619 preconstricted preparations, ATP and UTP failed to elicit a significant relaxatory response.

Prior exposure to α,β-meATP (100 μM) produced desensitisation and abolished subsequent contractions to ATP (3 mM) in the PCDA, while a significant ATP-evoked contraction (54 ± 7 % KCl response) was measured in the PPLV. Contractions to α,β-meATP and ATP were significantly attenuated by the P2 antagonist pyridoxalphosphate 6-azophenyl 2,4-disulphonic acid (10 μM) in both arterial and venous segments (F values 36.75-327.12, $P<0.001$).

In conclusion, these data show that $P2X_1$-like receptors, sensitive to α,β-meATP, mediate contraction in both the PCDA and PPLV. In addition, α,β-meATP-insensitive P2Y-like receptors mediate contraction in the PPLV, but not in the PCDA. Purine or pyrimidine nucleotides failed to evoke a relaxation. Thus, these data show a novel spectrum of P2 receptors in blood vessels of the pig trotter, with a heterogeneous expression of the receptors between artery and vein.

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75P DOES ENDOGENOUS SUBSTANCE P MODULATE BAROREFLEX GAIN IN THE RAT?

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Baroreceptor afferent stimulation releases substance P (SP) in the nucleus tractus solitarius (Morilak *et al.*, 1988) and SP and SP-receptor antagonists applied to this area of the rat brain stem cause changes in blood pressure (see Seagard *et al.*, 2000). However, there is no direct evidence whether endogenous SP participates in baroreflex regulation of blood pressure in the rat. We have investigated this by pharmacologically targeting three separate steps in the SP signalling pathway during vasoactive drug-induced manipulation of baroreceptor activity.

Male Wistar rats (300-400 g, n = 22) were anaesthetised with urethane (1.25 $g.kg^{-1}$ i.p.). Blood pressure was recorded from the right femoral artery and the right internal jugular vein was cannulated for infusion of drugs. Baroreflex gain was assessed in terms of heart rate change per mm Hg change in blood pressure during stepwise elevations of pressure by 25-100 mm Hg with phenylephrine (25-200 $nmoles.min^{-1}$ i.v.) and stepwise reductions in pressure by 25-80 mm Hg with sodium nitroprusside (7-60 $nmoles.min^{-1}$ i.v.). Statistical significance of differences ($P < 0.05$) was calculated using Welch's *t*-test.

Resting mean blood pressure and heart rate were 108 ± 5 mm Hg and 387 ± 7 bpm respectively (means ± S.E.M.). Baroreflex gain was similar in response to both elevated and reduced blood pressures and the pooled mean value was –0.49 ± 0.04 $bpm.mmHg^{-1}$. As the actions of neurally released SP appear to be mediated in part through nitric oxide (Hughes *et al.*, 1990), we inhibited synthesis of endogenous nitric oxide using N^G-nitro-L-arginine methyl ester (30 $mg.kg^{-1}$ i.v., n = 7). This produced a significant increase in baroreflex gain from –0.37 ± 0.06 $bpm.mmHg^{-1}$ to –0.86 ± 0.20 $bpm.mmHg^{-1}$ ($P<0.03$). We next assessed involvement of intracerebral neurokinin-1 (NK-1) receptors in the baroreflex by administration of the NK-1 antagonist SR 140333 ((S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2]octane chloride; 360 $nmoles.kg^{-1}$ i.v., n = 8) and tested the effect of the capsaicin analogue resiniferatoxin (4 doses of 0.3 $ug.kg^{-1}$ i.v. at 4 min intervals, n = 7). Neither treatment caused any change in baroreflex gain ($P>0.1$).

Our findings do not support the view that endogenous SP normally modulates the arterial baroreflex in the rat. They are however consistent with the possibility that endogenous nitric oxide acts as an inhibitory modulator of this reflex, independent of SP.

We thank Dr J.E. Brelière (Sanofi) for SR 140333.

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76P DOES ENDOGENOUS SUBSTANCE P MODULATE BAROREFLEX GAIN IN THE RAT?

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We assessed the involvement of intracerebral neurokinin-1 (NK-1) receptors in the baroreflex by administration of the NK-1 antagonist SR 140333 (360 $nmoles.kg^{-1}$ i.v., n = 8) and the effect of blocking synaptic SP release by administration of the capsaicin analogue resiniferatoxin (4 doses of 0.3 $ug.kg^{-1}$ i.v. at 4 min intervals, n = 7). Neither treatment caused any significant change in baroreflex gain ($P > 0.1$). Lastly, as the actions of neurally released SP appear to be mediated in part through nitric oxide (Hughes *et al.*, 1990), we inhibited nitric oxide synthase using N^G-nitro-L-arginine methyl ester (30 $mg.kg^{-1}$ i.v., n = 7). This treatment produced a significant increase in baroreflex gain from –0.37 ± 0.06 $bpm.mm\ Hg^{-1}$ to –0.86 ± 0.20 $bpm.mm\ Hg^{-1}$ ($P < 0.03$).

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77P AN ANIONIC BACKGROUND CONDUCTANCE (I_{AB}) IN GUINEA PIG ISOLATED VENTRICULAR MYOCYTES AND ITS MODULATION BY TEFLUTHRIN

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Recently a novel anionic background conductance (I_{AB}) has been recorded from rat isolated ventricular myocytes and suggested to play a role in modulating ventricular action potential duration (APD; Spencer et al., 2000). The present study was performed in order to determine (*i*) whether I_{AB} exists in guinea pigs, another widely studied species, and (*ii*) whether I_{AB} possesses any interesting pharmacology that may discriminate it from other anionic conductances.

Hearts of male guinea pigs (400-500g) were retrogradely perfused at 37°C and myocytes isolated using a similar enzymatic dispersion procedure to that described by Levi & Issberner, 1996. Myocytes were then used in whole-cell voltage-clamp experiments, under conditions which allowed sole movement of anions, and I_{AB} elicited by membrane potential 'saw-toothed' ramps between –90 mV and +70 mV (ramp rate of 0.32 Vs^{-1}) from a holding potential of –50 mV. Data was analysed using pClamp v. 6.0 (Axon Instruments, Inc.).

I_{AB} was found to be present in every guinea pig myocyte tested (n=20). Experiments, using these cells revealed that I_{AB} was highly permeable to NO_3^- 0.84 ± 0.07pA/pF (n=9, mean ± standard error of the mean; throughout) at +60mV and impermeable to aspartate (0.49 ± 0.001pA/pF at +60mV; n=6). The mean membrane capacitance of cardiac myocytes was 245 ± 10pF (n=20), indicating that the magnitude of I_{AB} was small when compared to the swelling- and cAMP- activated Cl^- conductances present in these cells.

Significantly, the pyrethroid tefluthrin (10 μM), known to modulate the activity of a number of cardiac cationic conductances (Spencer *et al.*, 2001), caused a marked enhancement of I_{AB}. Using NO_3^- as the charge carrier it was found that tefluthrin activation of I_{AB} was independent of membrane voltage. This effect was quantified by fitting exponentials (using the Chebyshev technique) to the difference I_{AB} currents obtained in the absence and presence of the pyrethroid. Such analysis revealed an 81.8 ± 4.3% (n=5) increase in the current density at an example membrane voltage of +60 mV. Unlike other cardiac Cl^- currents (Mulvaney *et al.*, 2000) I_{AB} was found to be insensitive to 50 μM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS; 4.59 ± 0.51% (n=5)).

Here we report for the first time an activator of I_{AB} and demonstrate that this current exists in guinea pig ventricular myocytes. On the basis of these preliminary experiments it appears that I_{AB} in the rat and guinea pig is similar.

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78P HYPOPHOSPHORYLATION OF CYTOSKELETAL AND MYOFIBRILLAR PROTEINS CORRELATES WITH HYPERCONTRACTILITY OF REPERFUSED CARDIOMYOCYTES

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Prolonged ischemia followed by reperfusion increases cardiomyocyte contractility at diastolic Ca^{2+} levels. Protein dephosphorylation is implicated, since the chemical phosphatase 2,3-butanedione monoxime reproduces the injury while protein phosphatase inhibitors confer protection. This study aimed to identify the endogenous phosphatase involved, and its site(s) of action.

Adult rat cardiomyocytes were subjected to *in vitro* ischemia/reperfusion by pelleting under N_2 at 37°C for 1h, then resuspended into normoxic Tyrode solution (15 min at pH 6.4, then 15 min at pH 7.4; 'reperfusion'). Contractility was evaluated as tendency to undergo Ca^{2+}-independent rigor contracture at low MgATP concentration. Finally, myofibrillar and cytoskeletal protein fractions were isolated for western analysis using phosphoserine-specific antibodies.

At 15-100 μM MgATP cells subjected to ischemia/reperfusion underwent rigor more readily than controls (e.g. 82±4% *vs* 47±9% respectively, at 50 μM MgATP; n=5, $p < 0.05$), ruling out enhanced sensitivity of myofilaments to Ca^{2+} as the primary cause of hypercontractility. Rather, cell injury must reflect Ca^{2+}-independent enhancement of myofibrillar activation and/or degraded mechanical resistance of the cytoskeleton. Okadaic acid (OA; 1 nM) abolished this increase in propensity of ischemic/reperfused cells to undergo rigor, implying protection at a dose selective for protein phosphatase PP2A rather than PP1.

By contrast, pharmacological activation of protein kinase families A (8-bromo-cAMP, 2 mM) or C (PMA, 0.1 μM) conferred no protection. Hypercontractility in ischemic/reperfused cells correlated with OA-sensitive serine dephosphorylation of several myofibrillar and cytoskeletal proteins (apparent molecular masses 32, 49, 64, 100, 119 and 137 kD). These have been immunoprecipitated for identification by microsequencing.

We conclude that hypercontractility of reperfused cardiomyocytes is Ca^{2+}-independent and coincides with serine dephosphorylation of specific myofibrillar/cytoskeletal proteins, probably by protein phosphatase 2A.

79P INDUCTION OF HYPERTROPHIC RESPONSIVENESS TO NEUROPEPTIDE Y IS MEDIATED BY THE CARDIOMYOCYTE Y_5 RECEPTOR IN THE SPONTANEOUSLY HYPERTENSIVE RAT

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Increased plasma levels of neuropeptide Y (NPY) occur in hypertension and correlate with severity of left ventricular hypertrophy (LVH) *in vivo* (Hulting *et al.,* 1990). NPY does not initiate protein synthesis in freshly isolated cardiomyocytes *in vitro*, but augments 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). Hypertension is evident in this model at 11 weeks of age (195±11 mmHg, mean ± s.e.m., n=8), while cardiac hypertrophy, which occurs in response to pressure overload, is present at 16 weeks, as evidenced by increased heart: body mass ratio, (0.00555±0.00005 $mg.g^{-1}$, mean ± s.e.m., n=95 v 0.00520±0.00005 $mg.g^{-1}$, mean ± s.e.m., n=86; $p < 0.05$) compared to the age-matched normotensive Wistar Kyoto strain (WKY).

To determine whether NPY-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 male SHRs (8, 12, 16, 20 and 24 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 and antagonists (Millar *et al.,* 1994). Data are given as mean values ± s.e.m, and 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.

NPY (10^{-8} M) increased cellular protein mass after 24 hours in cardiomyocytes from SHRs at 16 weeks by 9.2±2.1% (n=7; $p<0.05$). The peptide did not increase cellular protein mass significantly in SHRs at 20 weeks and was devoid of effect in SHRs at 8, 12 and 24 weeks and in WKY cells at all ages. De *novo* protein synthesis was increased maximally in SHRs at 12, 16 and 20 weeks ($p<0.05$; n=7) in response to NPY by 12.6±2.1% (10^{-6} M), 20.1±4.2% (10^{-8} M) and 9.4±1.8% (10^{-7} M), respectively. No increases were evident in SHR cells at 8 and 24 weeks or in WKY cells at any age.

PYY_{3-36}, which displays selectivity for Y_2 and Y_5 receptors relative to other Y receptor subtypes, increased *de novo* protein synthesis maximally at 10^{-7} M by 16.2±5.1% (n=4; $p<0.05$) in SHRs at 16 weeks. Similarly, the Y_5-selective agonist, D-Trp-34 NPY (Parker *et al.*, 2000), increased *de novo* protein synthesis maximally at 10^{-6}M by 17.8±5.2% (n=7; $p < 0.05$) in cardiomyocytes from SHRs at 16 weeks. LPY ($\leq 10^{-6}$ M), which displays some selectivity for Y_1 and Y_4 receptors over other Y receptor subtypes, did not increase *de novo* protein synthesis in cardiomyocytes from either strain at 16 weeks. The Y_1-selective antagonist, VD-26 (2×10^{-7} M), and the Y_2-selective antagonist, BIIE 0246 (2×10^{-7} M) (Doods *et al.*,1999), did not alter *de novo* protein synthesis *per se* and did not attenuate responses to NPY (10^{-7} M) and PPY_{3-36} (10^{-7} M), or to the phorbol ester, phorbol 12 myristrate-13 acetate (10^{-7} M), employed as a negative control.

In conclusion, these data indicate that hypertrophic responsiveness to neuropeptide Y, as evidenced by increased synthesis of *de novo* protein, is induced transiently in cardiomyocytes from the SHR subsequent to the onset of hypertension. This effect of the peptide is mediated by the neuropeptide Y Y_5 receptor subtype.

This study was funded by the British Heart Foundation (FS:98012)

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80P ANGIOTENSIN II REDUCES INFARCT SIZE IN RAT ISOLATED PERFUSED HEARTS PARTLY VIA STIMULATION OF AT_1 RECEPTORS

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Angiotensin II (AngII) reduces infarct size in isolated perfused rat hearts after ischaemia-reperfusion (I/R) either as a preconditioning stimulus (Liu et al., 1995) or when present throughout I/R (Headley et al., 2000). In the latter study, reperfusion was performed for 30 min suggesting that AngII is protective in the early stage of injury. However, it is not clear if AngII limits infarct size with increased reperfusion time. In this study, we examined the effect of AngII on infarct size and contractile function in rat hearts subjected to 2 h reperfusion. In addition, the AT_1 receptor antagonist valsartan was used in an attempt to characterise the receptor subtype responsible for the infarct limiting action of AngII.

Hearts from male Wistar rats (250-400g) were perfused via the aorta at constant pressure (60mmHg) with Krebs solution (mM: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; Mg_2SO_4, 1.2; $NaHCO_3$, 25) gassed with 95% O_2/ 5% CO_2. Left ventricular developed pressure (LVDP) was measured with a pressurized (5-10 mmHg) balloon inserted into the left ventricle and connected to a pressure transducer. Coronary flow (CF) was measured with a Transonic flow probe on the aortic inflow connected to a T206 flow meter. Mechanical function was recorded during an initial 30 min aerobic perfusion. Hearts were then subjected to 30 min global, normothermic (37°C), no-flow ischaemia followed by 2 h reperfusion. Electrical pacing was applied at 5 Hz during periods of aerobic perfusion. Infarct size (% of the left ventricle) at the end of reperfusion was determined from triphenyltetrazolium chloride staining. Vehicle (n = 7), AngII (10 nM, n = 7) or AngII (10 nM) + valsartan (1 μM, n = 6) were infused for 10 min before ischaemia and throughout reperfusion. Values (mean ± s.e.mean) obtained after 2 h reperfusion were compared among vehicle and AngII groups by analysis of variance with Dunnett's *post hoc* test. Results were taken to be significant if $P < 0.05$.

After 30 min of aerobic perfusion, there were no differences in LVDP or CF between the control and AngII + valsartan groups, the control values being 99±10 mmHg and 15±1 $ml.min^{-1}$, respectively. Prior to ischaemia, addition of AngII significantly decreased LVDP to 88±8 mmHg and CF to 13±1 $ml.min^{-1}$. After 2 h of reperfusion, LVDP and CF in control hearts were reduced by 60 and 79%, respectively, from pre-ischaemic values. Compared with control hearts, neither of the drug-treated groups affected the recovery of contractile function following reperfusion. Infarct size was significantly reduced in those hearts receiving AngII (12±2% LV) when compared to controls (23±3% LV). However, valsartan in combination with AngII, partially reversed this effect returning values to near control levels (18±3% LV).

AngII significantly reduced infarct size in hearts after 2 h reperfusion. Thus, Ang II-mediated limitation of infarct size is retained throughout 2 h of reperfusion and appears to be mediated, in part, via the AT_1 receptor subtype. Although AngII may be acting as a preconditioning stimulus, it is notable that infarct size was limited despite the presence of AngII throughout reperfusion.

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81P ANGIOTENSIN TYPE I RECEPTOR ANTAGONIST LOSARTAN, AT THERAPEUTIC CONCENTRATIONS, BINDS TO THROMBOXANE RECEPTORS IN HUMAN CARDIOVASCULAR AND RENAL TISSUE.

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Angiotensin II is a potent vasoconstrictor peptide mediating its effect via the angiotensin type I (AT_1) receptor (Dietz *et al.*, 1998). Orally administered AT_1 receptor antagonist losartan inhibits angiotensin II vasoconstriction in the human forearm (Baan *et al.*, 1996). However, evidence suggests additional mechanisms may be involved in the anti-hypertensive action of losartan. This is highlighted by the increased release of prostaglandin I_2 in cultured vascular smooth muscle cells by 10nM losartan (Jaiswal *et al.*, 1991) and the inhibition of thromboxane A_2 contraction in canine coronary artery (Li *et al.*, 1997). Therefore, the aim of this study was firstly, to determine using radioligand binding if losartan competes for thromboxane (TP) receptors in human coronary artery. Secondly, using *in-vitro* receptor autoradiography, to establish if 1μM losartan, a concentration within the therapeutic range (Munafo *et al.*, 1992), competes for TP receptors in non-diseased and diseased human cardiovascular and normal renal tissue.

Human cardiac and renal tissues were obtained with local ethical approval. Following optimisation of binding conditions (Katugampola & Davenport., 2000), 30μm cryostat sections of human coronary artery were incubated with the thromboxane receptor agonist [^{125}I]-BOP (0.1nM) (Morinelli et al., 1989) in the presence of 50nM-50μM losartan. Non-specific binding was defined using 1μM SQ29548 (Monshizadegan *et al.*, 1992). For autoradiographical visualisation, sections of human tissue were incubated with 0.1nM [^{125}I]-BOP in the absence or presence of SQ29,548 (1μM) or losartan (0.1μM).

Losartan competed for [^{125}I]-BOP binding with a dissociation constant (K_D) of 4.2 ± 0.22μM (n=4 individuals, mean ± s.e. mean) in non-diseased coronary artery. Autoradiography revealed that 0.1μM losartan, a concentration lower than achieved following oral dosing, competed for TP receptors in cardiac tissue obtained from hearts of patients transplanted for both dilated cardiomyopathy and ischaemic heart disease. Binding was localised to cardiac myocytes.

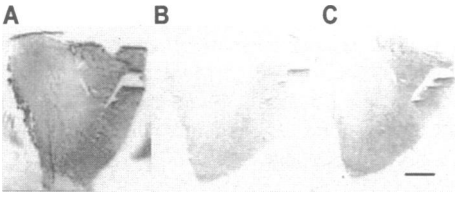

Figure 1. (A) Total binding in the presence of 0.1 nM [^{125}I]-BOP (B) non-specific binding in the presence of 1 μM SQ29,548 (C) losartan competing [^{125}I]-BOP binding site at 0.1 μM. Scale bar = 2 mm.

Losartan also competed for TP receptors in human saphenous vein graft and atherosclerotic coronary artery with binding to both the thinned media and proliferated intimal smooth muscle layers. In human renal tissue, 0.1μM losartan competed for TP receptors in glomeruli and arcuate arteries of the cortex (Figure 1). The ACE inhibitor captopril, the non-selective angiotensin receptor antagonist saralasin, and the angiotensin type II receptor agonist CGP42112 did not compete for [^{125}I]-BOP binding, indicating the unique profile of losartan.

We have demonstrated the dual receptor (AT_1 and TP) binding properties of losartan at therapeutic concentrations to human cardiac blood vessels, renal blood vessels and cardiac muscle. This may contribute to its overall therapeutic profile in the treatment of hypertension and heart failure.

Losartan was a gift from Merck & Co., Inc, New Jersy, U.S.A.

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82P COMPARISON OF THROMBOXANE (TP) AND ANGIOTENSIN (AT_1 & AT_2) RECEPTORS IN HUMAN SAPHENOUS VEIN; DENSITY AND PHENOTYPIC CHANGES ASSOCIATED WITH VEIN GRAFT DISEASE.

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The saphenous vein is widely used as a vessel for bypass graft surgery (Rosenfeldt *et al.*, 1999). However, angiotensin II, thromboxane and endothelin-1, potent vasoconstrictors with smooth muscle cell mitogenic properties, have all been implicated in vein graft spasm and stenosis. Endothelin receptors have been well characterised in normal and diseased saphenous vein (Maguire & Davenport, 1999). Therefore, our aim was firstly to determine the localisation of angiotensin II, AT_1 and AT_2, and thromboxane TP receptors in normal saphenous vein (SV). Secondly, to identify alterations in receptor density and distribution of receptor subtype that may occur with the phenotypic changes of smooth muscle cells (contractile to synthetic) in diseased saphenous vein graft (SVG).

Human normal SV were obtained (with local ethical approval) from 8 patients undergoing coronary artery bypass graft surgery. Samples of occluded SVG were from 10 patients undergoing heart transplantation for ischaemic heart disease. Following optimisation of binding conditions, 30μm cryostat sections of vessels were incubated with the angiotensin receptor antagonist, ^{125}I-(Sar^1, Ile^8) angiotensin II (0.2nM) or the thromboxane TP receptor agonist, [^{125}I]-BOP (0.1nM) for 60 and 30 min respectively for quantitative *in-vitro* receptor autoradiography. The concentration of radioligand selected labelled approximately 33% of the receptors. Developed images were quantified using computer-assisted densitometry (Davenport *et al.*, 1995) with relative receptor density measured in amol mm^{-2}. Receptor densities were compared using unpaired Student's *t*-test (significance value P<0.05). Sections were stained with haematoxylin and eosin to facilitate histological examination.

We identified TP, AT_1 and AT_2 receptors in the medial and intimal smooth muscle layers of both normal SV and SVG (Figure 1A and 1B). However, total TP receptor density was 3-5 fold greater than angiotensin II receptor density in both vessel types. TP receptor density was significantly upregulated in both the media and intima

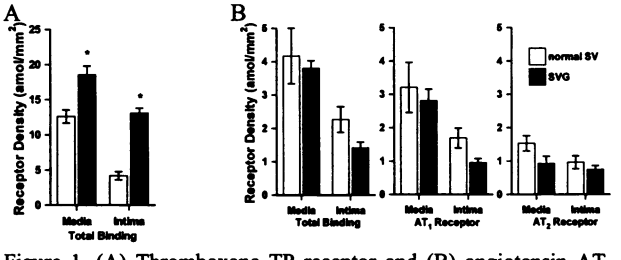

Figure 1. (A) Thromboxane TP receptor and (B) angiotensin AT_1 and AT_2 receptor distribution on the medial and intimal smooth muscle layers of human normal SV and SVG. *P<0.05 compared to normal SV (n=8-10)

of SVG (~3-fold increase in the intimal layer), compared with normal SV (Figure 1A). In SVG, significantly fewer (P<0.05) AT_1receptors were present in the intima (Figure 1B), compared to the media. Whether this decrease in proliferative AT_1 receptors is a consequence of graft age or an adaptive response to circulating levels of angiotensin II in disease, is not known.

Using quantitative receptor autoradiography we have shown, for the first time, the relative densities of TP and angiotensin II receptors in normal SV and SVG obtained from the same sample of patients. Interestingly, TP receptor density was greater than angiotensin II receptor density in both vessels. Additionally the significant increase in TP receptors in SVG suggests the potential of TP receptor antagonist to reduce vein graft occlusion. Furthermore, the differential alteration of TP and angiotensin II receptor subtypes highlights the complexity of the phenotypic changes associated with vein graft disease.

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83P AN INVESTIGATION INTO THROMBOXANE A_2 AND LIPOXIN A_4 SIGNALLING IN PRIMARY HUMAN MESANGIAL CELLS

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Thromboxane (TX) A_2 acts as a mesangial and renal cell vasoconstrictor, leading to a decrease in glomerular filtration rate (Remuzzi *et. al.*, 1992). It is the most abundant eicosanoid synthesised in glomerulonephritis (GN), thereby exacerbating the inflammatory response associated with GN. During the resolution phase of GN, lipoxin (LX) A_4 biosynthesis increases. Through pleiotropic effects on mesangial cells LX can act as 'braking signals' on inflammatory cascades (O'Meara & Brady, 1997). The aim of this study is to characterise TXA_2 and LXA_4 receptor mediated signalling pathways in primary cultures of human mesangial cells (hMC's).

The human thromboxane receptor (TP) exists as two isoforms, TPα and TPβ. We have found expression of both the α and β isoforms of TP in hMC's with 1.5±0.1 (n=3) fold greater levels of TPα mRNA relative to TPβ. Expression of both isoforms was detected by immunofluoresence and shown to occur predominantly in cell membranes. U46619, the stable TXA_2 mimetic, induced calcium mobilisation in hMC's illustrating that the main signalling pathway used by TP is through activation of phospholipase C.

Prostacyclin significantly decreased (from 88.8±5 to 32.71±1; p=0.02; n=3) but did not abolish, U46619 mediated calcium mobilisation. Since prostacyclin desensitises the α but not the β isoform (Walsh *et. al.*, 2000) these data are consistent with the activity of a TP receptor independent of TPα.

Thus far, one form of the lipoxin A_4 receptor (LXR) has been identified. Stimulation of hMC's with LXA_4 neither mobilised calcium nor attenuated U46619 mediated calcium mobilisation. However, in hMC's, LXA_4 inhibits forskolin induced cAMP accumulation, illustrating that the LXR in hMC's most likely couples to $G_{\alpha i}$. Although the primary TXA_2 and LXA_4 signalling cascades differ activation of extracellular signal regulated kinase (ERK) was observed in hMC in response to TXA_2 and LXA_4.

Taken together these data illustrate that in hMC's signalling via the TP receptors is mediated primarily through calcium mobilisation. In contrast, signalling via the LXR does not evoke calcium mobilisation and is coupled to inhibition of cAMP production whereas both TP and LXR couple to ERK activation in hMC's.

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84P INVESTIGATION OF CO-POLYMER FILMS AS DRUG DELIVERY SYSTEMS FOR PREVENTION OF VASCULAR SMOOTH MUSCLE CELL PROLIFERATION

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Restenosis refers to the re-occlusion of a blood vessel following procedures such as percutaneous transluminal coronary angioplasty (PTCA). The cascade of growth factors and cytokines released in response to the initial injury stimulate the inappropriate migration and proliferation of vascular smooth muscle cells into the intima (Schwartz, 1998).

This study explores the possibility of using polymer films to deliver, in a controlled manner, the anti-mitotic agent colchicine, to prevent the proliferation of smooth muscle cells. To this end, we have prepared a series of co-polymers with different hydrophilic/hydrophobic ratios using N-isopropylacrylamide (NIPAA), a hydrophilic non-toxic, biocompatible, thermoresponsive polymer (Piskareva *et al.*, 1999; Gorelov *et al.*, 1997) and N-tertbutylacrylamide (NTBA), a hydrophobic monomer.

Uptake into cells and release from films was measured using [ring C, methoxy-^{3}H-colchicine]. Colchicine (100nmol/film) was incorporated into NIPAA/NTBA co-polymer films (5% w/w polymer/ethanol, 5μm thick) and release into tissue culture medium was measured at 37°C. Proliferation of bovine aortic smooth muscle cells (BASMC, passage numbers 5-11) was assessed by measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation.

Uptake of colchicine by BASMC was linear for up to 8h, reaching saturation at 24h. Levels of drug in the cells reached 1.5 fmol/μg protein (150 fmol/10^5 cells) after 48h exposure to 24μM colchicine.

Exposure of cells to a range of colchicine concentrations (30nM-1μM) reduced proliferation significantly, with 30nM colchicine reducing the level of proliferation to 53.7 ± 5.5% (mean ± S.E.M., n=3, $p<0.05$) of untreated cells. Inclusion of colchicine (1μM) in the medium at the time of seeding reduced adhesion of BASMC to 23.3 ± 0.7% (mean ± S.E.M., n=3, $p<0.05$) of control levels. The extent of colchicine release from co-polymer films was reduced by incorporation of NTBA: amounts released into 1 ml after 48h by 85:15, 65:35 and 50:50 (NIPAA:NTBA) films were 25, 15 and 0.5nmol, respectively. When solutions containing colchicine eluted from co-polymer films were applied to BASMC in culture, significant inhibition of proliferation occurred: solutions containing 30nM colchicine reduced proliferation to 43.5 ± 3.6% of control levels (mean ± S.E.M., n=3, $p<0.05$).

In conclusion, this study has shown that colchicine, incorporated into NIPAA/NTBA copolymer films, when released in a controlled manner, retains its biological activity.

Supported by Enterprise Ireland Grant No. .

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85P INVESTIGATION OF THE ACCESSIBILITY OF A MEMBRANE TRANSLOCATING SEQUENCE GRAFTED ONTO POLYSTYRENE PARTICLES AND BIOAVAILABILITY OF A RADIOLABELLED MTS DRUG CONJUGATE *IN VIVO*

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The unidirectional translocation of thousands of distinct proteins across intracellular membranes is mediated by membrane translocation sequences (MTS). MTSs are recognised by acceptor proteins that aid in routing the pre-protein from the translocation machinery into the membrane of intracellular organelles and consist on average of ~15 amino acid residues (Lindgren et al, 2000). Studies of MTS peptides suggested that the length and properties of flanking peptide sequences (the functional cargoes) do not affect the peptide-transporting activities (Du et al, 1998). Coupling of a specific ligand to vaccines, drugs or particulate systems can be a powerful tool to route compounds to a certain target cell population (Frey et al, 1999).

In this study, the high affinity of biotin for streptavidin was exploited for the linkage between a biotinylated MTS peptide and streptavidin-coated polystyrene particles. The aim of this study was to probe the accessibility of cell membranes using an MTS ligand adsorbed to fluorescent particles (in-vitro) and bioavailability of a radiolabelled MTS drug conjugate in a rat model (in-vivo).

Biotin-labelled MTS ligand (15mer,D form) was adsorbed to 0.3μm streptavidin-coated fluorescent polystyrene particles and binding and/or uptake was investigated in Caco-2 cells grown on coverslips and mouse intestinal loops. Samples were examined using confocal microscopy. Evaluation of systemic bioavailability of a tritium-labelled MTS drug conscious fasted Wistar rats and the resulting plasma samples were analysed using a scintillation counter.

Fluorescent polystyrene particles with adsorbed MTS ligand exhibited higher binding and uptake into Caco-2 cells and in M cells in the mouse loop models compared to control biocytin particles. The ^{3}H MTS drug conjugate showed absolute tritium bioavailability in excess of a 1.5 fold increase compared to administration of a control, in the in-vivo model. Both control and MTS drug conjugates rapidly crossed the gastrointestinal barriers into systemic circulation with the MTS sequence enhancing delivery of the drug as indicated by increased bioavailability.

The peptide probes designed for this study demonstrated the potential use of MTS sequences to target drugs and particulates in the gastrointestinal tract. These signal sequences may prove useful in future vaccine and oral drug delivery targeting programs. Investigation of translocation of the particle batches across polarised Caco-2 cells is ongoing and synthesis of derivatives of the MTS sequence is being investigated.

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86P ANALYTICAL METHOD DEVELOPMENT FOR THE DETERMINATION OF COMPACTED DNA AND PROTAMINE SULPHATE IN LIPOPLEXES.

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Analytical methods were established to determine the concentration of DNA and protamine sulphate (PS) in cationic lipid/DNA complexes (lipoplexes).

Lipoplexes have been successfully used in *in vitro* and *in vivo* gene delivery. These formulations are prepared by pre-stabilisation and compaction of the DNA with PS before addition of the lipids. The use of protamine can reduce the size of the complex and enhances the homogeneity. The determination of DNA and protamine sulphate concentrations is essential for the characterisation and determination of consistency of such formulations.

When bound to protamine sulphate, the DNA is not available to bind with Picogreen® reagent commonly used with nucleotide fluorescent detection. Procedures to decomplex the DNA from protamine sulphate and the subsequent effect on the assay were assessed. Sodium dodecyl sulphate (SDS) and low molecular weight (LMW) heparin were evaluated as decomplexing agents. Calibration curves were constructed using both uncomplexed and complexed DNA with varying ratios of PS:DNA studied. The appropriateness of the Picogreen® Assay was further assessed by carrying out matrix interference studies. In addition, a protein assay using Coomassie Plus® reagent with detection by UV-Vis spectrometry was modified for the determination of the concentration of PS.

The decomplexing of PS:DNA with 1% SDS and 0.1% LMW heparin gave comparable results. The uncomplexed DNA and complexed DNA gave different linear standard curves; complexed DNA giving a lower gradient than uncomplexed DNA with either of the decomplexing agents (Figure 1). However, the use of >3.5%v/v of the decomplexing agent in the final test solution resulted in quenching of the fluorescence signal. The standard curves for 2:1, 1:1 and 0.87:1 PS:DNA were similar in gradient. An increased gradient was seen for calibration curves of 0.6:1 and 0.2:1 PS:DNA.

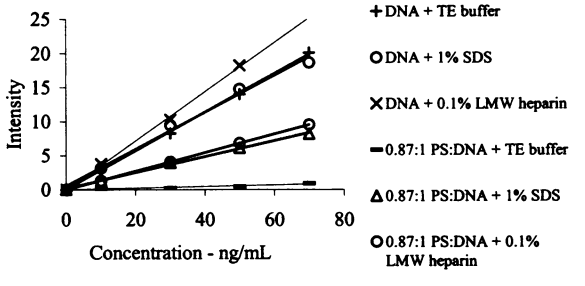

Figure 1. Comparison of the calibration curves of DNA and 0.87:1 PS:DNA in 1% SDS, 0.1% LMW heparin and TE buffer with analysis by the PicoGreen® assay.

Matrix interference studies revealed that at pH<7 low DNA recovery was obtained and at pH>7 recoveries greater than 100% were observed. All investigations suggested that it is optimal to use a standard curve of the same complex ratio and pH when measuring unknown DNA concentrations of a known PS:DNA ratio. The Coomassie Plus® reagent was suitable for protamine sulphate with decomplexation of the PS:DNA complex unnecessary for this assay.

The determination of concentrations of DNA in PS:DNA complexes has been achieved by decomplexation with 1% SDS or 0.1% LMW heparin. Linear calibration curves were achieved, however, it is essential that the calibration standards are prepared in the same matrix and with the same ratio of PS:DNA.

87P ATP INDUCES A BIPHASIC DILATOR RESPONSE IN MESENTERIC VESSELS FROM SPRAGUE DAWLEY AS WELL AS WISTAR RATS

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Previously we have shown that the phosphonucleotide ATP induces a biphasic dilator response in isolated mesenteric vascular bed preparations from Wistar rats (Stanford and Mitchell, 1998). The first phase is transient, well studied in the literature and mediated by nitric oxide and/or prostacyclin. The mechanisms, mediators and/or pathways mediating the subsequent and sustained phase are still the subject of investigation. It is known that biological responses can vary between strains of laboratory rats. Thus, when others were using a Sprague Dawley strain of rat in our group for unrelated projects, we took tissue and compared responses to ATP with those we had previously found using Wistar rats.

Male Wistar or Sprague Dawley rats (250-300g) were anaesthetised with sodium pentobarbitone (100 $mgkg^{-1}$; *i.p.*) and sacrificed by cervical dislocation. The mesenteric artery was cannulated and the mesentery excised. The bed was perfused at a constant rate with Kreb's buffer heated to 37°C and gassed with 95% O_2, 5% CO_2. Test drugs were added to the Krebs buffer. Perfusion pressure, measured by an arterial cannula, was raised to approx. 120 mmHg by methoxamine ($1x10^{-6}M$ – $1.2\ x10^{-5}$). The effects of 1-3µl volume injections of ATP ($1x10^{-7}$ and $3x10^{-7}$ M) were recorded.

The mesenteric blood vessels from either Wistar (1st phase EC_{50} $3.26x10^{-10}M$, 2nd phase EC_{50} $3.54x10^{-8}M$) or Sprague Dawley (1st phase EC_{50} $1.30x10^{-10}M$, 2nd phase EC_{50} $2.72x10^{-7}M$) strains of rat demonstrated the same biphasic response to ATP (Figure 1).

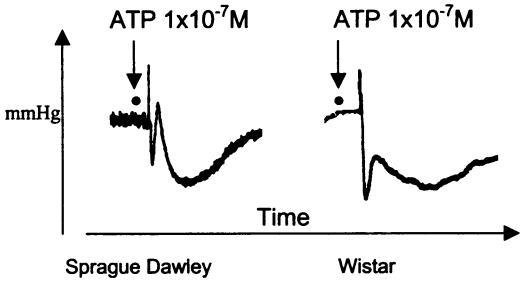

Figure 1. Effect of ATP ($1x10^{-7}$mol) on perfusion pressure of the rat isolated mesenteric artery bed from Sprague Dawley (left) or Wistar (right) rats. Data shown are representative traces and similar results were obtained in tissue from at least 3 other rats.

This data shows that the biphasic vasodilator response induced by ATP is not specific to the Wistar strain and adds weight to the notion that it has importance in vascular physiology.

Stanford S.J & Mitchell J.A, (1998) *Br J. Pharmacol.* **125**, 94P

88P VASCULAR RESPONSES TO METHOXAMINE IN ISOLATED PERFUSED MESENTERIC ARTERIAL BEDS FROM ENDOTOXAEMIC RATS

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In conscious rats, 2h and 24h after the onset of continuous infusion of lipopolysaccharide (LPS), there is reduced pressor and mesenteric vasoconstrictor responsiveness to methoxamine (ME) given either as a 3 min i.v. infusion (Waller *et al.*, 1994) or as bolus i.v. injections (Tarpey *et al.*, 1998). However, isolated mesenteric vascular beds taken from LPS-infused rats show normal responses to bolus doses of ME (Tarpey & Randall, 1998). Since the mode of administration of a contractile agonist (bolus vs infusion) may influence responsiveness *in vitro*, the aim of the present study was to measure responses to continuous infusion of increasing concentrations of ME in mesenteric vascular beds isolated 2h and 24h after the onset of LPS infusion in rats. For comparison, responses to bolus doses of ME were also established in different preparations.

Under anaesthesia (fentanyl and meditomidine; 300µg kg^{-1} of each i.p.) male Sprague-Dawley rats (300-419g) had i.v. catheters implanted, 24h before i.v. infusion of saline (0.4ml h^{-1}, Sal) or LPS (150µg kg^{-1} h^{-1}; *E. Coli* serotype 0127:B8) (Waller *et al.*, 1994). After either 2h or 24h infusion animals were anaesthetised with sodium pentobarbitone (<200mg kg^{-1} i.v.) and killed by decapitation. Mesenteries were removed and prepared as described previously (Ralevic & Burnstock, 1988). The preparations were perfused with Krebs' solution, to which was added ME in cumulative concentrations between 1 and 100µM. In separate preparations dose-response relationships were measured for ME given as bolus injections (50pmol-5µmol). Responses were measured as the increase in tone above baseline, and analysed by ANOVA, with Tukey's multiple comparison post-hoc test. Data are presented as means ± s.e.mean.

The maximum response (R_{MAX}) to cumulative concentrations of ME was not significantly different after 2h LPS infusion (n=8) compared to 2h saline (n=9) (96.63±15.56mmHg and 99.44±9.33mmHg respectively), but was significantly ($P < 0.05$) reduced following 24h LPS (n=8) compared to 24h Sal (n=9) (64.38±9.05mmHg vs 106.72±9.58mmHg respectively). There were no significant differences between EC_{50} values for 2h Sal, 24h Sal, 2h LPS and 24h LPS, which were 14.9±6.1µM, 7.3±1.6µM, 7.9±2.1µM and 10.9±2.0µM respectively.

Vasoconstrictor responses to bolus doses of ME were not different after either 2h (n=8) or 24h (n=9) LPS infusion compared to the corresponding saline groups (2h, n=8; 24h, n=9). R_{MAX} values were 125±18mmHg, 112±27mmHg, 116±13mmHg and 102±10mmHg respectively. pD_2 values were 7.0±0.1, 7.2±0.2, 7.2±0.1 and 7.1±0.1 respectively.

The mechanism(s) underlying the findings of LPS-induced hyporesponsiveness to ME given *in vitro* as infusions but not as bolus injections needs further investigation, but may relate to impaired availability of, or sensitivity to, Ca^{2+} (Martinez *et al.*, 1996).

The financial assistance of the British Heart Foundation is gratefully acknowledged.

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89P MECHANISMS OF TESTOSTERONE-INDUCED VASORELAXATION IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

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The mechanisms of vasorelaxation to testosterone are still unclear. A recent study demonstrated that vasorelaxation to testosterone is mediated via nitric oxide (NO) (Chou *et al.*, 1999). In contrast, Yue *et al.* (1995) showed that testosterone-mediated responses were unaffected by a NO synthase inhibitor. Honda *et al.* (1999) reported that testosterone causes vasorelaxation by activating K^+ channels. The aim of this study was to examine the roles of NO and K^+ channels in testosterone-induced vasorelaxation in the rat mesenteric arterial bed.

Male Wistar rats (250-350 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and exsanguinated. The mesenteric arterial bed was isolated (McCulloch *et al.*, 1997) and perfused with oxygenated Krebs-Henseleit solution plus 10 μM indomethacin. Following 30 min equilibration, methoxamine (1 - 100 μM) was added to increase perfusion pressure (100 - 150 mmHg). In other experiments, 60 mM KCl was added to induce tone to examine the effects of high extracellular K^+ on responses to testosterone (McCulloch *et al.*, 1997). Testosterone was added cumulatively to the perfusion fluid (100 pM - 10 μM). In some experiments the endothelium was removed by perfusion with distilled water for 10 minutes. The vasorelaxant effects of testosterone were also assessed in the presence of 300 μM N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, 300 μM tetrabutylammonium chloride (TBA), a selective inhibitor of K^+ channels, 10 μM glibenclamide, an ATP-sensitive K^+ channel inhibitor, or 100 nM charybdotoxin (ChTx), an inhibitor of large-conductance calcium-activated K^+ (BK_{Ca}) channels; all drugs were added 30 min before testosterone. Maximal responses (R_{max}) are expressed as mean with s.e.mean and pEC_{50} values are expressed as mean with 95% confidence intervals (CI). Values between two groups were compared by the Student's *t*-test for unpaired values.

Testosterone (100 pM – 10 μM) caused concentration-related vasorelaxations of methoxamine-induced tone (pEC_{50}=9.95 (9.33-10.56), R_{max}=62.0±1.7 %, n=6). Both addition of L-NAME and removal of the endothelium significantly ($p<0.01$) reduced the potency and maximal responses to testosterone (L-NAME: pEC_{50}= 8.76 (8.52-9.00), R_{max}=51.5±1.2 %, n=6; endothelial denudation: pEC_{50}=8.12 (7.45-8.79), R_{max}=50.7±2.6 %, n=5). Vasorelaxation to testosterone was abolished by 60 mM KCl and a contractile response was revealed (pEC_{50}=7.66 (7.23-8.10), R_{max}=-19.8±2.4 %, $p<0.001$, n=5). Testosterone-induced vasorelaxation was sensitive to TBA, and once again vasoconstriction was uncovered (R_{max}= -25.3±5.9 %, $p<0.001$, n=5) Glibenclamide had no effects on testosterone-induced responses (pEC_{50}=9.29 (8.90-9.68), R_{max}= 64.0±1.6 %, n=6). The presence of ChTx significantly ($p<0.05$) inhibited vasorelaxation to 1 μM testosterone (control: 63.3±9.9 %, n=6; ChTx: 11.9±12.7 %, n=3).

The results of the present study demonstrate that, in the rat mesenteric arterial bed, testosterone causes potent vasorelaxations, which are partly endothelium- and NO-dependent. A high concentration of extracellular K^+ inhibited vasorelaxations to testosterone, suggesting, therefore, that testosterone induces vasorelaxations primarily by increasing K^+ efflux. The inhibitory effects of TBA and ChTx on testosterone-induced responses suggest that vasorelaxation to testosterone involves BK_{Ca} channel activation.

This work is funded by The Thai Government.

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90P CYTOKINE-INDUCED APOPTOSIS OF HUMAN CULTURED VENOUS SMOOTH MUSCLE CELLS

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Apoptosis of vascular smooth muscle cells (SMCs) is increasingly linked to the pathology of atherosclerosis. In vitro, apoptosis of SMCs can be induced by inflammatory cytokines relevant to atherosclerotic processes (Geng *et al.*, 1996). Here we have characterised the effects of such cytokines on apoptosis of SMCs derived from human saphenous vein. This vessel type is susceptible to occlusion, due to atherosclerosis, when used as a conduit in coronary artery bypass surgery.

Saphenous vein was cultured as described previously (Stanford *et al.*, 2000). SMCs were treated for either 24 or 48 hours with IL-1β (10ng/ml), TNFα (10ng/ml) or IFNγ (100ng/ml) alone, or in combination. At the end of experiments cells were lysed and apoptosis was assessed using an ELISA (Boehringer Mannheim) to determine levels of histone-associated mono- and oligonucleosomes as described previously (Fortenberry *et al.*, 1998).

At either 24 or 48 hours (Figure 1) IL-1β alone, or in combination with TNFα, inhibited SMC apoptosis. Neither TNFα (24 hours) nor IFNγ (24 and 48 hours) alone had any effect. The inhibitory effect of IL-1β on apoptosis was reversed by IFNγ and IFNγ in combination with TNFα actually increased apoptosis above basal levels. For each cytokine treatment, effects were greater at 48 than at 24 hours.

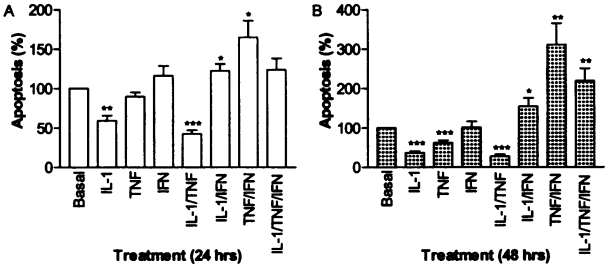

Figure 1. Effect of IL-1β (10ng/ml), TNFα (10ng/ml) and IFNγ (100ng/ml) on apoptosis (% of basal) of SMCs at A) 24 hours and B) 48 hours. Data represents mean±sem, n=9. One sample t-test (basal vs. treatments): *P<0.05, **P<0.01, ***P<0.0001.

Here we report that apoptosis of venous SMCs in culture can be modulated by cytokines. In general IL-1β was found to be inhibitory whereas TNFα and IFNγ acted synergistically to potentiate apoptosis. The signal transduction pathways and second messengers involved remain to be determined. This data adds to our knowledge of the complex interactions that take place between cytokines and SMCs at the site of inflammation.

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This work was funded by grants from the BHF and the Wellcome Trust.

91P THE EGF RECEPTOR TYROSINE KINASE INHIBITOR AG1478 PREVENTS α2 ADRENOCEPTOR-MEDIATED VASOCONSTRICTION, AND ERK ACTIVATION IN PORCINE PALMAR LATERAL VEIN

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Previous studies have shown that α2 adrenoceptors mediate vasoconstriction through the Erk-MAP kinase pathway (Roberts, *in press*). How α2 adrenoceptors stimulate the Erk signal transduction pathway is not clear. G protein-coupled receptors can activate Erk by transactivation of the epidermal growth factor (EGF) receptor and hence activation of the associated tyrosine kinase (Zwick *et al.*, 1999). Therefore, the aim of this present study was to determine whether EGF receptor transactivation could be involved in α2 adrenoceptor-mediated vasoconstriction.

Porcine palmar lateral veins were dissected into 5 mm segments and mounted in a tissue bath containing Krebs-Henseleit buffer maintained at 37°C, and gassed with 95% O2/ 5% CO2. Contractions were measured using an isometric force transducer linked to a MacLab data acquisition system. After reproducible responses to 60 mM KCl were obtained, cumulative concentration-response curves to the selective α2-adrenoceptor agonist UK14304 were obtained in the absence or presence of AG1478, an inhibitor of EGF receptor tyrosine kinase (Levitzki & Gazit, 1995; 0.1–1 μM in 0.1 % DMSO added 1 h beforehand). Alternatively, segments were exposed to a single, maximum concentration of UK14304 (10 μM), in the absence or presence of 1 μM AG1478. After contractions had reached a plateau (3-4 min), tissues were removed from the tissue baths, rapidly frozen, then homogenised. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose by Western blotting. Levels of phosphorylated and total Erk were determined by immunoblotting. Bands were measured by densitometry.

UK14304 produced large contractions of the porcine palmar lateral vein (maximum 72.7 ± 6.2 % 60 mM KCl response, mean ± s. e. mean, n=6). This contraction was significantly inhibited in the presence of 0.1 μM AG1478 (52.0 ± 3.1 % KCl response) or 1 μM AG1478 (29.1 ± 8.0 %; both $p<0.05$ vs UK14304 alone, ANOVA followed by Bonferroni, n=6), indicating that α2 adrenoceptor-mediated contractions are dependent upon EGF receptor tyrosine kinase activity. Contractions to a single concentration of UK14304 (10 μM) were associated with an increase in Erk phosphorylation (178.4 ± 18.5 % of basal, $p<0.05$, Student's paired t-test, n=6). Levels of total Erk were unchanged (117.3 ± 9.1 % of basal, n=6). This demonstrates that α2 adrenoceptor-mediated vasoconstriction is associated with activation of Erk. The phosphorylation of Erk induced by 10 μM UK14304 was significantly inhibited in the presence of 1 μM AG1478 (117.0 ± 7.3 % basal, $p<0.05$ Student's paired t-test vs UK14304). In conclusion, these data suggest that α2 adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein is dependent upon activation of Erk through EGF receptor transactivation.

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Supported by The Wellcome Trust.

92P EFFECTS OF CYCLOOXYGENASE INHIBITION ON ENDOTHELIUM DEPENDENT RELAXATION IN RAT AORTA

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Cyclooxygenase (COX) is accepted to be a potential enzymatic source of reactive oxygen species and is capable of generating oxidant stress conditions e.g. during ageing (Heymes *et al.*, 2000). The isoprostanes, in particular 8-epi $PGF2_{\alpha}$ can be generated through both COX-independent and dependent mechanisms with the latter reported for the rat aorta (Wagner *et al.*, 1997). With this in mind the aim of the present study was to characterise the effects of COX inhibition on rat endothelium dependent relaxation (EDR) and to determine if inhibition of either isoform affected blood vessel reactivity.

EDR (mediated by acetylcholine, ACh) of isolated aortic rings (Male Wistar rats, 400-450g) was measured by isometric force displacement in tissues pre-contracted with 0.1 μM phenylephrine (PE) and cumulative dose response curves to ACh (0.001 – 30 μM) were recorded. Maximal percentage relaxations (R_{max}), relative to 30 μM ACh were used for analysis. In some experiments, endothelium was removed mechanically and endothelium independent relaxation was measured (in pre-contracted tissues) as maximal percentage relaxations to glyceryl trinitrate (GTN). All COX inhibitors were dissolved in DMSO. Data were analysed using the Students' t-test. Results are expressed as mean ± s.e.m., with $p<0.05$ considered as significant.

When rings were pre-incubated with the non-selective COX-1 inhibitor, indomethacin (10 μM) for 30 min, EDR was significantly improved (R_{max} control 76.7 ± 6% vs pre-incubated 103.4 ± 5.7%, $p<0.05$, n=4). When rings were pre-incubated with the COX-2 selective inhibitor, nimesulide (10 μM) for 30 min, R_{max} was not significantly different from that seen in untreated aorta (R_{max} control 94.3 ± 3.1% vs 97.7 ± 1.7%, $p>0.05$, n=4). When tissue was pre-incubated with the COX-1 selective inhibitor, SC560 (10 μM), R_{max} of treated tissue was significantly improved (R_{max} control 96.1 ± 2.2% vs treated 109.1 ± 1.9%, $p<0.001$, n=7). In experiments with denuded rings maximal relaxation to GTN was not altered when tissue was exposed to indomethacin. (R_{max} control 105.2 ± 14.1% vs treated 135.6 ± 7.8%, $p>0.05$, n=4).

In conclusion, indomethacin produced a significant potentiation of EDR, suggesting that a COX derived vasoconstrictor is active in the tissue. Pre-treatment with nimesulide did not have a significant effect on EDR, discarding the possibility of COX-2 activity. Pre-treatment of the tissue with SC560 significantly potentiated EDR, suggesting that COX 1 is the prevalent isoform active in EDR. This finding is consistent with the report that a COX derived vasoconstrictor prostanoid is present in rat aorta.

Supported by the Irish Health Research Board.

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93P THE EFFECT OF THE STATINS ON PROSTACYCLIN RECEPTOR SIGNALLING.

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Prostacyclin (prostaglandin (PG) I_2) plays a major role in the maintenance of vascular homeostasis. It signals through its specific prostacyclin receptor (IP) to mediate activation of adenylyl cyclase and, in certain cell types, phospholipase C activation. We have previously shown that the IP is isoprenylated by the attachment of a carbon 15 farnesyl isoprenoid and that this attachment is necessary for efficient IP-mediated cAMP generation and intracellular calcium mobilization (Hayes *et al.,* 1999). In preliminary studies, inhibition of isoprenylation by lovastatin, a HMG-CoA reductase inhibitor, significantly decreased both IP mediated cAMP generation and intracellular calcium mobilization.

Statins, widely used in the prevention of coronary heart disease, lead to reductions in low-density lipoprotein cholesterol reducing the incidence of stroke and total mortality. Statin therapy has the potential to result in pleiotropic effects not directly associated with their cholesterol lowering properties and therefore, may inadvertently target isoprenylated proteins. We sought to investigate the effects of statins on IP signalling in more detail. In the current study, we investigated the effects of lovastatin and cerivastatin on [^{3}H]iloprost binding and on cAMP generation and $[Ca^{2+}]_i$ mobilization by the IP receptor in response to its selective agonist cicaprost.

Signalling by the mouse (m) and the human (h) IP over-expressed in human embryonic kidney (HEK) cells and by the hIP receptor, endogenously expressed in human erythro-leukemia 92.1.7 (HEL) cells were examined. The statins did not affect mIP and hIP radioligand binding ($p > 0.05$) but significantly reduced cAMP generation (inhibitory concentration at 50% inhibition (IC_{50}) values: Lovastatin, 3.5 μM (mIP); 0.8 – 0.85 μM (hIP); Cerivastatin, 80 nM (mIP); 50 – 60 nM (hIP)) in a time and concentration dependent manner. Also, $[Ca^{2+}]_i$ mobilization by the mIP and hIP were significantly reduced (IC_{50} values: Lovastatin, 4.2 μM (mIP); 2.6 – 3.1 μM (hIP); Cerivastatin, 90 nM (mIP); 63 – 65 nM (hIP)). Signalling by the non-isoprenylated β_2 adrenoceptor or the human thromboxane A_2 receptor (TP) isoforms were not affected by statin pre-treatment ($p > 0.05$). IP receptor mediated desensitisation of h.TPα signalling was reduced by statin pre-treatment (Lovastatin, 10 μM, $p < 0.05$; Cerivastatin, 0.5 μM, $p<0.05$).

In conclusion, these data suggest that statin therapy may interfere with IP receptor signalling *in vivo.* Therefore, in clinical settings where elevated levels of statins occur, the statins may interfere in IP receptor signalling and therefore, with IP regulated vascular hemostasis.

Statistical analysis: Students' t test (unpaired).

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94P CHARACTERISATION OF CHEMOKINE TRIGGERED INTERNALISATION AND PHOSPHORYLATION OF THE CHEMOKINE RECEPTOR CCR5

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The G-protein coupled receptor CCR5 has been shown to be an important receptor for the chemokines MIP-1α, MIP-1β, RANTES and MCP-2 (Samson et al., 1996). More recently, CCR5 has been shown to be a critical component, alongside CD4, of the receptor complex allowing human immunodeficiency virus type 1 (HIV-1) entry into target cells (Alkhatib et al., 1996). CCR5 expression level plays a critical role for HIV infections, a lower CCR5 level leading to a higher immunity against HIV I infection. Therefore the mechanisms of receptor internalisation and subsequent downregulation are important. In this study we, therefore, investigated the effects of a number of chemokines on several processes linked to CCR5 activation, including receptor phosphorylation and receptor internalisation in stably transfected CHO cells.

CCR5 was stably expressed in CHO (Chinese hamster ovary) cells (CHO.CCR5). CCR5 Immunoprecipitation and Western blot were carried out using a method described by Xiao et al. 1999. Phosphorylation of the receptor was determined after immunoprecipitation and Western blot as a shift in mobility, the phosphorylated receptor migrating more slowly on the SDS-PAGE than the un-phosphorylated form. Treatment with phosphatase 1 abolished the phosphorylation and the different migration behaviour. Internalisation assays were performed using flow cytometry. Cells were treated with chemokines (50nM) and cell surface expression of the receptor was determined using a monoclonal antibody against CCR5 and compared to untreated cells stained in the same assay as control. In some experiments, cells were pre-treated with pertussis toxin (100ng/ml) for 18 hours or with wortmannin (1μM) for 1 hour before assaying. Data were analysed using GraphPad Prism and statistical analyses were made using Students-t-test.

MIP-1α, MIP-1β and RANTES could induce phosphorylation of CCR5 in stably transfected CHO cells. Receptor phosphorylation was not inhibited by pre-treatment with pertussis toxin. We have previously shown that MCP-2 is able to activate CCR5 in [^{35}S]GTPγS-binding assays (Mahmoud et al., 2000), but neither MCP-2, nor MCP-3 or MCP-4 were able to induce receptor phosphorylation.

Internalisation of CCR5 was determined for a range of chemokines using flow cytometry analyses. MIP-1α and RANTES induced receptor internalisation by up to 50 percent, whereas MIP-1β, MCP-2, MCP-3 and MCP-4 only induced very low internalisation (Table 1). Internalisation was de-creased by pertussis toxin treatment for MIP-1α and RANTES. Uncoupling of receptor and G-protein, therefore, affects internalisation of the receptor. Internalisation for MIP-1β, MCP-2, MCP-3 and MCP-4 was significantly increased by wortmannin treatment. Wortmannin treatment may inhibit phosphatidylinositol 4-kinase (PI4-kinase) and subsequently increase the formation of clathrin coated pits on the cell surface (Feniger-Barish et al. 2000). This study shows that MIP-1α, RANTES and MIP-1β are potent agonists for CCR5 for phosphorylation and internalisation. MCP-2, MCP-3 and MCP-4 are only able to induce receptor internalisation after wortmannin treatment but are not able to induce receptor phosphorylation.

Table 1: Cell surface CCR5 in CHO.CCR5 cells: effects of chemokines (expressed as mean±SEM (n), **=p<0.05, relative to treated control)

chemokine	treated control	PTX	wortmannin
MIP-1α	52.3±4.0 (4)	98.2±4.0 ** (6)	61.1±4.1 (8)
MIP-1β	85.8±5.3 (4)	not done	61.1±4.1** (8
RANTES	54.4±3.1 (5)	83.7±4.7 **(12)	39.1±11.5 (5)
MCP-2	85.5±6.8 (6)	81.2±4.4 (8)	67.8±3.8 ** (7)
MCP-3	85.9±5.5 (7)	90.0±5.1 (7)	67.2±3.8 ** (6)
MCP-4	79.2±2.5 (6)	95.4±2.9 (9)	70.0±3.0 ** (5)

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The financial support of the BBSRC is gratefully acknowledged.

95P CHARACTERISATION OF [^{35}S]GTPγS BINDING TO CHO CELL MEMBRANES EXPRESSING HUMAN CCR4 RECEPTORS

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The CCR4 receptor is a high affinity receptor for the chemokines TARC (Thymus and Activation Regulated Chemokine) and MDC (Macrophage Derived Chemokine) (Imai *et al.*, 1998). Activation of the CCR4 receptor by TARC and MDC has been shown to elicit cellular calcium mobilisation and chemotaxis (Langham *et al.*, 2001). However, there is no report that directly measures CCR4-mediated G protein activation. We have used CCR4 CHO cell membranes to investigate agonist-induced G protein activation in [^{35}S]GTPγS binding filter washing (FW) and scintillation proximity (SPA) assays.

CCR4 CHO cells were cultured in NUT.MIX F-12/HAMS with 10% foetal calf serum and 0.4mgml^{-1} G418. For GTPγS binding studies, cell membranes were prepared and assayed as described by Hall *et al.* (1999), with the following modifications: assay buffer contained 1(SPA) or 10 (FW) mM $MgCl_2$, 100 mM NaCl and 2 μM GDP. These conditions yielded the highest human MDC stimulation of [^{35}S]GTPγS binding. 5 (FW) or 10 (SPA) μg membranes were used per reaction. Results were expressed as a fold of basal [^{35}S]GTPγS binding and intrinsic activity expressed as a fraction of the maximal human MDC response. For inhibition studies, G protein antagonist concentration response curves were carried out in the presence of 10 nM human MDC. Data are expressed as mean ± s.e.mean from 4 - 10 experiments.

Human MDC was defined as a full agonist and stimulated basal [^{35}S]GTPγS binding by 4.0 ± 0.30 fold (FW) or 2.3 ± 0.10 fold (SPA). Human TARC, mouse MDC and mouse TARC had lower intrinsic activities, whereas human MIP-3β had no activity up to 100 nM. The G protein inhibitors (suramin and NF-023) inhibited both stimulated and basal [^{35}S]GTPγS binding. pIC50s of suramin and NF-023 were 5.68 ± 0.02 (FW) or 6.28 ± 0.05 (SPA) and 5.25 ± 0.05 (FW) or 4.89 ± 0.09 (SPA), respectively.

Table 1. Effects of CCR4 agonists on [^{35}S]GTPγS binding.

	pEC50 Filter wash	Intrinsic activity Filter wash	pEC50 SPA	Intrinsic activity SPA
Human MDC	8.63 ± 0.07	1.00	8.55 ± 0.11	1.00
Human TARC	7.92 ± 0.12	0.40 ± 0.03	8.48 ± 0.08	0.61 ± 0.04
Mouse MDC	8.34 ± 0.04	0.50 ± 0.01	-	-
Mouse TARC	8.47 ± 0.12	0.47 ± 0.02	-	-

MDC and TARC caused CCR4-mediated G protein activation in membranes from CCR4 CHO cells. Similar data were obtained for human MDC and human TARC in FW and SPA [^{35}S]GTPγS binding assays. [^{35}S]GTPγS binding represents an alternative functional test for the human CCR4 receptor.

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96P CHARACTERISATION OF AGONIST-INDUCED INHIBITION OF CYCLIC AMP ACCUMULATION IN CHO CELLS EXPRESSING HUMAN CCR4 RECEPTOR

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Macrophage derived chemokine (MDC) and thymus- and activation-regulated chemokine (TARC) are agonists at the CCR4 receptor and stimulate Ca^{2+} mobilisation and chemotaxis in transfected cell lines (Imai *et al.*, 1998). Their effect on cyclic AMP (cAMP) accumulation has not previously been reported. Fluorescence polarisation (FP, Checovitch et al., 1995), FlashPlate (Kariv *et al.*, 1999) and AlphaScreen™ (LOCI™, Patel *et al.*, 2000) cAMP assays were investigated for measuring inhibition of forskolin-induced cAMP accumulation at the human CCR4 receptor.

Assays were carried out following the manufacturers' recommended instructions. Human CCR4-transfected CHO cells were maintained in NUT.MIX F-12/Hams with 10% foetal calf serum and 0.4 mg.ml^{-1} G418. Cells (7500 – 25000 cells/well) were incubated with or without forskolin and/or agonist at room temperature for thirty minutes in 384 well microtitre plates prior to cell lysis and measurement of cAMP concentration. Data are expressed as mean ± s.e.mean from 3-6 experiments. Z factor = 1-(((3 * SD of sample) + (3 * SD of control))/(mean of sample – mean of control)).

The midpoint of the cAMP standard curves were 580 ± 48 fmol/well for AlphaScreen, 883 ± 108 fmol/well for FP and 1417 ± 93 fmol/well for FlashPlate. Basal levels of cAMP were 50-500 fmol/well (cell number dependent) following a thirty minute incubation. 10μM forskolin submaximally stimulated cAMP concentrations by at least 10-fold. The Z Factor (Zhang *et al.*, 1999) was used to determine the robustness of each assay using 10μM forskolin and an EC_{80} concentration of MDC. The Z factor was 0.62 ± 0.04 ('excellent' > 0.5) for the AlphaScreen assay, 0.16 ± 0.22 for FP and -1.6 ± 0.19 for FlashPlate. The AlphaScreen assay was used for subsequent work. The pEC_{50}s of chemokine receptor ligands were 9.5 ± 0.09 for MDC, 10.1 ± 0.07 for TARC ($p < 0.01$, paired student t-test) and <6.5 for RANTES, I-309, MIP-3β and IP-10. The maximal inhibition of 10μM forskolin-stimulated cAMP by MDC was 75.1 ± 3.4% and 74.8 ± 5.6% for TARC.

We have demonstrated that MDC and TARC inhibit cAMP accumulation in a CCR4 receptor transfected CHO cell line. The affinity of MDC has previously been reported to be 3-fold higher than TARC in a binding assay (Imai *et al*, 1998). In this cAMP accumulation assay the apparent potency of MDC was lower than TARC. We are presently evaluating the reasons for this.

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97P DESENSITISATION OF THE α ISOFORM OF THE HUMAN THROMBOXANE A_2 RECEPTOR BY PROSTAGLANDIN D_2 RECEPTOR (DP)

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The prostanoids thromboxane (TX)A_2 and prostaglandin (PG)D_2 mediate opposing actions in platelets and vascular and non-vascular smooth muscle. TXA_2 is a potent stimulator of platelet shape change, aggregation, constriction of vascular and bronchial smooth muscle, whereas PGD_2 is a potent inhibitor of platelet aggregation. TXA_2 and PGD_2 mediate their actions by interacting through their specific cell surface G-protein receptors (GPCRs) termed TP and DP, respectively. Alternatively mRNA splicing of the human TP gene, within exon 3, gives rise to two isoforms termed TPα and TPβ that differ exclusively in their carboxyl cytoplasmic (C) tail regions.

The aim of this study was to investigate the effects of stimulation of DP on signalling by TP in both human platelets and human embryonic kidney (HEK) 293 cells over-expressing the individual TPα and TPβ isoforms. The experimental procedures carried out in the study included assessment of TP expression levels by radioligand [^{3}H]SQ29,548 binding studies, and assessment of TP signalling by analysis of agonist-mediated intracellular calcium mobilisation ($[Ca^{2+}]_i$), measurement of IP_3 levels and agonist-mediated TP phosphorylations (Walsh *et al.*, 2000). Data are presented as the mean value plus or minus the standard error of the mean (± S.E.M; n = 4 - 5). Statistical analyses were performed using the unpaired Students't-test.

Efficient mobilisation of $[Ca^{2+}]_i$ was observed in platelets in response to the TP agonist U46619 (1 μM; $\Delta[Ca^{2+}]_i$ = 156 ± 6.35nM). Pre-stimulation of platelets with the DP agonist BW245C (1 μM) for 1 min greatly reduced subsequent U46619-mediated $[Ca^{2+}]_i$ mobilisation ($\Delta[Ca^{2+}]_i$ = 29.4 ± 1.1 nM, p< 0.001).

This DP-mediated desensitisation of TP signalling in platelets was prevented by pre-treatment with the cAMP-dependent protein kinase (PK)A inhibitor, H-89 (U46619 mediated $\Delta[Ca^{2+}]_i$ = 144 ± 7.5 nM in the presence of 10 μM H-89), but was unaffected by the PKC inhibitor GF 109203X (U46619 mediated $\Delta[Ca^{2+}]_i$ = 20.4 ± 2.3nM in the presence of 50 nM GF 109203X). We further investigated if DP-mediated desensitisation specifically targets TPα, TPβ or both in HEK 293 cells and compared it to that which occurs in platelets.

Our results conclude that signalling by TPα ($\Delta[Ca^{2+}]_i$ = 12 ± 7.3 nM), but not TPβ ($\Delta[Ca^{2+}]_i$ = 138 ± 15.9 nM), was subject to DP-mediated desensitisation. Furthermore, this DP-mediated desensitisation of TPα signalling occurred in a PKA-dependent, PKC-independent manner. $TP^{\Delta 328}$, a truncated variant of TP containing only those residues common to both TPα and TPβ, was insensitive to prior DP stimulation (U46619-mediated $\Delta[Ca^{2+}]_i$ = 163 ± 7.9 nM following pre-stimulation with BW245C: p > 0.91), indicating that the C-tail of TPα contains the target-site(s) for DP-mediated desensitisation. We further established that mutation of Ser^{329} to Ala^{329} within a consensus PKA sequence within the unique C-tail region of TPα, to generate $TP\alpha^{S329A}$, rendered it insensitive to BW245C-mediated desensitisation (U46619-mediated ($\Delta[Ca^{2+}]_i$ = 260 ± 2.9 nM following pre-stimulation with BW245C; p > 0.15).

In conclusion TPα, but not TPβ, is subject to DP-mediated desensitisation and that this occurs in a PKA dependent, PKC independent manner, and that the PKA target site of TPα is Ser^{329}.

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98P MAPK ACTIVATION THROUGH THE HUMAN THROMBOXANE A_2 RECEPTOR α AND β ISOFORMS

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Both thromboxane (TX) A_2 and the F2 isoprostane 8-epi-prostaglandin (PG) $F_{2\alpha}$ stimulate vascular smooth muscle cell (SMC) constriction and hypertrophy/proliferation in a number of species. TXA_2- and 8-epi$PGF_{2\alpha}$-mediated mitogenic signalling have not been studied in detail in human SMC. In this study, the effect of the TXA_2 receptor (TP) agonist, U46619 and 8-epi$PGF_{2\alpha}$ on TP-mediated mitogen-activated protein kinase (MAPK) signalling in the immortalised human uterine ULTR SMC line (Perez-Reyes *et al.*, 1992) was examined.

Employing an *in vitro* myelin basic protein (MBP) phosphorylation assay and phosphorylation-specific anti-ERK1/2 antibodies, U46619 mediated a time dependent activation of extracellular-signal regulated kinase (ERK) with maximal ERK activation observed at 5 min ($P \leq 0.05$ relative to basal levels). Moreover, 8-epi$PGF_{2\alpha}$ mediated a time dependent activation of ERK, with maximal ERK activation observed at 10 min ($P \leq 0.01$ relative to basal levels). A concentration dependent activation of ERK in response to U46619 and 8-epi$PGF_{2\alpha}$ was observed with maximal ERK activation observed at 100-300 nM ($P \leq 0.01$ relative to basal) and 300 nM ($P \leq 0.01$ relative to basal), respectively. Both U46619 and 8-epi$PGF_{2\alpha}$ mediated c-jun N-terminal kinase (JNK) activation with maximal activation at 20, and 10 min, respectively ($P \leq 0.05$ relative to basal). Whereas U46619-mediated ERK and JNK activation was abolished by the TP antagonist, SQ29,548 (1μM, 1 min; $P \leq 0.01$ relative to U46619 induced ERK activation), 8-epi$PGF_{2\alpha}$-mediated ERK and JNK activation was partially inhibited ($P \leq 0.05$ relative to 8-epi$PGF_{2\alpha}$-mediated ERK/JNK activation).

The MAPKK1/2 inhibitor PD98059 inhibited U46619- and 8-epi$PGF_{2\alpha}$-mediated ERK activation ($P \leq 0.05$ relative to U46619/8-epi$PGF_{2\alpha}$-mediated ERK activation). Both U46619- and 8-epi$PGF_{2\alpha}$-mediated ERK activation were inhibited by the protein kinase (PK) C, PKA and phosphoinositide 3-kinase inhibitors GF 109203X ($P \leq 0.05$ relative to U46619/8-epi$PGF_{2\alpha}$-mediated ERK activation), H-89 ($P \leq 0.05$ relative to U46619/8-epi$PGF_{2\alpha}$-mediated ERK activation) and wortmannin ($P \leq 0.05$ relative to U46619/8-epi$PGF_{2\alpha}$-mediated ERK activation), but was unaffected by pertussis toxin ($P \geq 0.05$ relative to U46619/8-epi$PGF_{2\alpha}$-mediated ERK activation). U46619- and 8-epi$PGF_{2\alpha}$-mediated ERK activation involves transactivation of the epidermal growth factor receptor ($P \leq 0.01$ relative to U46619/8-epi$PGF_{2\alpha}$ -mediated ERK activation).

In humans, TXA_2 signals through two splice variants, TPα and TPβ, which differ exclusively in their C-terminal intracellular tail regions. Using human embryonic kidney (HEK) 293 cell lines stably over-expressing either TPα or TPβ, we have shown that both TP isoforms independently activate ERK ($P \leq 0.01$ relative to basal) and JNK ($P \leq 0.05$ relative to basal). 8-epi$PGF_{2\alpha}$ mediated ERK and JNK activation through SQ29,548 sensitive TPα and TPβ.

These findings provide the first evidence that both TPα and TPβ activate the ERK and JNK signalling cascades. Statistical analyses were performed using the Student unpaired t-test.

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99P MAP KINASE PATHWAY SIGNALLING IS ESSENTIAL FOR EXTRACELLULAR MATRIX DETERMINED MAMMARY EPITHELIAL CELL SURVIVAL

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Mammary epithelial cells in a variety of primary cell culture models require both growth factors and specific extracellular matrix (ECM)- attachment for survival. Cells cultured in the absence of either of these signals will die by the programmed cell death, apoptosis. Whilst the signalling pathways involved in the transduction of the survival effects of growth factors are fairly well characterised the anti-apoptotic pathways induced by ECM are less well understood.

The aims of our study, therefore, are to investigate possible signalling moieties involved in ECM- and growth factor-mediated mammary epithelial cell survival. It is also of interest to investigate any possible interactions between the different pathways involved.

The methods used include standard western blotting and immunocytochemical analyses as well as qualitative and quantitative measures of cell death (e.g. Hirt (1967) and Farrelly *et al.* (1999)).

Here we demonstrate that ECM activates the Erk 1/ Erk 2 (p42/44 MAPK) pathway and that inhibition of this signal, by PD 98059 (Alessi *et al.* (1995)), leads to apoptosis in these cells (4.8 fold induction ± 0.3, $p<0.001$). Associated with this cell death is a possible compensatory signalling through the p38 MAP kinase pathway. This provides evidence for a hitherto undescribed Erk 1/ Erk 2 to p38 MAP kinase pathway 'cross-talk'.

We also show that insulin is essential for the survival of these cells and that inhibition of this signalling, by LY294002 (Vlahos *et al.* (1994)), also leads to apoptosis (7.4 fold induction ± 0.7, $p<0.001$). The cell death associated with inhibition of MAP kinase signalling however, occurred in the presence of insulin that activates the classical PI-3 kinase-dependent Akt/ PKB survival signals and Akt phosphorylation. Cell death induced by inhibition of the MAP kinase pathways did not affect Akt phosphorylation and may, thus, be independent of PI-3 kinase signalling.

Thus we conclude that both these signalling pathways are essential for mammary epithelial cell survival but that there does not appear to be any interaction between them.

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100P EVALUATION OF PHOSPHODIESTERASE-3 IN NORMOXIC AND HYPOXIC HUMAN PULMONARY ARTERY SMOOTH MUSCLE CELLS

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The development of pulmonary hypertension in the chronic hypoxic rat is associated with a decrease in cyclic nucleotides (MacLean *et al.*, 1996). This corresponds to an increase in phosphodiesterase (PDE) activity (MacLean *et al.*, 1997). We have focused our current studies on PDE3, which is a cGMP-inhibited cAMP specific PDE expressed as two isoforms (PDE3A and PDE3B). The aim of this study was to establish a cellular model to investigate the expression of PDE3 in cultured human pulmonary smooth muscle cells chronically exposed to hypoxia.

Human pulmonary artery smooth muscle cells (hPASMC) were maintained in hypoxic (550mbar, 10% O_2, balanced with N_2) or normoxic conditions for 14 days. Cultured hPASMC were also treated with and without 100μM of the membrane permeable analogue of cAMP, 8-Bromoadenosine 3'5'-Cyclic Monophospate (8-Br-cAMP), for 24 hours. RT-PCR using specific oligonucleotide primers for PDE3A and PDE3B allowed amplification of corresponding products. Equal loading of 200ng total RNA was confirmed by RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Total PDE activity was measured using a two step radioactive assay that monitors the conversion of [^{3}H] cAMP into ^{3}H adenosine. The cAMP assay was carried out in the presence and absence of the PDE3 inhibitor SKF94836(10μM).Results are expressed throughout as percentage of control ± standard deviation, and significance by student t-test.

No hypoxia-induced changes were observed in transcripts for PDE3B (100% ± 4%, n=3, NS), or G3PDH (100% ± 1%, n=3, NS). However the transcript for PDE3A was increased (190% ± 12%, n=3, $p<0.05$) after 14 days of hypoxia. This was correlated with an increase in cAMP PDE activity (147% ± 7%, n=3, $p<0.001$), which was abolished by the addition of 10μM SKF94836 to the PDE assay (115 ± 25, n=3, NS), showing the increased activity was due to PDE3. HPASMC treated with 100μM 8Br-cAMP for 24 hours mimicked the effect of hypoxia by increasing PDE3A transcript level (130% ± 8%, n=3, $p<0.05$), but not PDE3B (103% ± 2%, n=3, NS) or G3PDH (100% ± 2%, n=3, NS). An increase in cAMP PDE activity (195% ± 42%, n=3, $p<0.05$), which was reduced by 10μM SKF94836 (107% ± 15%, n=3, NS) was also seen.

These results show that PDE3A and PDE3B are expressed in cultured human smooth muscle cells. We have demonstrated that chronic hypoxia in hPASMC is associated with an increase in PDE3 cAMP activity, which can be accounted for by the *de-novo* expression of PDE3A. The 8Br-cAMP also induced an increase in PDE3A transcript and PDE3 activity, thereby demonstrating a role for the cAMP pathway in regulating PDE3A expression in hPASMC.

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101P THE EFFECTS OF A SELECTIVE COX-2 INHIBITOR GR253035X IN RAT MODELS OF INFLAMMATORY HYPERALGESIA ASSESSED USING BEHAVIOURAL READOUTS AND BY QUANTITATION OF SPINAL IMMUNOCHEMISTRY

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Since the discovery of a second cyclooxygenase (Cox-2) isoform, research has centred on the separation of the anti-hyperalgesic effects of non steroidal anti-inflammatory drugs (NSAIDs) from their side effects (Jouzeau et al. 1997). Cox-2, induced during inflammation, is now thought to be the relevant target for the anti-inflammatory and anti-hyperalgesic effects of NSAIDs (Kawai 1998). We have identified a selective Cox-2 inhibitor, GR253035X (8-Acetyl-3-(4-fluorophenyl)-2-(4-methanesulphonylphenyl)-imidazo(1,2-1) pyridine), using an *in-vitro* human cell based assay. Selectivity was determined as being greater than 571. In the same assay, selectivity of both celecoxib and rofecoxib was 25 and 3125 respectively. The analgesic and anti-inflammatory activity of GR253035X has been evaluated in rat models of inflammatory hyperalgesia induced by intraplantar Freunds Complete Adjuvant (FCA, 100µl 1mg/ml Mycobacterium tuberculosis, Sigma). Celecoxib and rofecoxib were used as comparitors. We also investigated the effect of GR253035X on spinal c-fos levels following intraplantar carrageenan (2%, 100µl. Sigma). Immunohistochemistry was performed using a method similar to that of Buritova, et al. (1997). Male random hooded rats (170-200g) were fasted overnight for all studies. Hyperalgesia was assessed using a dual channel weight averager (Clayton et al., 1997). Paw oedema was assessed using a plethysmometer.

When administered one hour prior to intraplantar FCA, GR253035X produced a dose related reversal of the decrease in weight bearing on the inflamed paw six hours post FCA (ED_{50} 0.3mg.kg^{-1} po). This compares favourably with celecoxib (ED_{50} 3mg.kg^{-1} po) and rofecoxib (ED_{50} 0.5mg.kg^{-1} po) in the same model. Inhibition of the associated paw oedema was noted with GR253035X (20% 1mg.kg^{-1} po), celecoxib (18% 10mg.kg^{-1} po) and rofecoxib (16% 10mg.kg^{-1} po.).When administered 2 days post FCA, GR253035X, celecoxib and rofecoxib produced a dose-related reversal of the decrease in weight bearing on the inflamed paw six hours post dose (ED_{50}'s 1.9, 6.6 and 1mg.kg^{-1} po, respectively). Furthermore, GR253035X (3mg.kg^{-1} po) inhibited neuronal activation in the spinal cord, assessed using *c-fos* immunohistochemistry, in regions implicated in nociceptive transmission (laminae I-II 37%, V-VI 40% and VII-X 24%) three hours post carrageenan.

In conclusion, GR253035X, a potent and selective Cox-2 inhibitor, produced anti-hyperalgesic activity in rat models of inflammatory hyperalgesia, and compares favourably with the effects observed with celecoxib and rofecoxib. In addition, GR253035X reduced spinal *c-fos* levels, an indirect marker of neurones implicated in the nociceptive transmission (Chapman & Besson, 1997).

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102P THE nNOS INHIBITOR, GW289013, INHIBITS NEUROGENIC VASODILATION IN THE ANAESTHETISED RAT

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Nitric oxide derived from the neuronal isoform of nitric oxide synthase (nNOS) is known to have a number of important physiological functions in the CNS, including mediating vasodilation and maintenance of cerebral blood flow. nNOS has also been identified in the cell bodies of trigeminal afferent neurones within the trigeminal ganglion (Alm *et al.*, 1995) and the spinal trigeminal nucleus (Dohrn and Beitz, 1994) and so may also have a pathophysiological role in migraine headache. GW289013 (5-(3-Aminomethyl-phenyl)-3-methyl-pyridin-2-ylamine) is a selective nNOS inhibitor with IC50s against human recombinant nNOS, eNOS and iNOS isoforms, 1.1 µM, 16 µM and 33.5 µM, respectively. (Dawson, unpublished data). In this study we have used intravital microscopy to determine the effect of GW289013 on neurogenic dural blood vessel dilation evoked by electrical stimulation of perivascular trigeminal nerves and on vasodilation evoked by acetylcholine.

Male Sprague Dawley rats (300-350g) were anaesthetised using pentobarbitone (65mgkg^{-1} i.p.). The left femoral vein and artery were cannulated for anaesthetic maintenance, drug administration and cardiovascular measurements. Rats were placed in a stereotaxic frame and the middle meningeal artery (MMA) viewed through a closed cranial window using an intravital microscope and video dimension analyser to measure artery diameter. A bipolar electrode was placed approximately 200µm from the vessel. Four identical stimulations (50-300µA, 5Hz, 1ms pulse width, 10s duration) were made at 25 minute intervals and each was preceded five minutes earlier by acetylcholine. (3.0µg kg^{-1} i.v.). Five minutes after the first (control) stimulation each animal was infused with GW289013 (2mgkg^{-1} min^{-1}), for 10 min. Immediately following the infusion and 5 min after the last stimulation blood samples were removed, centrifuged, and the plasma aspirated and frozen. Each animal was then killed by cervical dislocation and the brain rapidly removed and snap frozen in liquid nitrogen.GW289013 concentration was later determined in plasma and brain tissue.

Plasma concentration of GW289013 indicated an elimination half life of approximately 1 h. The mean brain concentration was 5.5 ± 1.5 µg.kg^{-1} 75 min after the infusion was started (n=6). GW289013 had no significant effect on mean arterial blood pressure or on vessel diameter *per se*, but caused a significant inhibition of neurogenic vasodilation at 10 min (40.6 ± 6.0 %), 35 min (31.0 ± 5.7 %) and 60 minutes (38.0 ± 4.2 %) after completion of the infusion, compared to the pre-treatment control stimulation (n=6, P<0.02).

Intravenous injection of acetylcholine (3 µg kg^{-1} i. v. bolus) caused a decrease in mean arterial blood pressure (39.8 ± 0.5 %, n=6) which returned to baseline within 2 min. The same dose of acetylcholine caused increases in dural artery diameter of 71.7 ± 7.3 %, n=4 (vehicle group) and 73.8 ± 15.3 %, n=6 (GW289013 treated group). Neither GW289013 nor vehicle caused significant inhibition of the MMA dilatory response to acetylcholine at 5 min (77.4 ± 15.6 % and 74.2 ± 8.1 %, respectively), 30 min (65.8 ± 4.3 % and 70.9 ± 1.1 %, respectively) or 55 minutes (63.0 ± 4.1 % and 71.8 ± 7.1 %, respectively) after infusion.

These results imply that neurogenic vasodilation may be inhibited as a consequence of nNOS inhibition and thus nNOS inhibitors may have therapeutic potential for the treatment of migraine.

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103P NEUTROPHIL ACTIVATION BY FMLP AND IL-8:DIFFERENCES IN INVOLVEMENT OF RHOA

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Neutrophil extravasation in the lung during inflammation can occur by two pathways: one that is dependent on the neutrophil integrin complex, CD11/CD18, and one that is CD11/CD18-independent. Using an *in vitro* system, we examined neutrophil migration across human pulmonary endothelial cells and demonstrated that the chemoattractants FMLP and IL-8 elicit different migratory responses: activation of normal neutrophils to migrate by FMLP resulted in CD11/CD18-dependent migration whereas migration in response to IL-8 was CD11/CD18-independent.

The present study aimed to test the hypothesis that the signal transduction pathways mediating CD11/CD18-dependent and -independent neutrophil migration are distinct. The involvement of the small GTPase, RhoA, in FMLP and IL-8 activation of neutrophils was examined by quantifying its membrane translocation. Peripheral blood neutrophils were isolated from normal, healthy volunteers and were activated by exposure to either FMLP or IL-8. Following activation, neutrophils were lysed by sonication and ultracentrifugation yielded membrane pellets. Proteins in the membrane preparations were resolved using SDS-PAGE and RhoA was quantified using immunoblotting followed by enhanced chemiluminescence detection.

Compared to unactivated neutrophils, membrane translocation of RhoA was increased 1.7- and 3.8-fold in FMLP-activated neutrophils after 1- and 5 minutes, respectively. In contrast, no increase in RhoA translocation was observed in neutrophils activated by IL-8.

These results demonstrate differences in RhoA involvement in neutrophils stimulated by FMLP and IL-8 that may reflect differences in the signal transduction pathways mediating CD11/CD18-dependent and -independent migration.

104P PRE-EXPOSURE TO FMLP CAUSES NORMAL NEUTROPHILS TO MIMIC NEUTROPHILS FROM PATIENTS WITH ACUTE PULMONARY INFECTION

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During pulmonary infection, neutrophils (PMNs) migrate out of the bloodstream into the airspace where their function is to eliminate invading pathogens. Migration can occur by either of two mechanisms, one that involves the PMN CD18 integrin adhesion complex (CD18-dependent migration) or one that does not require CD18 (CD18-independent migration).

Using an *in vitro* model that supports both migratory mechanisms, we have recently demonstrated that the bacterial chemoattractant, f-met-leu-phe (FMLP) stimulates CD18-dependent migration of normal PMNs whereas migration to the chemoattractants, IL-8 and LTB_4, occurs by the CD18-independent mechanism (Mackarel *et al.*, Am. J. Respir. Cell Mol. Biol. 2000; 23: 154-161). In contrast, PMNs from patients with acute pulmonary infection were found to migrate to all three stimuli using the CD18-dependent pathway.

The aim of this study was to examine whether the switch from CD18-independent to -dependent migration observed with PMNs from acutely infected patients may be due to exposure to bacterial products such as FMLP in the bloodstream. To do this, normal PMNs were pre-incubated ± FMLP and the CD18-dependency of migration to IL-8 and LTB_4 was determined.

In the absence of FMLP pre-treatment, migration to IL-8 and LTB_4 was predominantly CD18-independent with 68.5±6.2%, and 73.8±9.7% of PMNs migrating using this route (n=4). Pre-exposure to FMLP decreased the amount of migration occurring by the CD18-independent pathway to 37.9±5.9% and 40.3±5.1% for IL-8 and LTB_4 respectively ($p<0.05$).

These results demonstrate that exposure of normal PMNs to FMLP causes them to 'switch' from CD18-independent to CD18-dependent migration.

Mackarel *et al.*, Am. J. Respir. Cell Mol. Biol. 2000; 23: 154-161

105P GREMLIN INHIBITS DNA SYNTHESIS AND PROLIFERATION IN HUMAN MESANGIAL CELLS BY A BMP INDEPENDENT MECHANISM

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Gremlin is a member of the TGF-beta superfamily of secreted proteins, and has been described in embryogenesis including limb bud development and neural crest cell differentiation (Hsu *et al.*, 1998; Zuniga *et al* 1999). It is believed to function as a bone morphogenetic protein (BMP) antagonist by heterodimerising with BMP 2, 4 and 7, thus preventing the BMPs from interacting with their receptor.

We previously showed that mesangial cell gremlin mRNA levels were induced by high glucose, cyclic mechanical strain, and TGF-beta1 in vitro, and gremlin mRNA levels were elevated in the renal cortex of rats with streptozotocin-induced diabetic nephropathy in vivo (McMahon *et al.*, 2000).

In this study we wished to investigate the consequences of increased gremlin expression in diabetic nephropathy.

Gremlin was overexpressed in primary human mesangial cells by transfection of the mammalian expression vector pCDNA6-V5His using the cationic polymer polyethylenimine. By using the empty vector as a control, we showed that overexpression of gremlin in mesangial cells resulted in an attenuated response (at least 50%, n=5) to fetal calf serum (10%) stimulated proliferation. This effect was not reflected by inhibition of either PDGF (10ng/mL) or EGF (50ng/mL) induced proliferation. Addition of human recombinant BMP-2 (100ng/mL) and BMP-4 (100ng/mL) also decreased PDGF (10ng/mL) stimulated mesangial proliferation (BMP-2 and BMP-4 reduced proliferation by at least 50%, n=3 and 25%, n=2, respectively), and unlike the BMP antagonist noggin (250ng/mL), Gremlin did not inhibit this effect. Furthermore, noggin did not affect the anti- proliferative action of gremlin.

In conclusion, these results suggest that gremlin inhibits proliferation through a mechanism that is independent of its known target, BMP. The anti- proliferative effects of Gremlin in mesangial cells may have consequences in renal hypertrophy, one of the earliest abnormalities of diabetic nephropathy.

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106P EFFECTS OF BUDESONIDE AND IMM125 ON ANTIGEN-INDUCED AIRWAY INFLAMMATION AND HYPERRESPONSIVENESS TO ADENOSINE (ADO) IN ACTIVELY SENSITISED BROWN NORWAY (BN) RATS

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We have demonstrated a marked augmentation of the bronchoconstrictor response to ADO in actively sensitised (AS) BN rats challenged with ovalbumin (OA). The augmented response is mast cell mediated and shows many similarities to the response to ADO seen in asthmatics (Hannon *et al.*, 2001). To further explore the mechanism of the up-regulation of the response to ADO, we have investigated the effects of pretreatment with budesonide, an established anti-inflammatory therapy for asthma, and IMM125, an analogue of the immunosuppressant cyclosporin (Keller *et al*, 2000).

BN rats, AS to OA, were challenged either with OA (0.3 $mgkg^{-1}$) or saline i.t. and 2.5 h later anaesthetised and set-up for measurement of airway resistance and cardiovascular parameters (Hannon *et al*, 2001). ADO and comparator bronchospasmogens were given i.v. in a sequence starting 3 h post challenge. In separate experiments, rats (n = 8-10 per group) were challenged either with OA (0.3 $mgkg^{-1}$) or saline i.t. and 24 h later bronchoalveolar lavage (BAL) performed. Differential leukocyte cell counts in BAL fluid were measured using an automated cell analysing system (Cobas Helios). Eosinophil peroxidase (EPO), myeloperoxidase (MPO) activities and protein concentrations in BAL fluid were determined using standard photometric assays.

Bronchoconstrictor responses to ADO (0.3 & 1 $mgkg^{-1}$), 3 h post OA challenge, were reduced dose-dependently by budesonide or IMM125 (0.01-1 $mgkg^{-1}$) given i.t. 25 and 1 h before the challenge. Bronchoconstrictor responses to methacholine (MCh; 3 & 10 μgkg^{-1}) were unaffected by either agent, whereas those to bradykinin (BK; 30 & 100 μgkg^{-1}) were unaffected by IMM125 but reduced dose-dependently by budesonide (Fig. 1). In AS animals challenged with saline, budesonide and IMM125 (1 $mgkg^{-1}$) induced qualitatively and quantitatively similar changes in sensitivity to higher doses of (3 & 10 $mgkg^{-1}$), MCh and BK.

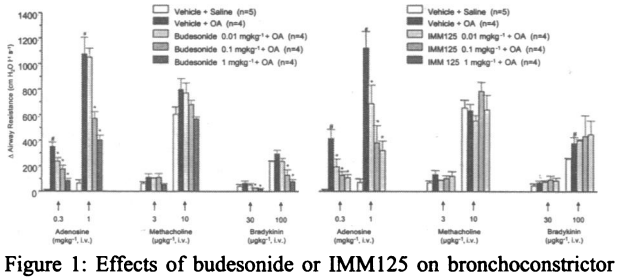

Figure 1: Effects of budesonide or IMM125 on bronchoconstrictor responses to ADO, MCh or BK established 3 h post challenge with saline or OA (0.3 $mgkg^{-1}$) in AS BN rats. Mean values ± s.e. means of n experiments are presented. #,*P < 0.05, that the value differs from the equivalent control.

Challenge with OA (0.3 $mgkg^{-1}$) led to increases in the BAL fluid leukocyte numbers, EPO and MPO activities and protein concentration measured 24 h post challenge. Pretreatment with budesonide (1 $mgkg^{-1}$) induced significant reductions in eosinophil (-59 %), neutrophil (-73 %) and lymphocyte (-49 %) numbers and inhibition of protein concentration (-60 %) and MPO activity (-50 %). IMM125 (1 $mgkg^{-1}$) had no significant effect on any of these parameters.

Thus, budesonide and IMM125 block the augmented response to ADO in sensitised rats challenged with OA. In the case of budesonide the effect is associated with a powerful, generalised anti-inflammatory effect. With IMM125, the effect is seen at doses which are not anti-inflammatory and may reflect direct suppression of mast cell mediator release (Hultsch *et al.*, 1998).

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107P HYPERRESPONSIVENESS OF LUNG PARENCHYMAL STRIPS TO ADENOSINE RECEPTOR AGONISTS INDUCED BY ANTIGEN CHALLENGE IN ACTIVELY SENSITISED BROWN NORWAY (BN) RATS

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A marked augmentation of the bronchoconstrictor response to adenosine (ADO) is seen in actively sensitised BN rats challenged with ovalbumin (OA); the augmented response is a consequence of mast cell activation leading to the release of 5-HT which in turn induces bronchoconstriction (Hannon et al., 2001). Parenchymal strips removed from sensitised rats challenged with OA also show hyperresponsiveness to ADO and responses are mast cell dependent (Hannon et al., 2001). We now report a preliminary pharmacological analysis of the augmented response to ADO in the parenchymal strip.

BN rats (200-300 g) were sensitised to OA (Hannon et al., 2001) and challenged with saline or OA, 0.3 mg kg^{-1} given intratracheally, 3 h prior to death. The lungs were perfused *in situ* with Krebs' solution prior to slices (10-12 mm long, 3 mm thick) being cut from the major lobe. Tissues were set-up for recording isotonic tension in Krebs' solution at 37 °C bubbled with carbogen. An initial contraction to bethanechol 100 μM was obtained. After repeated washing, a single concentration of an ADO receptor agonist was tested per strip. After further washout a cumulative concentration-response curve (CRC) to 5-HT (1-100 μM) was generated. Finally, a cumulative CRC to bethanechol (1-100 μM) was established. Antagonists were incubated with the tissue for 30 min prior to eliciting a single response to NECA (100 μM) and a CRC to 5-HT performed.

Tissues from sensitised rats challenged with saline responded only weakly to ADO despite showing robust responses to 5-HT (Fig. 1). In contrast, responses to ADO on strips taken from animals challenged with OA were significantly greater than those seen in animals challenged with saline despite responses to 5-HT being significantly reduced (Fig. 1).

Augmentation of the responses to NECA, a broad-spectrum ADO receptor agonist, was also seen in strips from rats challenged with OA. In contrast, neither CPA, CGS 21680 nor Cl-IB-MECA (A_1, A_{2A} and A_3 selective, respectively), used at 100-fold their K_i concentrations at the relevant sites (Jacobson et al., 1995), induced contraction of strips removed from OA-challenged animals (Fig. 1). Neither DPCPX (A_1 selective) nor ZM 234185 (A_{2A} selective) each at a concentration of 100 nM, affected the response to NECA (100 μM). Similarly, even at 900 μM, the broad spectrum ADO receptor antagonist, 8-SPT, failed to block the response to NECA.

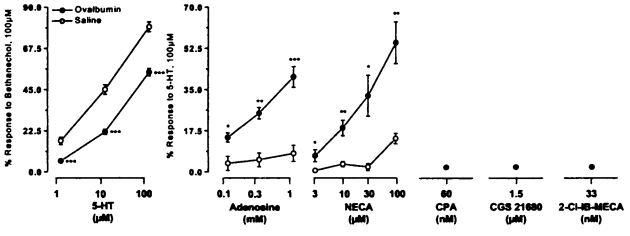

Figure 1: Effects of 5-HT and ADO receptor agonists on lung parenchymal strips from sensitised BN rats challenged with saline or ovalbumin. Values are means ± s.e. means of 4-5 individual experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ that the value differs significantly from the saline control.

Thus, the augmented responses to ADO and NECA seen in lung parenchymal strips from sensitised animals challenged with OA seem not to be mediated by A_1, A_{2A} or A_3 receptors. Although logically, the A_{2B} receptor should mediate the response, the unequivocal demonstration of the fact must await the availability of potent and selective antagonists at this site.

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108P INFLUENCE OF THE Thr164Ile POLYMORPHISM IN THE β_2-ADRENOCEPTOR ON THE EFFECTS OF β-AGONISTS ON HUMAN LUNG MAST CELLS

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The β_2-adrenoceptor (β_2-AR) is polymorphic at a number of loci and these polymorphisms may influence receptor function (Green *et al.*, 1995). Studies in transfected cell systems have shown that one polymorphism, thr164ile, in the fourth transmembrane domain of the β_2-AR alters ligand binding and impairs receptor coupling to G protein (Green *et al.*, 1995). The aim of the present study was to establish whether this polymorphism influences the ability of β-AR agonists to inhibit histamine release from human lung mast cells (HLMC).

Human lung tissue was obtained from surgical resections. For genotypic analysis, genomic DNA was extracted from a small amount of the lung tissue and genotype was determined by polymersae chain reaction-restriction fragment polymorphism (PCR-RFLP) essentially according to the method described by Aynacioglu *et al.* (1999). The rest of the tissue was physically and enzymatically disrupted to generate mast cell suspensions and these cells were used for histamine release experiments. Cells were incubated (10 min) with or without a β-AR agonist (0.1 nM-10 μM) before challenge with anti-human-IgE (1/300) for 25 min. Histamine release was determined by an automated fluorometric technique. Statistical treatments involved the use of Student's t test.

53 lung preparations were genotyped of which 50 were found to be homozygotes (thr164thr) and 3 were heterozygotes (thr164ile). None of the preparations were found to be homozygous for ile. These data are in general keeping with the findings of others (Liggett *et al.*, 1998). Attempts were made to correlate function with genotype. Both isoprenaline (ISO) and salbutamol (SAL) inhibited histamine release in a dose-dependent manner although SAL, relative to ISO, was a partial agonist. Maximal responses (Emax) and potencies (pD_2) for the inhibition by agonists, in both homozygotes (n=50) and heterozygotes (n=3), were obtained by model fits to the mean data. The parameter values together with s.e. in their estimates are found in the table (asterisk denotes that values are significantly different in heterozygotes compared to homozygotes with $P<0.01$ at least).

	Homozygotes		*Heterozygotes*	
Agonist	*Emax*	*pD_2*	*Emax*	*pD_2*
ISO	50±2	8.7±0.1	52±2	8.2±0.1*
SAL	45±1	7.3±0.1	36±1*	6.7±0.1*

These data indicate that ISO and SAL are more potent and that SAL is more efficacious in homozygous HLMC preparations than in heterozygous preparations. Thus, the thr164ile polymorphism in the β_2-AR could influence the extent to which β-AR agonists inhibit the responses of HLMC.

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109P EFFECT OF PYCNOGENOL ON MAST CELL HISTAMINE RELEASE

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Pycnogenol (PYC) is a mixture of bioflavonoid compounds from Pinus maritima bark with high ability to scavenge free radicals (Packer *et al.*,1999). Earlier studies have shown that a metabolic activity which causes free radical formation can also stimulate histamine release from mast cells (Di Bello *et al.*, 1998). Compound 48/80 and ionophore A-23187 are both active histamine releasers, with ability to stimulate free radical formation. We have therefore investigated if PYC can inhibit histamine release induced by these secretagogues. In addition, we have compared its activity with disodium cromoglycate (SCG), which is a known inhibitor of histamine release from mast cells.

Aliquots of mast cell suspensions (approximately 106/ml) were incubated at 37°C for 20 minutes with a range of PYC concentrations and challenged with ionophore A-23187 (5 μmol/l) or compound 48/80 (0.2 μg/ml). Histamine release in the supernatant was estimated as described earlier (Sharma & Jande,1989).

Mean (± SEM; n=8) IC50 values (μg/ml causing 50% inhibition of unblocked histamine release) for PYC were 53.56 ± 4.82 and 73.66 ± 4.77 for compound 48/80 and ionophore A-23187 respectively and were similar to those obtained with SCG. These results suggest that PYC can effectively inhibit histamine release from mast cells. Both compound 48/80 and ionophore A- 23187 have the ability to generate free radicals which are inducers of histamine release from body cells. It there-fore appears likely that inhibition of histamine release by PYC was due to its ability to scavenge free radicals.

While the availability of free calcium is essential for histamine release by ionophore A-23187 and compound 48/80, it is not yet known if PYC has a direct effect on calcium transport across the cell membrane and/or mobilisation of intracellular calcium from stores in the mast cell. Earlier studies have shown that SCG, which prevents histamine release from mast cells, is also an inhibitor of free radical formation; and others have recently demonstrated that oxygen free radicals can effectively alter calcium handling by body cells (Goldhaber & Qayyum, 2000).

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Dr. O.P. Gulati, Horphag Research, Switzerland kindly provided Pycnogenol.

110P PHARMACOLOGICAL DIFFERENCES BETWEEN ATYPICAL β-ADRENOCEPTORS IN RAT AORTA AND β_3-ADRENOCEPTORS IN RAT COLON

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We have previously reported that the selective β_3-adrenoceptor antagonist, SR 59230A (Manara *et al.*, 1996), at concentrations up to 1 μM, had no effect on atypical β-adrenoceptor-mediated relaxation in noradrenaline-preconstricted rat aorta (Brawley *et al.*, 2000). Effects of higher concentrations of SR 59230A were not reported in that study since preliminary results had shown that these caused a reduction in the size of the noradrenaline pre-constriction (unpublished). We have therefore carried out further studies in rat aorta using SR 59230A and the selective β_3-adrenoceptor agonist, BRL 37344 (Arch et al, 1984). Experiments were also carried out in rat colon, a tissue known to contain β_3-adrenoceptors (McLaughlin & MacDonald, 1990).

Male Wistar rats (200 - 250 g) were stunned and killed by cervical dislocation before removal of the thoracic aorta or distal colon. Ring preparations (aorta) or longitudinal segments (colon) were suspended in Krebs physiological saline solution (PSS) gassed with 95/5 % O_2/CO_2. at 37 °C for isometric recording. Aortic rings were pre-constricted with phenylephrine (0.6 μM) and distal colon segments were contracted with KCl (30 mM) before carrying out cumulative concentration-response curves (CRCs) to relaxants. In antagonist studies, tissues were incubated with antagonist for 30 min. before constricting. Values are mean±s.e.mean. Statistical analysis was carried out using Student's t test. BRL 37344 produced concentration-dependent relaxation of rat aorta (% maximum relaxation, 101±4 ; pEC_{50} 4.64±0.05, n=6) and rat colon (% maximum relaxation, 89±2; pEC_{50} 8.11±0.03, n=6). The relaxation was much slower in colon (up to 25 min to reach plateau) than in aorta (up to 8 min).

Pre-incubation with SR 59230A (30 nM – 1 μM) had no effect on CRCs to BRL 37344 in aorta (e.g. pEC_{50}: SR 59230A, 1 μM, 4.67±0.05; n=6, P>0.05). SR 59230A itself produced a slow (up to 25 min to reach plateau) concentration-dependent relaxation of phenylephrine-constricted aortic rings (% maximum relaxation, 102±3; pEC_{50}, 5.47±0.03; n=6).

Pre-incubation with SR 59230A (1 μM) produced a 93-fold shift of the BRL 37344 CRC in colon (pEC_{50}: SR 59230A, 6.14±0.06; n=6, P<0.001) with no change in the maximum response (SR 59230A, 92±4; P>0.05) giving an estimated pA_2 value of 7.97. SR 59230A also produced a slow, concentration-dependent relaxation of KCl-contracted colon (% maximum relaxation 99±6; pEC_{50}, 4.99±0.05; n=4).

These results show pharmacological differences between the atypical β-adrenoceptors of rat aorta and the β_3-adrenoceptors of rat colon. BRL 37344 and SR 59230A appear to mediate effects other than previously reported at β_3-adrenoceptors.

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McLaughlin, D.P. & MacDonald, A. (1990) *Br. J. Pharmacol.*, **101**, 569-574.

111P SR59230A IS NON-SELECTIVE BETWEEN BETA-ADRENOCEPTOR SUBTYPES IN MOUSE COLON CIRCULAR SMOOTH MUSCLE

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Recent studies suggest that β_3-adrenoceptors (β_3AR) are involved in sympathetic relaxation of gastrointestinal tissues (Manara *et al.*, 1996). Although selective β_3AR agonists are available, there are few antagonists. One potential candidate is SR59230A (SR; Manara *et al.*, 1996: pA_2 β_1=7.31±0.97, β_2=6.63±0.06, β_3=8.76±0.17). However, a wide range of pK_B values have been reported for SR at the β_3AR (e.g. Kaumann *et al.*, 1996, Galitzky *et al.*, 1997), suggesting that perhaps SR is not as selective as originally proposed. This has particular relevance for those who use SR to determine whether β_3AR signalling is present (Gao *et al.*, 1998). In the present study, the βARs present on murine colon were characterised using SR as an antagonist.

Mouse (CD1, dispatched by schedule 1 technique) colon rings (~ 5mm) were suspended in oxygenated (95% O_2 /5% CO_2) Krebs solution at 37°C, under 1g loading force. After attainment of a stable contraction to 80mM KCl (~ 1.5 hrs), 10μM phentolamine, 0.3μM desipramine and 30μM corticosterone were added to block α-AR and catecholamine uptake. Antagonists were incubated for 90min and cumulative agonist concentration-effect (E/[A]) curves were constructed. Agonist effects were normalised to the maximal relaxation caused by 100mM papaverine. For all groups, n=5-13.

CGP20712A, a selective β_1AR antagonist, had a flat Schild slope parameter (b=0.40±0.09) and a pA_2 of 8.40±0.06, suggesting the presence of the β_1AR and another receptor at a similar location. However, use of a selective β_2AR antagonist (ICI-118,551: 30nM), or agonist (terbutaline), provided no evidence of an important role for this subtype. SR (1μM) alone shifted the isoprenaline E/[A] curve in parallel with a pA_2 of 7.12±0.19 and in the presence of 0.1μM CGP20712A and 30nM ICI 118,551 (to block $\beta_{1/2}$ subtypes), a pK_B of 6.12±0.07 (b=0.98±0.12) was estimated with 1-10μM SR. 0.3μM SR failed to significantly shift CL316,243 (selective β_3AR agonist) E/[A] curves, consistent with a pA_2 <7. In a within-experiment comparison, 1μM SR shifted isoprenaline E/[A] curves with a pA_2 of 6.47±0.14 but failed to produce a shift in the presence of $\beta_{1/2}$AR blockade.

The pA_2 estimated for SR against isoprenaline (7.12±0.19) was lower than that expected at the β_3AR and closer to that reported for the β_1AR by Manara *et al.* (1996) and, in the presence of combined $\beta_{1/2}$ blockade, was lower still (6.12±0.07). In addition, the failure of 0.3μM SR to shift the CL316,243 E/[A] curve confirms that, in this assay, SR is a less potent and selective β_3AR antagonist than previously reported. Isoprenaline-induced relaxation of the mouse isolated colon appears to be mediated predominantly via β_1- and β_3-AR.

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112P PHARMACOLOGY OF CGP 12177 IN CHO CELLS TRANSFECTED WITH THE HUMAN β_2-ADRENOCEPTOR.

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The β_1/β_2-adrenoceptor antagonist CGP 12177 is a compound with high affinity for the β_2-adrenoceptor (Bylund *et al.*, 1994). This high affinity, together with its hydrophilicity, has made CGP 12177 an important probe for cell surface β_2-adrenoceptors. In the present study we have evaluated the effect of CGP 12177 on cyclic AMP response element (CRE) - mediated gene transcription responses in CHO-K1 cells.

CHO-K1 cells expressing the human β_2-adrenoceptor at 300fmol/mg protein and a secreted placental alkaline phosphate (SPAP) reporter gene under the trancriptional control of six CREs (McDonnell *et al.*, 1998) were used in the present study. Measurements of ^{3}H-cyclic AMP (cAMP) accumulation and SPAP secretion were made as described previously (McDonnell et al., 1998).

CGP 12177 acted as a partial agonist (EC_{50} 0.20 ± 0.02 nM; n=14) in stimulating CRE-dependent SPAP secretion yielding a maximum response that represented 64.9 ± 2.89 % (n=11) of the maximum response to 10 μM isoprenaline. Consistent with these responses being mediated by β_2-adrenoceptors, the SPAP response to isoprenaline (K_D = 1.00 ± 0.27nM; n=9) and CGP 12177 (K_D = 0.32 ± 0.01 nM; n=3) were antagonised by ICI 118551. CGP 12177 (100nM) was able to antagonize the responses to higher concentrations of the full agonist isoprenaline in a manner consistent with its partial agonist action. The dissociation constant of CGP 12177 (K_D) was 2.62 ± 0.89 nM (n=8). CGP 12177 produced a significant ($p < 0.05$) but small stimulation of cAMP accumulation (EC_{50} = 0.76 ± 0.02 nM; maximum response = 6.50 ± 0.37 % of the response to 10μM isoprenaline; n=3). Preincubation with CGP 12177 (100nM) produced a parallel shift in the concentration-response curve to isoprenaline (K_D 0.12 ± 0.02 nM; n=10). Pre-treatment with PD 98059 (MEK-1 inhibitor) reduced the SPAP responses to both isoprenaline (by 29.2 ± 1.29% n=3) and CGP 12177 (20.1 ± 3.43% n=3) suggesting that activation of the MAP kinase pathway is responsible for part of the agonist responses. Pertussis toxin (PTX) pre-treatment had no effect on the isoprenaline (97.2 ± 1.73%) or CGP 12177 (102.6 ± 6.9%) induced SPAP responses (n=3) suggesting Gi coupling is not involved.

The data obtained in the present study suggest that CGP 12177 can exhibit partial agonist activity in systems that are amplified by the signal transduction pathway (e.g. SPAP gene transcription versus cyclic AMP accumulation). The difference in apparent K_D values obtained for antagonism of isoprenaline-stimulated SPAP secretion and cyclic AMP accumulation by CGP 12177 suggests that the β_2-adrenoceptor exists in different affinity/G-protein coupled states. The first signals via cAMP and is responsible for *circa* 70% of the isoprenaline-stimulated SPAP response. The remaining 30% of the response to isoprenaline appears to be mediated via a PTX-resistant MAP Kinase pathway for which CGP 12177 has a 23 fold lower affinity.

JGB holds a Wellcome Trust Clinical Training Fellowship.

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113P HOMOLOGY MODELLING OF THE 11β-HYDROXYSTEROID DEHYDROGENASE ISOZYMES*

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Two 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes have been described. 11β-HSD type I has been implicated in the pathology of non-insulin dependent diabetes mellitus, neurodegeneration, hypertension, obesity and fertility and has therefore been identified as a good candidate for targeted drug design. Current successful drug design techniques are structure-based. These techniques require knowledge of the 3D structure of the binding site of the enzyme. The 3D structure of these isozymes has not yet been experimentally elucidated. Fortunately, the isozymes belong to the short chain dehydrogenase (SDR) family that is characterised by a highly conserved domain structure despite low sequence identities. The 3D structure of several of these homologous SDR proteins has been determined by X-ray crystallography.

The aim of this study was to build homology models of both isozymes in preparation for site-directed design of small molecule leads. 11β-HSD type II was modelled in order to explore the diversity and selectivity between the isozymes.

The comparative protein modelling method of Sali and Blundell (1993) was used as implemented in the program, MODELER. This method generates 3D models of proteins from a multiple sequence alignment of the target sequence with homologous proteins whose structures are known. Multiple models were generated for each isozyme based on four different alignments and templates and by varying the optimisation level. Regions that were poorly modelled were identified. An iterative approach to improving the accuracy of the models was adopted until the accuracy converged.

Whilst such approaches provide insight into the accuracy of model geometry, they are unable to predict whether an endogenous ligand would fit into the binding site. A method to determine binding site accuracy by docking known ligands is described (Luty *et al.*, 1995, Stouten *et al.*, 1993).

Stereochemical assessment of the models was performed using PROCHECK (Laskowski, 1993). Features assessed include bond lengths and angles, dihedral angles, phi-psi angle distribution on the Ramachandran plot (Ramachandran *et al.*, 1963) and non-bonded interactions. The geometry of the best models produced is comparable to that found in high resolution X-ray structures. Cortisol and cortisone were successfully docked into models of both isozymes generated from two of the alignments. These models had similar stereochemical accuracy. The best fit, measured as the mean potential energy change (ΔPE), was observed in models generated from the first alignment. The ΔPE was –70 and –65 kcal/mol for cortisol and cortisone respectively (first alignment), compared with –50 and –59 kcal/mol (second alignment) for 11β-HSD type I models. Docking of known ligands is therefore essential in identifying accurately modelled binding sites.

The authors thank Prof. J. Seckl, Department of Medical Sciences, University of Edinburgh, for providing the isozyme sequences.

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114P DIFFERENTIAL EXPRESSION OF GENES IN A PEYER'S PATCH M CELL CO-CULTURE MODEL BY TOGA™.

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Peyer's Patches (PP) in the GI tract consist of dome-shaped aggregates of lymphoid follicles that are covered by the follicle associated epithelium (FAE) that contains M cells. M cells are specialised lymphoid epithelial cells that can transport particulate matter from the lumen to B and T cells in the lymphoid follicle in order to initiate mucosal immunity and thus are potential targets for vaccine delivery. A modified co-culture of the human intestinal carcinoma (Caco-2) and lymphocyte (Raji B) cell lines has been developed which displays morphological and transport features expected in the human M cell phenotype (Gullberg *et al.*, 2000). The model may have potential for investigating targeted vaccine delivery. The aim of this work was to use the TOGA™ (TOtal Gene Expression Analysis) system to identify the gene's which are over-expressed in the co-culture as compared to normal Caco-2 cells and to validate and correlate the expression of those genes with ones detected in human PP tissue.

Co-cultures were grown as previously described (Gullberg *et al.*, 2000), along with normal Caco-2 monolayers and Raji B cells. Total RNA was extracted using a standard TriReagent (GibcoBRL) protocol. TOGA™ analysis, sequence verification and validation was carried out as previously described (Sutcliffe *et al.*, 2000). *In-situ* hybridisation (ISH), using dioxygenin labelled antisense sequences to the cloned mouse homolgues, and immunohistochemistry were carried out on mouse and human PP cryosections respectively.

TOGA™ analysis of the co-cultures revealed 30 up-regulated genes, 9 of which are novel. 12 genes were found to be epithelial- specific. A number of the up-regulated genes in the co-culture were found to code for membrane-bound proteins and receptors, while others coded for transcription factors and signalling proteins (Table 1).

Table 1. List of some of the genes overexpressed in the co-culture model

Gene	Fold Increase	Type
Known Sequence 4	120.0	Signalling
Known Sequence 6	4.5	Nutrient transporter
Known Sequence 7	2.1	Receptor
Known Sequence 38	19.5	Cell adhesion
Known Sequence 42	2.4	putative GPCR
Novel sequence 18	2.6	unknown
Novel sequence 34	12.2	unknown

ISH on mouse PP cryosections confirmed that many of the genes isolated from the co-culture were epithelial cell-specific *in vivo*. Expression was cross-confirmed with immunohistochemistry on human PP cryo- and paraffin-sections.

In conclusion, the co-culture appears to provide a useful model of the human M-cell phenotype. TOGA™ is a valuable gene-expression tool that may permit apical membrane receptors on human M cells to be screened with ligands in order to enable antigen delivery using targeted particles.

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115P ANALYSIS OF DIFFERENTIAL EXPRESSION OF GENES IN *IN VIVO* PEYER'S PATCH M CELL MODELS BY TOGA™.

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One of the major obstacles to the study of Peyer's Patch (PP) M cell gene expression is the isolation of viable cells in sufficient quantity and purity from animals models so as to detect M cell specific genes. The use of a M cell-like co-culture system goes some way towards aiding this problem (see accompanying Abstract), but it is still an *in vitro* model. The aim of this work was to modify and develop various methods of isolation of PP's and FAE from BALB-c mice, and to use TOGA™ (TOtal Gene Expression Analysis) to identify the genes which were up-regulated in the test samples over the controls. The use of *Salmonella* treatment to increase the number of M cells within mouse PP to increase the probability of detection of M cell specific genes was also investigated.

Whole PPs (PPW), normal villi (V) and mesenteric lymph nodes (LN) were dissected out from BALB-c mouse gut. A number of PP's were placed in RNAlater™ and individual domes (PD) were micro-dissected out under a dissecting microscope. FAE fractions and normal epithelial (NE) cell layers were extracted using 40mM EDTA in a HEPES buffer and agitation with a stirrer bar on a magnetic plate (modified from Bjerknes and Cheng, 1981). To increase the % of M cells in BALB-c mouse PP the mice were treated by oral gavage with $5x10^9$ *Salmonella typhimurium* SL3261 *aroA*⁻ in 200µl LB broth (modified from Savidge *et al.*, 1991). FAE and NE fractions were also isolated from such salmonella treated mice. M cell numbers were measured by confocal microscopy using M cell specific stains (FITC-UEA-1).TOGA™ analysis was carried out as previously described (Sutcliffe *et al.*, 2000) on total RNA (TRIreagent) isolated from the different cell models

TOGA™ results showed that the intensity of gene expression in PD was greater than or equal to that in PPW, indicating that the micro-dissection of PPW into PD is likely to have enriched for FAE specific genes. In addition, with 2 exceptions, all genes found to date that were up-regulated in the PD were also up-regulated in the FAE, while 30 validated genes were isolated that were only increased in the FAE (Table 1). This supports the notion that FAE purification from PPW by EDTA incubation can enrich for FAE specific genes. The salmonella treatment was shown to increase M cell numbers in BALB-c mouse PP by 73% ($p<0.01$ by two-sample t-test). Salmonella treated mouse FAE showed up-regulation of 15 genes not found in untreated FAE, which may be due to the increased # of M cells, or the possibly reaction of the PP to the bacteria.

Table 1: No. of validated upregulated genes as compared to control V/NE

Fraction type	# of genes
Up in Purified FAE, not PD or LN	30
Up in purified FAE and PD not LN	8
Up in purified FAE, PD and LN	15
Uniquely up in salmonella enriched FAE	15

In conclusion the use of TOGA™ highlights the benefits of micro-dissection techniques and bacterial enrichment methods in the study of M cell biology.

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116P SCREENING PHAGE DISPLAY LIBRARIES *IN VIVO* FOR THE IDENTIFICATION OF INTESTINAL TARGETING LIGANDS

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Oral delivery of vaccines giving rise to a mucosal immune response is highly desirable, as many pathogens invade via mucosal surfaces. M-cells are professional antigen sampling cells that are found in the epithelium of the gut associated lymphoid tissue or Peyer's patch. The transcytotic capacity of M cells and the downstream processing of antigen sampled would suggest that targeting vaccines to M cells would enhance oral immunisation (Foster *et al*, 1998). However, to date no human M-cell marker has been identified as a target for delivery of vaccines and/or other drugs through the M-cell route. The aim of this study was to investigate the use of *in vivo* Phage Display technology for the identification of novel targeting ligands for M cells of Peyer's patch.

The key advantage of this technique is that peptides are selected on the basis of interaction with target receptors *in situ*. Phage display technology has been used previously for the identification of peptide ligands that target tumor vasculature (Arap *et al,* 1998) and vasculature of different organs (Rajotte *et al,* 1998).

A 12-mer peptide phage display library displaying up to 10^9 phage clones was administered into rat intestinal loops that contained at least one Peyer's patch (n=5). Following a 2-hour incubation bound phage were amplified in *E. coli* and isolated by PEG precipitation. A total of four "biopanning" cycles were performed. In addition, *in vitro* ELISA-based screening was performed to examine binding to intestinal PP and non-PP tissue homogenates of several species. DNA from Peptides were synthesised for use in binding, uptake and immunohistochemical studies.

All clones selected demonstrated broad species specificity including human intestinal cell lines. There was no difference in binding of phage clones to PP compared with non-PP tissue. Thirty unique sequences were identified. Many of the clones were selected in more than one rat used in the screening cycles. Analysis of the peptide sequences revealed that several peptides contained common tripeptide motifs. Two peptides shared a tetrapeptide motif. Searches were performed using the Swissprot database to identify possible homologues for each peptide sequence.

Several potential target receptors were identified but need confirmation. The 30 unique peptides were synthesised with various tags for use in binding and uptake studies aimed at designing vaccine delivery technologies. In addition, stabilised versions of these peptides were synthesised. Immunohisto-chemistry was carried out on human intestinal tissue using synthesised peptides and binding to epithelium was demonstrated. Peptides with high binding affinities (K_d <30µM) were conjugated to fluorescent-loaded particulate systems to examine drug delivery capabilities in cell and animal models.

Here we have described the use of *in vivo* phage display technology as a method for identification of targeting ligands to sites in the gastrointestinal tract. The technology has provided us with potential ligands for targeting for drug or vaccine delivery by the oral route.

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117P MODULATION OF INNATE AND ACQUIRED IMMUNE RESPONSES WITH CPG MOTIFS FROM BACTERIAL DNA

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Synthetic oligodeoxynucleotides (ODN), that include cytidine-phosphate-guanosine (CpG) unmethylated motifs found in bacterial DNA are potent adjuvants for boosting immune responses to third part antigens (Gurunathan *et al.*, 2000; McCluskie *et al.* 2001). However their mechanism of action are not fully understood. The aim of this study was to examine the capacity of different CpG containing ODN for their ability to modulate innate and acquired immune responses in mice.

Bone marrow derived immature dendritic cells (DC) were prepared by cultivating murine bone marrow cells for 10 days with granulocyte monocyte-colony stimulating factor (GM-CSF). Immature DC and murine macrophages (J774 macrophage cell line) were incubated with CpG-containing or control ODN (0.4-10μg) or LPS (1μg) for 4 or 24 hours. The supernatants were assessed for chemokines, MIP-1α, MIP-1β, RANTES and MIP-2 and cytokines IL-12, IL-10 and TNF-α by enzyme linked immunosorbent assay (ELISA). DC maturation was determined by assessing the expression of B7-1, B7-2, CD40, MHC class II and CCR5 by FACscan analysis. Induction of acquired immune responses was assessed by immunization of mice with the antigen, keyhole limpet haemocyanin (KLH; 1 μg) in the presence of CpG-containing or control ODN (50 μg per mouse). T helper (Th) subtypes induction was determined by assessing IFN-γ and IL-5 production by lymph node and spleen cells in response to antigen stimulation *in vitro*. Antibody responses were determined by ELISA.

CpG-ODN induced the production of MIP-1α, MIP-1β, RANTES, MIP-2, IL-12 and TNF-α from DC and J774 cells. CpG-ODN enhanced CD40 and B7.1 expression on DC by 28% and 32% respectively; MHC class II and B7.2 expression was upregulated to a lesser extent. The *in vivo* studies demonstrated that CpG induced T cell responses to an unrelated antigen after a single immunization. KLH-specific T cell cytokine production in spleen cells or cells draining lymph nodes was enhanced in mice immunized with KLH in presence of the CpG-ODN. Anti-KLH serum IgG was also augmented by the CpG-ODN. The subclass of antibody demonstrated a predominant IgG2a response, but this was affected by the route of immunization.

The data demonstrate that CpG-ODN are capable of enhancing adaptive immune responses, in particular immune responses of the type 1 T cell subtype. This may be explained by the ability of CpG-ODN to stimulate DC maturation and to enhance production of the regulatory cytokine, IL-12 and the chemokines, MIP-1α, MIP-1β and RANTES, which stimulate the differentiation and recruitment of Th1 cells.

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118P EVIDENCE THAT LEPTIN ACTS AS A SHORT TERM SATIETY FACTOR

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We have recently reported that i.p., but not s.c., administration of leptin produces a short lasting decrease in food intake in hungry rats and suggested that leptin released from the stomach during a meal may play a role as a short-term peripheral satiety factor (Patel and Ebenezer, 2001). The present study was undertaken to provide further support for this hypothesis.

Experiment 1. Male Wistar rats (b.wt. 250 – 320g, n=16) were fasted for 22h and injected i.p. with either vehicle (n=8) or leptin (10μg kg^{-1}; n=8). Immediately after injection they were placed separately into experimental cages with free access to food. Food intake was measured as described previously (Ebenezer, 1992). The animals had free access to water at all times. Experiment 2. Male Wistar rats (b. wt. 260 – 310g, n=7) were deprived of water for 16h each day. Prior to experimental sessions they were injected i.p. with either vehicle or leptin (10μg kg^{-1}) and put into experimental cages with free access to water. Water intake was measured as described previously (Ebenezer et al., 1992). A cross-over experimental design was used with each rat receiving both treatments. Experiment 3. Male Wistar rats (b.wt. 230 – 280g, n=8) were fasted for 22h and injected i.p. with either vehicle or leptin (10 μg kg^{-1}). Immediately after injection they were placed separately in recording cages were they had free access to food and water. The behaviour of the rats was recorded on video tape and analysed off-line. The duration the animals engaged in each of five behaviours, i.e. feeding, drinking, motor'activity, grooming, and resting, were monitored in 5 min bins. Each rat received both treatments in a cross-over experimental design. The cumulative data from all 3 experiments were analysed by using appropriate 2-tailed t-tests.

The results obtained in Experiment 1 show that leptin (10 μg kg^{-1}) Injected i.p. significantly decreased cumulative food intake at 15 and 30 min after injection. The most pronounced effect was during the first 15 min (Mean food intake (g) ± s.e. mean: vehicle = 4.3 ± 0.5g, leptin = 2.5 ± 0.3g, $p<0.01$). By contrast, leptin (10 μg kg^{-1}; i.p.) had no effect on water intake (Experiment 2; Mean water intake (ml) ± s.e. mean at 15 min: vehicle = 11.9 ± 0.9ml, leptin = 11.7 ± 0.8ml, n.s.). The results obtained in Experiment 3 show that compared to vehicle treatment, leptin (10 μg kg^{-1}; i.p.) significantly increased the cumulative time the rats spend feeding, and grooming during the first 15 min after injection (Mean duration (min) ± s.e.mean spent (a) feeding: vehicle 9.8 ± 0.24 min, leptin 8.8 ± 0.4 min, $p<0.05$; (b) grooming: vehicle 0.29 ± 0.05 min, leptin 0.62 ± 0.1 min, $p<0.05$). The duration the animals spent engaging in the other behaviours was not significantly altered by leptin.

The results confirm and extend our previous observations (Patel and Ebenezer, 2001) and show that i.p. administration of leptin (10μg kg^{-1}) reduces food intake in 22h food-deprived rats. Moreover, this effect appears to be behaviourally specific, as leptin does not decrease water consumption. Furthermore, leptin (10μg kg^{-1}) produced alterations in the behaviour of the rats during the first 15 min after administration (i.e., a reduction in time spent feeding and a decrease in time spent grooming) which is consistent with the actions of a short-acting satiety factor (see Antin et al., 1975). Thus, the present results lend further support to the suggestion that leptin acts as a short-term peripheral satiety factor (Patel and Ebenezer, 2001).

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119P NICOTINE DEPENDENCE AND THE CONTINGENT NEGATIVE VARIATION IN RATS

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A number of behavioural studies have shown that animals become dependent on nicotine after chronic exposure to the drug (e.g. Hall and Morrison, 1973). The aim of the present study was to investigate whether dependence to nicotine could be demonstrated using an electrophysiological measure known as the Contingent Negative Variation (CNV). The CNV is a slow negative wave that can be recorded from the scalps of conscious human subjects or rats during the interval between a warning stimulus (S1) and a response stimulus (S2) (see Ebenezer, 1986a). In general, drugs that increase arousal increase the magnitude of the CNV and drugs that decrease arousal decrease it magnitude (see Ebenezer, 1986b).

Male Wistar rats (n=12) were chronically implanted under Equithesin anaesthesia with silver-silver chloride electrodes. The active electrode was located over frontal cortex and the reference electrode was located over ipsilateral occipital cortex (Ebenezer, 1986a). EEGs were recorded by means of a.c. amplifiers (time constant = 15s). Each rat was fasted for 22h a day and placed separately in an operant chamber. When tone S1 (100ms, 1400Hz) was paired with the automatic delivery of food reinforcement (S2), a negative shift developed in the electroencephalogram (EEG) during the S1-S2 interval. Twenty-five trials were presented to a rat in a single session and averaged using the technique of signal averaging. Experiment 1: In this experiment, the S1-S2 interval was 2s. The rats (n=8) were injected s.c. with either saline (n=4, Group A) or nicotine (0.4 mg kg^{-1}; n=4, Group B) 10 min prior to each of 10 daily training sessions. On day 11, both groups of rats were injected s.c. with saline 10 min prior to the CNV session. Experiment 2. Rats (n=4) received training sessions in the S1-S2 paradigm until they generated steady CNVs. No drug treatment was given prior to each of the training sessions. In this experiment, the S1-S2 interval was 3s. CNVs were recorded from the rats following s.c. injection of saline. The rats were then injected twice daily with nicotine (0.4 mg kg^{-1}, s.c.) for the next 5 days. At the end of this period CNVs were recorded from the rats after they had received saline (s.c.).The results from Experiment 1 show that when nicotine was replaced with saline the mean CNV area of the rats (Group B) was significantly reduced (paired t-test) compared with the mean CNV area obtained under nicotine [Mean CNV area ± s.e.mean after (a) nicotine (day 10) = 94.8 ± 6.9 μV.s, (b) saline (day 11) = 45.4 ± 17.6 μV.s, $p<0.02$]. By contrast, there was no significant change in the mean CNV area in the rats (Group A) that received saline on days 10 and 11. Statistical analysis of the results obtained in Experiment 2 (paired t-test) shows that the mean CNV area recorded under saline following chronic treatment with nicotine was significantly reduced compared with that recorded under saline prior to drug treatment [Mean CNV area ± s.e.mean (a) prior to chronic nicotine = 211.0 ± 22.3 μV.s, (b) after chronic nicotine = 153.8 ± 17.6 μV.s, $p<0.05$]. The results from Experiment 1 & 2 suggest that regardless of whether the rats have been (a) trained to generate CNVs under nicotine or (b) chronically exposed to nicotine after they had been trained to generate CNVs, withdrawing nicotine results in a reduction in their magnitude. It is suggested that the rats become dependent on nicotine to maintain an optimal level of arousal, and when nicotine is withdrawn their level of arousal is altered so that they cannot attend to the S1-S2 paradigm as previously. The present results suggest that the CNV may provide a useful elctrophysiological means of assessing dependence to nicotine and other psychoactive drugs.

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120P EFFECTS OF FORSKOLIN ON ADIPOSE TISSUE cAMP ACCUMULATION DURING CHRONIC ETHANOL TREATMENT

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We have previously shown that chronic ethanol drinking significantly decreases the level of adipose tissue hormone-sensitive lipase (HSL) activity in parallel with falls in tissue cAMP. (Shih & Taberner, 2000). During withdrawal there were rebound increases in HSL and cAMP levels. Since chronic ethanol has been reported to down-regulate adenyl cyclase (Tabakoff *et al.*, 1995), we have studied the direct effect of forskolin on cAMP accumulation *in vitro* in adipose tissues from ethanol-treated mice.

Adult male CBA/Ca mice were given either water (control) or 24% (v/v) ethanol as sole drinking fluid (CED) for up to 4 weeks (Jelic *et al.*, 1998), or acute ethanol (0.1 or 2.5 g kg^{-1}), or saline was injected i.p. 1h prior to removal of brown and white adipose tissue for cAMP assays, as described previously (Shih & Taberner, 1997). Forskolin (100 M) was added to isolated fat cells to stimulate cAMP production. Differences between groups (means ± s.e.mean) were analysed by Student's t test.

Ethanol added to the incubation medium had no effect on basal or stimulated cAMP at concentrations up to 200 mM. The 2.5 g acute dose markedly suppressed forskolin-stimulated cAMP accumulation in both tissues (results are shown in Table 1). Short-term CED had no effect on cAMP levels, whereas the 28 day regime reduced stimulated cAMP in both brown and white fat. There was a rebound increase in basal cAMP in brown fat after withdrawal from CED. The results demonstrate that brown and white fat respond differently to ethanol, and that prolonged exposure to ethanol differentially affects basal and stimulated cAMP levels in the two tissues.

Table 1 Treatment group	Brown fat Basal cAMP	Brown fat Stimulated cAMP	White fat Basal cAMP	White fat Stimulated cAMP
Control untreated	1.45± 0.09	15.8 ± 3.4	2.19±0.16	16.4±3.9
0.1 g /kg ethanol	1.21± 0.13	12.3 ± 1.4	7.52±0.78**	22.4±4.4
2.5g /kg ethanol	1.02±0.05**	1.41±0.25**	2.25±0.08	6.87±0.82**
CED 5 day	1.97±0.11	14.7 ± 2.5	1.98±0.07	18.4±2.69
CED 28 day	1.07±0.05**	2.11±0.29**	3.54±0.54**	8.85±1.31**
CED 28 day, 9h withdrawal	6.05±0.72¶¶	16.4±2.7	2.20±0.34	20.6±4.3

cAMP: nmol.mg $protein^{-1}$ over 10 min.
Controls, n = 16, others, n = 8; $P < 0.01$ vs control **, CED ¶¶

From the time-dependence of the effects of CED on basal and stimulated cAMP in brown fat, and the rebound response seen in withdrawal, we conclude that the alterations in cAMP accumulation represent a direct adaptation of adenylyl cyclase to the continued presence of ethanol and are independent of an action at the G protein level.

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121P EFFECTS OF CHRONIC ETHANOL CONSUMPTION AND ABSTINENCE ON D1-LIKE RECEPTOR BINDING

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Our previous work showed increased locomotor stimulant effects of amphetamine, cocaine and nicotine when these drugs were administered repeatedly after chronic ethanol consumption (Manley and Little, 1997; Watson and Little, 1999). This study examines D1-like receptor binding within the projection areas of the mesolimbic dopamine system.

Male TO strain mice were housed in groups of 2-3, maintained on a 12h-light/12h-dark cycle (lights on 07.00h). Ethanol was administered for 21 days by liquid diet, on a schedule that produced physical dependence, as described previously (Manley and Little, 1997); ethanol intake was 24-31 g/kg/24h. Control animals were pair fed an isocalorific liquid diet. After cessation of the ethanol treatment all animals were placed on a normal laboratory chow diet.

Binding parameters of dopamine D1-like receptors were measured in saturation experiments using the D1-like receptor antagonist [^{3}H]-SCH23390. Cerebrocortical and striatal membrane preparations, obtained from mice (n=6/treatment group) at 24h, 6 days and 2 months after cessation of chronic ethanol consumption were suspended in 20 v/w of modified Tris-HCl buffer pH 7.4. Specific binding was measured by incubation with [^{3}H]-SCH23390 (150 pM to 4 nM)) for 2h at ambient temperature. Non-specific binding was measured by parallel incubation with 100 μm butaclamol.

Binding affinity for [^{3}H]-SCH23390 was significantly increased in the striatum at 24h ($P<0.02$), 6 days ($P<0.01$) and 2 months after withdrawal from ethanol treatment, compared with control values. No changes were observed in binding affinity in cerebrocortical preparations, or in Bmax in cortical or striatal tissues. This increase in D1-like receptor binding affinity may be an adaptive response to the reduction in the firing rate of VTA dopaminergic neurones demonstrated following withdrawal chronic ethanol consumption (Bailey et al., 1998). Synaptic efficiency would be maintained, and behaviour would therefore appear overtly normal, until drugs such as the psychostimulants were administered.

Table 1. Bmax (fmol/mg/protein) and Kd (nM) of [^{3}H]SCH23390 binding to D1 like striatal receptors, mean ± s.e.m.*P<0.01/**P<0.02 cf controls (Student's t-test).

Abstinence period:		24h	6 days	2 months
Controls	Kd	0.91±0.06	0.79±0.03	0.96±0.09
	Bmax	316.4±12.1	352.7±28.4	327.8±12.3
Chronic ethanol	Kd	0.51±0.02*	0.38±0.02**	0.50±0.02*
	Bmax	306.4±33.1	329.7±22.2	311.1±32.2

Table 2: Bmax (fmol/mg/protein) and Kd (nM) for [^{3}H]-SCH23390 binding to D1-like cerebrocortical receptors.

Abstinence period:		24 hours	6 days	2 months
Controls	Kd	1.12±0.05	0.98±0.08	1.07±0.07
	Bmax	243.7±47.7	191.4±31.1	207.5±15.7
Chronic ethanol	Kd	0.96±0.06	0.97±0.07	1.02±0.08
	Bmax	182.6±18.0	191.4±31.0	223.0±16.7

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122P BIOCHEMICAL CHARACTERISATION OF MICE TRANSGENIC FOR A MUTATION IN AMYLOID PRECURSOR PROTEIN (APP) KNOWN TO CAUSE FAMILIAL ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is characterised by a deficit in markers of the cholinergic system, including reduced choline acetyltransferase activity and pre- and postsynaptic receptor density (Francis et al., 1999). Tg2576 mice, which overexpress a transgene of human APP carrying the Swedish mutation (K670N, M671L), demonstrate age-dependant amyloid (Aβ) deposition, senile plaque formation and cognitive impairment (Hsiao et al., 1996). To investigate whether these mice also exhibit cholinergic dysfunction, regional biochemical markers of this system were determined.

Brains from Tg2576 mice and non-transgenic (non-Tg) littermates (20-35 g) of both sexes and 3 ages (4, 8 and 13 months) were dissected to yield cortex, hippocampus and striatum and stored frozen (-70° C) until assayed. Membranes were prepared as previously described (Minger et al, 2000), with slight modification, and incubated under the conditions in Table 1. Thus, membranes (200 l) were incubated with 25 l of a single concentration (approximately equal to the published K_d) of the appropriate radioligand and 25 l incubation buffer (total binding) or the appropriate displacer (non-specific binding). Binding was terminated by filtration through GF/C filters using a Skatron cell harvester and radioactivity determined by scintillation counting. Data were expressed relative to total protein using the Bradford method.

Cortical muscarinic M1 and M2 binding was significantly reduced (by 70% and 60% respectively) in Tg2576 mice compared to non-Tg littermates at 13 months of age (Table 1). There were no significant reductions in younger age groups, although a 25-30% decrease was seen in these receptors in 8 month old animals. No significant changes in binding to nicotinic receptor subtypes (α4β2 or α7) were seen.

In conclusion, although the loss of muscarinic receptors in the 13 month old Tg2576 mice exceeds that seen in AD, it does coincide with cognitive impairment and Aβ deposition in these animals (Hsiao et al, 1996).

Table 1. Comparison of binding to cholinergic receptor subtypes in 12 month old Tg 2576 mice and non-Tg littermates.

		Receptor binding non-Tg	Tg
Receptor	Incubation Conditions		
M1/ctx	5nM [^{3}H]pirenzepine, 100μM atropine sulphate, 1h, 25°C	591 ± 70	188 ± 34*
M2/ctx	4nM [^{3}H]AF-DX 384, 1μM atropine sulphate, 1h, 25°C	972 ±134	390 ± 62*
α4β2/ctx	400pM [^{3}H]epibatidine, 250nM cytisine, 4h, 25°C	126 ± 5	121 ± 8
α7/ctx	2.5nM [^{3}H]Methyllycaconitine, 1mM	79 ± 10	72 ± 3
α7/hip	nicotine, 2h, 25°C	78 ± 16	79 ± 22

Values are expressed in fmol/mg protein as mean ± s.e.m. for n=5-6 per group. * $p< 0.01$, Student's unpaired t-Test.

123P (-)BACLOFEN REDUCES [^{3}H]-DOPAMINE RELEASE FROM RAT VENTRAL TEGMENTAL AREA SLICES VIA $GABA_B$ RECEPTOR ACTIVATION

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The ventral tegmental area (VTA) contains the cells of origin of the mesoaccumbens dopaminergic system, which plays an important role in motor, cognitive and affective functions. Dopamine (DA) neurones in VTA are modulated by two GABA-containing pathways arising from interneurones and descending projections from the basal forebrain, innervating respectively $GABA_A$ and $GABA_B$ receptors in VTA (Johnson & North, 1992). *In vivo*, the $GABA_B$ agonist, baclofen, infused into the VTA causes a decrease in both axonal and somatodendritic DA release in the mesoaccumbens system (Yoshida *et al.*, 1994). In the present study we have developed a method to examine the effect of (-)baclofen on the electrically evoked release of [^{3}H]DA from rat VTA *in vitro*, as a preliminary to investigating its possible involvement in the suppression of addictive behaviours(Corrigall *et al.*, 2000).

The VTA was isolated from male Wistar rats (~250g) and cross-chopped into 250×250μm slices, which were then suspended in oxygenated Krebs-HCO_3^- containing ascorbic acid (0.3μM) and pargyline (10μM) and incubated with [^{3}H]DA (0.1μM) at 37°C for 30min. After rinsing in buffer additionally containing nomifensine (2.5μM), slices were transferred to chambers of a Brandel apparatus and superfused (0.5 ml.min^{-1}) for 35min before collection of consecutive 4.5min samples. [^{3}H]DA release was induced by electrical stimulation (20mA, 2Hz, 4min), which occurred 15 min after the pre-perfusion period (S1), and again 60min later (S2). Drugs were added 35min before S2. Release of radioactivity was expressed as fractional release (FR). Evoked release (S1 and S2) was calculated as the mean of the increased FR above baseline in the two fractions after the start of stimulation. Drug effects were assessed by comparing the ratio S2:S1 in the presence and absence of drug. Data were expressed as mean±s.e.mean and analysed using one-way ANOVA with post-hoc Dunnett's test.

Removal of Ca^{2+} (+ EGTA 1mM) or addition of tetrodotoxin (1μM) to the superfusion buffer (15min prior stimulation) abolished the electrically evoked [^{3}H]DA release. (-)Baclofen (0.1-100μM) dose-dependently reduced the evoked [^{3}H]DA release from VTA slices (EC_{50}: 0.103μM, 95%CI: 0.043-0.249, n=4-10). The $GABA_B$ antagonist CGP35348 (1μM, CGP) reversed the action of (-)baclofen (10μM) but did not produce any direct effects on the evoked [^{3}H]DA overflow (S2:S1, control:1.087±0.10, bacl: 0.731±0.08*, CGP+bacl: 0.926±0.14, CGP alone: 0.993±0.13, *P<0.05 vs control, n=6-10).

We have demonstrated that (-)baclofen can reduce the electrically evoked [^{3}H]DA release from slices of VTA in a concentration-dependent fashion and that this effect is mediated by $GABA_B$ receptor activation.

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124P $GABA_B$ RECEPTOR DISTRIBUTION IN HIPPOCAMPI RESECTED FROM PATIENTS WITH TEMPORAL LOBE EPILEPSY: ANTAGONIST BINDING

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Temporal Lobe Epilepsy (TLE) is a common seizure disorder which, when associated with hippocampal sclerosis (HS), is frequently resistant to drug therapy. Surgical resection of the hippocampus in such intractable cases of TLE has proven to be a highly effective way of suppressing the seizures. However, the basis for this effect is little understood although it seems reasonable to assume that changes in neurotransmitter action are involved. A decrease in $GABA_B$ receptor function has been reported in an animal model of TLE and this may contribute to the production of seizures (Mangan and Lothman 1996). In a previous study using resected hippocampal tissue from patients (Billinton et al 2001) it was also shown that there were selective regional decreases in the density of $GABA_B$ binding sites using the agonist ^{3}H-GABA as the receptor ligand. In the present study we have made a similar regional study but now using the high affinity antagonist, ^{3}H-CGP 62349 (0.5nM K_D Bittiger et al 1996) as the ligand to assess whether the pattern of changes are the same.

Brain hippocampal TLE specimens were frozen within 30 min of the surgical removal. The control specimens were collected 18-30 hours post-mortem and frozen straightaway. Each specimen was sliced (12 μm) on a cryostat at ~-20^{0}C and the sections collected on to electrostatically charged slides and stored at ---80^{0}C until the day of processing. Slides were removed from the freezer, dried at room temperature for 45 min and then incubated for 20 min in buffer solution (Tris-HCl 50 mM, pH 7.4 plus $CaCl_2$) and again for 60 min in fresh solution before drying under a stream of cool air for 30 min. Each section was then incubated for 60 min in solutions containing the receptor ligand, ^{3}H-CGP 62349 (0.125-8nM). Non-specific binding was determined by the addition of another $GABA_B$ antagonist, CGP 54626A (10 μM). The incubation solution was then aspirated and the sections washed twice in Tris buffer solution and distilled water. The sections were then dried under a stream of cool air for 30 min and apposed to autoradiography film. The films were developed after 21 days and the images analysed using an MCID imaging device. Material from fifteen TLE and ten post mortem controls were used for the study. Statistical analysis was performed using the Student's t-test (unpaired, two-tailed)

The number of specific ^{3}H-CGP 62349 binding sites was significantly reduced by 23+/- 5% in dentate gyrus (DG) of the TLE tissue compared with post mortem control. No significant changes were detected in any other region of the hippocampus. The binding affinity of ^{3}H-CGP 62349 was also increased in the DG with a 31% reduction in the K_D value. In other regions the K_D value was the same as controls. Whilst these data concur to some extent with those previously reported for the agonist, ^{3}H-GABA (Billinton et al 2001), there appeared to be less reduction in the density of the antagonist binding sites within the hippocampal regions of the TLE tissue. This may indicate that there are alterations in receptor coupling rather than any changes in the receptor protein *per se*.

We thank Novartis for ^{3}H-CGP 62349 and the National Society for Epilepsy for financial support.

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125P SPECTRUM OF ANTICONVULSANT ACTIVITY AGAINST SOMAN-INDUCED BURSTING IN THE GUINEA-PIG *IN VITRO HIPPOCAMPUS*

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

Neuronal activity in *str. pyramidale* of area CA1 was recorded extracellularly in 500 µm thick hippocampal slices isolated from male Dunkin Hartley guinea-pigs and maintained in a submersion type recording chamber at 31°C. Following positioning of the recording electrode, slices were monitored for at least 30 min for evidence of spontaneous activity (a criterion for slice rejection). Data are expressed as means ± s.e. mean.

Application of 0.1 µM soman led to the appearance of synchronised bursting activity in the majority of slices tested (latency to onset: 21.4 ± 2.5 min; discharge frequency 5-15 min after initial appearance of epileptiform activity: 1.8 ± 0.2 bursts/min; $n = 35$ slices).

Test compounds were applied to the slices 15 min after the appearance of soman-induced bursting, and their antiepileptiform effects were assessed by measuring the burst frequency during a 10 min epoch (30-40 min after drug application) and comparing it to the burst frequency over the 10 min epoch immediately before drug application. The table *(below)* summarises the effect of the various interventions (to facilitate comparisons, the concentration producing a 25%-75% reduction of burst discharge frequency is illustrated):

compound	concn. (µM)	burst rate (% of control)	no. of slices
cyclopentyladenosine	0.01	27.3 ± 4.9	4
atropine	0.3	61.2 ± 7.4	5
clozapine	3	52.7 ± 14.6	3
midazolam	10	26.5 ± 5.0	4
biperiden	10	44.0 ± 10.0	4
phenytoin	30	71.3 ± 36.4	3
untreated	-	123.8 ± 10.6	5

Gabapentin (300 µM; 3 slices) and the NK_1 antagonist, L-733,060 (10 µM; 4 slices), failed to inhibit soman-induced bursting: burst rates at 30-40 min were 163.3 ± 27.0% and 164.6 ± 35.6%, respectively, of pretreatment values.

These findings demonstrate that epileptiform bursting induced by soman can be inhibited by a variety of pharmacological interventions, with cyclopentyladenosine clearly possessing the most potent activity. With the caveat that the present *in vitro* model may not be predictive of *in vivo* anticonvulsant efficacy, the results provide further support for the evaluation of selective A1 adenosine receptor agonists in the management of OP-related seizures (Van Helden and Bueters, 1999)

Supported by the UK Ministry of Defence.

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126P EFFECT OF MAGNESIUM IONS ON BICUCULLINE-INDUCED EPILEPTIFORM ACTIVITY IN HIPPOCAMPAL ORGANOTYPIC SLICES AND MONOLAYER HIPPOCAMPAL NEURONAL NETWORKS

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There is a high incidence (>20%) of epileptic seizures in patients presenting with acute traumatic head injuries (Vespa *et al.*, 1999). Following the use of magnesium salts in the prevention and treatment of eclamptic seizures (Lucas *et al.*, 1993), and as a neuroprotective agent in stroke (Muir & Kennedy, 1995), its potential for the prevention of seizures in head trauma is currently being evaluated. This study investigated the effect of magnesium sulphate on bicuculline-induced epileptiform activity on two long-term cultured *in vitro* hippocampal preparations.

Primary dissociated Wistar rat hippocampal neurones from E18 foetuses were cultured on 64-channel multiple micro-electrode array plates (MMEAs) for 33-34 days *in vitro* to form monolayer hippocampal networks (MHNs) (Sokal *et al*, 2000). Organotypic hippocampal slice cultures (OHCs) were prepared from 6 day old male Wistar rats and cultured for 22-36 days *in vitro* (Gähwiler *et al*, 1997). Single-unit or multiple single-unit extracellular neuronal activity was recorded and analysed using *NeuroEXplorer* (Plexon Inc., USA). All data are presented as mean±SEM (OHCs n=6 cells/5 slices and MHNs n=25 cells/2 MMEAs). Statistical significance levels were determined using a two tailed Mann-Whitney U test.

Following recording of basal activity in 1mM Mg^{2+}, 10µM bicuculline methobromide evoked epileptiform-like activity, characterised by an increased firing rate and bursting activity, which was synchronised in multi-neuronal recordings (Sokal *et al*, 2000). Increasing Mg^{2+} ion concentration from 1-10mM produced a concentration-dependent decrease in firing rate (OHCs: EC_{50}=2.2mM; MHNs: EC_{50}=4.3mM). Bicuculline-induced bursting activity (a burst defined as a minimum of three spikes occurring with interspike intervals <300ms and a minimum duration of 10ms) was significantly decreased ($p<0.001$) by 4mM Mg^{2+} in both OHCs and MHNs from 8.1±3.0 to 2.4±0.6 bursts.min^{-1}; 23.3±4.0 to 10.1±1.2 bursts.min^{-1}, respectively.

Magnesium was found to abolish bicuculline-induced epileptiform activity in both monolayer and organotypic hippocampal cultures, the effects presumably mediated via blockade of NMDA receptor function and/or synaptic neurotransmission. This suppressive action of Mg^{2+} lies within the effective range used to reduce/abolish seizure activity in acute head injuries currently under clinical evaluation.

LJ is supported by a BBSRC-GlaxoWellcome Studentship.

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127P COMPARISON OF LEVETIRACETAM AND PHENYTOIN IN 6Hz VERSUS 60Hz ELECTROSHOCK SEIZURE MODELS

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The 6Hz model of electroshock seizures has recently been re-evaluated as a tool to investigate phenytoin-resistant seizures (Barton et al, 1999). These authors reported that 6Hz electrical stimulation in CD1 mice produced seizures that could be blocked by clonazepam, phenobarbital and valproic acid, but not by phenytoin. In addition, the authors reported that the new antiepileptic drug, s-levetiracetam, was effective in this 6Hz model, but not in the 60Hz maximal electroshock test.

In the present study, we examined 30 min pre-treatment (s.c.) with three antiepileptic drugs: valproic acid, phenytoin and s-levetiracetam (at doses shown in tables 1 & 2) on the seizures induced by corneal electroshock at 6 and 60 HZ in male NMRI mice (30g). Parameters used were 1) 6Hz for 3s, and 2) 60 Hz for 0.4s, at currents between 12 and 30mA.

Separate groups of 5 mice were used once at each current, and the number of animals responding with tonic hind-limb extension (within 10s) were counted. Fishers Exact Probability test was used to compare the effect of drug vs. vehicle.

The results (in tables 1 & 2) demonstrated that at both frequencies, s-levetiracetam (80 mg/kg) reduced seizure incidence at 15mA, but not at higher currents. This indicates that this drug can raise electroshock thresholds but is not active against maximal seizures. In addition, phenytoin (10 mg/kg) was effective at 6Hz (20mA) but not at 60Hz, indicating a greater sensitivity to phenytoin at that frequency.

Strain, or stimulator, differences and a different behavioural endpoint may explain the discrepancies between this study and that of Barton et al. However, in our hands this 6Hz model is more sensitive to phenytoin, and not resistant to it, and s-levetiracetam showed no distinction between the frequencies.

Barton, M.E., Wolf, H.H., White, H.S. (1999) *Soc. Neurosci. Abstr.* 25: 453.18

Table 1: No. of mice (out of 5) showing hindlimb extension in response to 6 Hz stimulation (* = P<0.05 c.f. vehicle)

Current	12mA	15mA	20mA	25mA	30mA
Vehicle	0	4	5	-	-
Phenytoin 5 mg/kg	-	-	0*	0	1
Phenytoin 10 mg/kg	-	-	0*	0	0
Saline	0	3	5	-	-
Valproate 80 mg/kg	0	3	5	-	-
Valproate 160 mg/kg	-	0	3	4	-
Saline	2	5	5	-	-
Levetiracetam 10 mg/kg	0	2	5	-	-
Levetiracetam 80 mg/kg	0	0*	5	-	-

Table 2: No. of mice (out of 5) showing hindlimb extension in response to 60 Hz stimulation (* = P<0.05 c.f. vehicle)

Current	12mA	15mA	20mA	25mA	30mA
Vehicle	0	3	5	-	-
Phenytoin 5 mg/kg	-	0	2	3	-
Phenytoin 10 mg/kg	-	-	0*	1	-
Saline	0	4	5	-	-
Valproate 80 mg/kg	0	4	5	-	-
Valproate 160 mg/kg	-	-	2	3	5
Saline	2	5	4	-	-
Levetiracetam 10 mg/kg	0	3	5	-	-
Levetiracetam 80 mg/kg	0	0*	5	-	-

128P THE $[K^+,Cl^-]$-COTRANSPORT INHIBITOR R(+)-DIOA SELECTIVELY INHIBITS GABA-STIMULATED $^{36}Cl^-$ ION INFLUX AT DIAZEPAM-INSENSITIVE HUMAN RECOMBINANT $GABA_A$ RECEPTOR SUBTYPES.

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Inhibitory $GABA_A$ receptors are pentameric assemblies of multiple subunits with integral chloride channels belonging to the ligand-gated ion channel receptor family. Benzodiazepine pharmacology is influenced by residues in both α and γ subunits and receptors can be broadly divided into diazepam-sensitive (α1,2,3,5) and insensitive subtypes (α4,6). The diuretic furosemide was previously shown to be a selective antagonist for diazepam-insensitive $GABA_A$ receptors, having highest affinity for α6-containing receptors but also inhibiting α4, with asparagine 265 in the β2/3 subunit contributing to its selectivity (Thompson *et al.*, 1999). Here we show another ion transport inhibitor also selectively inhibits diazepam-insensitive receptors.

Ltk^- cells stably expressing human recombinant $GABA_A$ subunits ($\alpha n\beta3\gamma2s$), grown in DMEM with 10% (v/v) FetalClone II serum, were seeded into 96 well plates at densities of $3\text{-}8x10^4$ cells/ml in a 200μl/well volume and grown for 7 days. Receptor expression was induced 24 hr prior to experiment with 1μM dexamethasone. Cells were washed with HEPES/Krebs buffer at room temperature (pH 7.4, 1M Tris) using a Dynatech 96 plate washer. Measurement of ^{36}Cl influx was performed in chloride-free buffer at 4°C, with acetate salts replacing chloride salts, and test compounds were added for a 30 sec preincubation using a Robbins Hydra 96 dispenser. At t=30 sec a second addition of ^{36}Cl ligand solution ± GABA was made and influx terminated at t=37 sec with 100μM picrotoxin stop buffer. Influx was determined by scintillation counting on a Packard TopCount.

Using a concentration of GABA eliciting a control (EC_{50}) response, the effects of a range of chloride transport inhibitors were examined on $GABA_A$ ion channel function. GABA stimulated ^{36}Cl influx into cells expressing α1-6$\beta3\gamma2s$ $GABA_A$ receptors in a concentration-dependent manner. EC_{50} values (μM) respectively were : α1 3.9±0.5 (n=13); α2 1.6±0.4 (n=12); α3 10±0.7 (n=12); α4 6.4±0.6 (n=17); α5 1.6±0.3 (n=17); and α6 2.8±0.5 (n=18). Agonist responses were specifically and concentration-dependently blocked by the competitive $GABA_A$ antagonist bicuculline (100μM) and the chloride channel blocker picrotoxin (100μM).

Furosemide selectively inhibited ^{36}Cl influx at $\alpha6\beta3\gamma2s$ receptors (IC_{50} 1.2±0.2μM, n=11) and to a lesser extent at $\alpha4\beta3\gamma2s$ receptors (IC_{50} 146±15μM, n=12) over $\alpha1\beta3\gamma2s$ (IC_{50} 2210±120μM, n=9). This subtype-selective inhibition by furosemide was independent of inhibition of the $[Na^+/2Cl^-/K^+]$ cotransporter, since another blocker, bumetanide, was without effect. DIOA ([(dihydroindenyl)oxy]alkanoic acid), an inhibitor of the $[K^+,Cl]$-cotransporter and the $[Cl^-/HCO_3^-]$ anion exchanger (Garay *et al.*, 1988) also exhibited subtype-selective inhibition of $GABA_A$ receptors but did not display the same degree of separation between $\alpha4\beta3\gamma2$ and $\alpha6\beta3\gamma2$. 10μM DIOA produced little inhibition of α1, 2, 3 or 5-containing receptors but inhibited $\alpha4\beta3\gamma2$ by 50±1.5% (n=12) and $\alpha6\beta3\gamma2$ by 70±3% (IC_{50} 4.5±0.5μM, n=12). Both furosemide and DIOA exhibited non-competitive inhibition, significantly decreasing the maximum response.

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Thompson, S.A. *et al.* (1999) *Mol. Pharmacol.* 55: 993-999.

129P ANXIOGENIC DRUGS INDUCE FOS EXPRESSION IN A SPECIFIC SET OF FOREBRAIN REGIONS IN THE RAT

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In a recent study we reported that pharmacologically diverse types of anxiogenic drugs activated a specific set of hindbrain areas that are also activated by various fearful stimuli (Singewald & Sharp, 2000). Here we tested whether there are also neural substrates within key forebrain areas of the fear/anxiety circuitry (Charney et al. 1998), that are commonly activated by anxiogenic drugs. To this end we administrated four pharmacologically diverse drugs at doses known to be active in various rodent animal models of anxiety. Fos expression was used as a marker of neuronal activation.

Male Sprague-Dawley rats (250-300g) were habituated to the procedures room for at least 24 h, and then were injected i.p. with one of the following: the α_2-adrenoceptor antagonist yohimbine (5 mg/kg), the benzodiazepine inverse agonist N-methyl-β-carboline-3-carboxamide (FG-7142, 7.5 mg/kg), the non-selective 5-HT_{2C} receptor agonist m-chlorophenyl piperazine (mCPP, 5 mg/kg), the adenosine antagonist caffeine (50 mg/kg), or saline vehicle. Two h later rats were anaesthetised with sodium pentobarbitone and perfused transcardially with 4 % paraformaldehyde. Brain sections were then processed for Fos immunocytochemistry as described previously (Singewald & Sharp 2000).

The main results are shown in Table 1. In brief, compared to saline controls all 4 drugs commonly increased Fos-like immunoreactivity (Fos-LI) in 7 out of 32 brain areas investigated: central nucleus of amygdala (cAMY), bed nucleus of stria terminalis (BNST), lateral septum, pre- and infralimbic cortex (IL-Ctx), paraventricular nucleus of the hypothalamus (PVN) and lateral hypothalamus. The effects were greatest in cAMY and PVN and smallest in lateral hypothalamus and lateral septum. In addition, all drugs but mCPP increased Fos-LI in the dorsomedial hypothalamus, cingulate cortex, medial and basolateral amygdala. No common effects were observed in other regions including the hippocampus or other parts of the hypothalamus.

Table 1. Effect of anxiogenic drugs on Fos-LI in rat forebrain.

	cAMY	PVN	BNST	IL-Ctx
Saline	3.2±0.7	3.9±0.9	1.9±0.4	5.0± 0.6
mCPP	17.3±2.3*	16.3±5.1*	7.5±1.0*	7.6±0.8$^+$
Caffeine	25.5±1.3*	26.4±5.6*	14.1±.3.0*	7.1±0.5$^+$
Yohimbine	30.7±2.1*	43.8±4.4*	14.5±2.3*	11.5±.2.6$^+$
FG-7142	32.7±2.1*	31.4±7.7*	14.5±2.4*	12.5±2.4$^+$

Values (means ± s.e.m, N=6-8) are counts of cells with Fos-LI/0.01 mm^2. $^+$ $p<0.05$, * $p<0.01$ vs saline, Kruskal-Wallis one-way ANOVA, Mann-Whitney U Test.

In summary, yohimbine, FG-7142, mCPP and caffeine commonly induced Fos-LI in a discrete set of hypothalamic areas, and limbic regions centred on parts of the amygdala, BNST, septum, and prefrontal cortex. These areas are recognised as major components of the fear/anxiety circuitry in the forebrain identified in behavioural models (Charney et al. 1998). Moreover, this circuitry has reciprocal connections with parts of fear/anxiety circuitry in the hindbrain, including the locus coeruleus and periaqueductal gray, that yohimbine, FG-7142, mCPP and caffeine also activate (Singewald & Sharp 2000). Therefore, an integrated forebrain/hindbrain neuronal system may process information that is common to both environmentally- and pharmacologically-evoked anxiety.

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130P MODULATION OF CYCLIN PROTEIN EXPRESSION DURING THE C6 GLIOMA G1 PHASE BY THE ANTICONVULSANT VALPROATE.

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Progression of the cell cycle is controlled by a complex network of cyclin proteins, cyclin-dependent kinases (cdks) and phophatases that regulate cdk activity. In general, cyclins are unstable, being transiently upregulated during cell cycle progression, which upregulates cdk activity at crucial cycle restriction points. This phenomenon is best characterised in *Saccharomyces cerevisiae* where cyclin-cdk interactions control G1-S and G2-M phase transitions.

As there has been little investigation of these interactions in mammalian systems, the current study aimed to elucidate cyclin/cdk regulation in the rat C6 glioma cell cycle. In addition, the effect of the anticonvulsant valproate on cyclin regulation was determined, since the teratogenic potency of this drug has been linked to cell cycle arrest (Bacon et al., 1998).

The C6 cell cycle was synchronised by mitotic selection, as verified by a surge of thymidine incorporation at the G1-S phase transition. In three separate and independent experiments, cells were harvested throughout the 12h G1 phase, and extracts subjected to immunoblotting with monoclonal antibodies. Immunoblot analysis revealed the appearance of cyclin D3 expression only in the late G1 phase, while cyclin D1 was expressed throughout the G1phase but was upregulated at the 4-5h time period. Following exposure to 2mM valproate for 48h, cyclin D3 expression was induced in asynchronous C6 glioma cells in comparison to cells treated with the vehicle (dimethylsulphoxide) alone. This was associated with precocious upregulation of cyclin D3 at 4-6h into G1– which coincides with the point in the cell cycle at which the drug mediates its antiproliferative effect. Valproate-induced upregulation of cyclin D3 was dose-dependent in the 1 to 3 mM range. In contrast, expression of the proliferating cell nuclear antigen (PCNA) was reduced by valproate, while the expression of the cdks was unaffected. These findings suggest that the antiproliferative and prodifferentiative actions of valproate may be mediated by perturbation of cyclin D3 expression during the G1 phase.

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131P EVIDENCE THAT OREXIN-A EVOKED GROOMING, BUT NOT WATER INTAKE, IN THE RAT IS MEDIATED BY THE OREXIN-1 (OX_1) RECEPTOR

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Orexin-A (OxA) is a potent agonist at the OX_1 and OX_2 receptor (OX_1R and OX_2R). We have shown previously that OxA elicits a marked grooming response after intracerebroventricular (icv.) administration to rats and that this response is blocked by the OX_1R antagonist, SB-334867 (Duxon *et al.*, 2000). The aim of this study was to further determine the role of the OX_1R in the OxA elicited grooming and water intake response in the rat using two novel pharmacological tools, the OX_1R antagonist SB-408124 and the OX_2R peptide agonist SB-668875 (Brough *et al.*, this meeting).

Male CD rats (200-250 g) were implanted with a unilateral cannula directed at the lateral ventricle, as described in Duxon *et al.* (2000), to allow icv. administration of OxA (3µg/5µl for grooming studies or 0.3-30µg/5µl for water intake studies), SB-668875 (0.3-30µg/5µl) or vehicle (water, 5µl) immediately prior to assessment of whole body grooming or water intake (two separate studies). The effect of SB-408124 was assessed upon OxA elicited grooming or upon OxA elicited water intake (1-10 mg/kg, po. and 30 mg/kg, po. respectively) by pretreating animals with this OX_1 antagonist 30 minutes prior to icv. injection. Grooming behaviour (duration and number of bouts) and water intake (mls consumed) were assessed in the hour following icv. dosing. N = 6-8 per behavioural group.

SB-408124 significantly inhibited OxA elicited increases in grooming time and grooming bouts (Figure. 1). OxA, at a dose

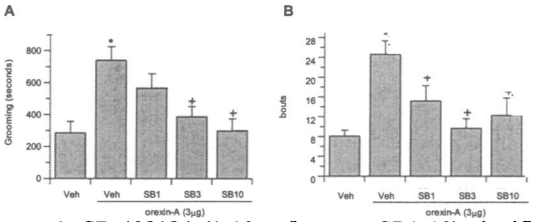

Figure 1. SB-408124 (1-10mg/kg, po., SB1-10) significantly antagonised ($^+P<0.05$ from veh/OxA) the significant (*P<0.05 from veh/veh) elevation in grooming time (A) and bouts (B) elicited by OxA.

(30µg) inducing a robust/significant increase in water intake (Veh=0.7±0.1ml, 30µg=2.6±0.3ml [P<0.05]), was unaffected by pretreatment with SB-408124 (30 mg/kg, po.; SB-408124/OxA=2.7±0.7ml). The OX_2-selective peptide, SB-668875 (0.3-30µg, icv.) had no effect on either grooming behaviour or water intake at any of the doses tested. This data further demonstrates that OxA elicited grooming is mediated by the OX_1R in the rat. Despite the higher dose of OxA required to elicit a significant increase in water intake (compared to grooming), dosing animals with a 30mg/kg dose of SB-408124 was still unable to antagonise this effect suggesting putative OX_2R involvement. However, the failure of SB-668875 to elicit an increase in water intake, at doses which increase locomotor activity (Gartlon *et al.*, this meeting), currently prevents OxA-induced increases in water intake from being ascribed to the OX_2 receptor.

Duxon, M.S., Stretton, J., Starr, K.S. *et al.* (2000) Psychopharmacology 153: 203-209

132P ROLE OF OX_1 AND OX_2 RECEPTORS IN MEDIATING THE MOTOR ACTIVITY RESPONSE TO THE OREXINS

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Orexin-A and -B are potent agonists at the OX_1 (pEC50: 8.0 and 7.3, respectively) and OX_2 (pEC50: 8.2 and 8.4, respectively) receptors (Smart *et al.*, 1999). We have shown previously that both orexins cause motor activation in rats and that orexin-B mediated hyperactivity is blocked by the OX_1 receptor antagonist, SB-334867 (Hagan *et al.*, 1999; Jones *et al.*, 2001). SB-668875 is a novel peptide agonist with selectivity for OX_2 over OX_1 receptors (pEC50: 7.84 and 5.83, respectively; Brough *et al.*, this meeting). Therefore, the aims of this study were to determine the motor activity response to SB-668875 and investigate the influence of the OX_1 receptor antagonist, SB-408124 (pKb = 7.78 [OX_1], 5.77 [OX_2], Brough *et al.*, this meeting), upon the motor activity response to the orexins and SB-668875.

Male CD rats (200-250 g) were implanted with a unilateral cannula directed at the lateral ventricle (anaesthesia was Domitor 0.4 mg/kg sc. and Sublimaze 0.45 mg/kg ip.). Habituated (>60 min) motor activity was recorded in an automated activity monitor (total beam breaks and total cage transits, see Jones *et al.* 2001) 60 min post-icv. administration. Peptides or saline (Veh) were injected icv. (5 µl). The response to orexin-A (1-10 µg) and SB-668875 (1-30 µg) was initially determined. For antagonist studies, rats received SB-408124 (10 mg/kg po.) or vehicle (1% MCW) 30 min prior to testing. In separate studies, Veh or orexin-A (3 µg), orexin-B [10 µg; Jones *et al.*, (2001)] or SB-668875 (10 µg) (n = 11-12 for each group) were administered immediately before testing.

Orexin-A increased beam breaks at all doses tested (eg. Veh = 649 ± 69; 1 µg = 1490 ± 133 [P<0.05]), but failed to significantly increase cage transits. SB-669975-DM increased beam breaks at 10 and 30 µg (eg. Veh = 866 ± 102; 10 µg = 1773 ± 237 [P<0.05]) and cage transits at 30 µg (Veh = 22 ± 6; 30 µg = 56 ± 11 [P<0.05]). SB-408124 did not reverse an increase in beam breaks or cage transits in response to orexin-A. Similarly, an SB-668875 mediated increase in beam breaks was not affected by SB-408124. In contrast, orexin-B caused an increase in beam breaks (Veh = 411 ± 93; 10 µg = 1085 ± 123 [P<0.05]) which was partially reversed by SB-408124 (797 ± 165 [P = 0.13]) and, further, an increase in cage transits (Veh = 7.8 ± 2; 10 µg = 24 ± 4 [P<0.05]) which was significantly reversed by SB-408124 (11 ± 4 [P<0.05]).

The motor activity response to orexin-A and orexin-B clearly differ. SB-408124 reversed orexin-B-induced hyperactivity, suggesting a role for OX_1 receptors in this response. However, SB-408124, which reverses orexin-A-induced grooming (Duxon *et al.*, this meeting) failed to block either orexin-A- or SB-668875-induced motor activation, suggesting that OX_1 receptors are not involved in this behavioural response. A role for OX_2 receptors is inferred, but confirmation of this requires an OX_2 selective receptor antagonist.

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133P CO-LOCALISATION OF NEUROPEPTIDE Y5 RECEPTORS ON NEURONS CONTAINING OREXIN A, OREXIN B OR MELANIN CONCENTRATING HORMONE IN THE RAT LATERAL HYPOTHALMUS

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Interactions between various centres within the hypothalamus are believed to play an important role in the central control of food intake. Leptin is believed to enter the brain at sites close to the arcuate nucleus (ARC; Kalra *et al.,* 1999), where neuropeptide Y (NPY) containing neurons are found. These project to the lateral hypothalamus (LH) where they innervate distinct populations of neurons expressing the peptides orexin and melanin concentrating hormone (MCH; Broberger *et al.*, 1998). To establish a link between the ARC and the LH, we examined the distribution of the NPY Y5 receptor, thought to be important in feeding, in the LH. A series of double labelling immunohistochemical studies were undertaken to determine the separate localisation of the NPY Y5 receptor with orexin A (OXA), orexin B (OXB) or MCH.

A polyclonal antibody against a unique, ten amino acid, synthetic peptide sequence, from NPY Y5 receptor was raised in a female Duncin-Hartley guinea pig (600g). The antiserum was purified by caprylic acid/ammonium sulphate precipitation. Adult male Sprague-Dawley rats (300-350g) were perfused transcardially with 4% paraformaldehyde. Brains were removed and sectioned to 80μm using a vibrotome (Leica VT 1000S, Leica Microsystems Ltd.). Sections containing hypothalamus (bregma −1.3 to −3.8mm; Paxinos & Watson, 1982) were incubated with OXA (1:350), OXB (1:350; Alpha Diagnostics, USA) or MCH (1:750; gift from Knoll Ltd) rabbit primary antisera along with NPY Y5 receptor (1:75) guinea pig antiserum, prior to addition of fluorescence-labelled secondary antibodies. Immunoreactivity was visualised using confocal microscopy (Leica TCS4D).

NPY Y5 receptors showed a strong, punctate immunoreactive signal, which was observed in all regions examined. It appeared to be concentrated in the distal cytoplasm and in fine processes. In some cells, the staining appeared to have a nuclear component. OXA and OXB immunoreactivity was detected in cell bodies throughout the hypothalamus. MCH showed fine granular staining which was only detected in the LH. This staining was distinctive, which would be appropriate for a protein associated with Golgi or endoplasmic reticulum. Thick, axonal type processes labelled for OXA, OXB or MCH. Co-localisation of NPY Y5 receptor immunoreactivity with that for OXA or OXB or MCH was observed in cell bodies. However, no co-localisation was seen in processes.

These results suggest that cells in the rat LH which express OXA, OXB or MCH may be downstream targets for NPY, possibly implicating these peptides in the leptin signalling system.

This work was funded by Knoll Ltd & University of Nottingham

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134P ELECTROPHYSIOLOGICAL EVIDENCE FOR 5-HT_2 RECEPTOR-MEDIATED CONTROL OF 5-HT CELL FIRING IN THE DORSAL RAPHE NUCLEUS OF THE ANAESTHETISED RAT

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The firing of 5-HT neurones in the midbrain raphe nuclei is regulated by $5HT_{1A}$ autoreceptors (Barnes & Sharp, 1999). Earlier electrophysiological studies by Garratt et al. (1991), found that the $5HT_2$ agonist 1-(2,5-di-methoxy-4-iodophenyl)-amino-propane (DOI) inhibited 5-HT cell firing in the dorsal raphe nucleus (DRN) *in vivo*, however the role of 5-HT_2 receptors could not be confirmed with the antagonists then available. Interestingly, new *in vitro* data suggest that DRN 5-HT neurones are inhibited by $5HT_{2A}$ and 5-HT_{2C} receptors located on adjacent interneurones (Lui et al., 2000). Here we sought to confirm the effect of DOI on 5-HT cell firing in the DRN *in vivo*, and test further the pharmacology of this effect.

Male Sprague-Dawley rats (240-320 g) were anaethetised by chloral hydrate supplemented with saffan. Single barrel glass electrodes (filled with 2 M NaCl and 2 % pontamine sky blue; 10-16 MΩ) were stereotaxically lowered into the DRN for extracellular recordings of single unit activity (Hajos et al., 1995). 5-HT neurones were identified on the basis of their firing characteristics, the inhibitory effect of 8-OH-DPAT, and the DRN location of the recording site. Drugs were injected via a tail vein. DOI was injected in doubling doses (10-100 μg/kg) at 120 s intervals with firing rate being measured in the final 60 s of each interval. In some experiments, 5-HT_2 receptor antagonists ritanserin (1 mg/kg), MDL 100907 (0.2 mg/kg) or SB 206553 (0.5 mg/kg) were injected 300 s prior to DOI (10-100 μg/kg). Data (n=5-8 rats/group) were analysed using 1 or 2 way ANOVA with appropriate post hoc tests.

DOI caused a dose-related inhibition of 5-HT cell firing (20-100 μg/kg; $p<0.0001$ versus pre-drug values, 1 way ANOVA). The effect of DOI was apparent at 20 μg/kg, and 100 μg/kg reduced firing to 20 % of pre-drug levels. The inhibitory effect of DOI was almost completely blocked by pretreatment with either the $5HT_2$ antagonist ritanserin ($p<0.0001$, 2 way ANOVA) or the selective $5HT_{2A}$ antagonist MDL 100907 ($p<0.0001$, 2 way ANOVA). The $5HT_{2B/2C}$ selective antagonist SB206553 also blocked the action of DOI although only at the lower doses ($p<0.0001$, 2 way ANOVA).

In summary, this study confirms earlier findings (Garratt et al., 1991) by showing that the 5-HT_2 agonist DOI inhibits the firing of 5-HT neurones in the rat DRN *in vivo*. Importantly, our experiments using selective 5-HT_2 receptor antagonists indicate that this effect is mediated by 5-HT_2 receptors, with the 5-HT_{2A} subtype playing a major role. The 5-$HT_{2B/C}$ subtype may also have minor involvement. Our data are consistent with recent *in vitro* electrophysiological evidence that DRN 5-HT neurones are regulated by $5HT_{2A}$ and 5-HT_{2C} receptors located on GABAergic interneurones in the DRN (Lui et al, 2000). Further investigation of the neuroanatomical substrate(s) underlying the 5-HT_2 receptor-mediated regulation of 5-HT cell firing *in vivo* is underway.

LJB is supported by a MRC Industrial Collaborative Studentship with Eli Lilly & Co.

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135P THE 5-HT_2 AGONIST DOI INCREASES THE EXPRESSION OF THE IMMEDIATE EARLY GENES ARC, HOMER, C-FOS AND ZIF-268 IN RAT BRAIN REGIONS *EX VIVO*

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The induction of immediate early genes (IEGs) by 5-HT agonists can be used as a measure of the genomic response to 5-HT receptor activation, and more specifically as a molecular anatomical marker of increased 5-HT function. Expression of the transcription factor IEG *c-fos*, and the effector IEG *arc*, increases after administration of 5-HT_2 receptor agonists such as 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI; Moorman et al., 1995; Pei et al., 2000). However, the relative sensitivity of these IEGs to 5-HT_2 receptor agonists is uncertain, as is the influence of these drugs on other IEGs. Here we test the effect of different doses of DOI on the expression of two transcription factor IEGs (*c-fos* and *zif 268*) and two effector IEGs (*arc* and *homer*).

Groups of male Sprague-Dawley rats (250-270 g) were injected s.c. with DOI (0.5, 2 or 4 mg/kg) or saline vehicle, and 1 h later brains were removed and frozen prior to sectioning. Sections were probed for IEG mRNA using in situ hybridisation (Pei et al., 2000). The relative abundance of the different IEGs was determined in 12-14 brain regions by densitometric quantification of the autoradiograms.

DOI caused a dose-related increase in the mRNA abundance of *arc* and *c-fos* in cortical regions, including those noted in Table 1. Interestingly, in cortical areas DOI also increased the expression of *homer* and *zif 268* (Table 1). Of the four IEGs tested, abundance of *arc* mRNA increased the most, with the greatest effect being observed in the frontal cortex. In contrast to cortical areas, IEG expression was not altered in the caudate putamen or regions of the hippocampus (Table 1).

Table 1. Effect of DOI on abundance of *arc*, *homer*, *zif 268* and *c-fos* mRNA in rat brain regions.

	DOI (mg/kg s.c.)	Frontal cortex	Parietal cortex	Caudate Putamen	CA1
Arc	-	100±12	100±16	100±14	100±15
	0.5	283±78	310±88[a]	102±8	87±9
	2	424±75[b]	396±17[b]	101±7	91±19
	4	330±12[a]	383±7[a]	89±5	34± 2
Homer	-	100±7	100±5	100±7	100±9
	0.5	123±6[a]	130±7[b]	116±6	115±2
	2	135±7[b]	131±9[a]	119±6	102±4
	4	144±5[b]	120±9	105±11	99±6
Zif 268	-	100±8	100±9	100±10	100±14
	0.5	113±10	125±14	104±6	91±6
	2	129±2[a]	130±9	95±5	89±6
	4	135±13	137±9[a]	99±5	77±10
c-fos	-	100±25	100±18	100±12	n.d.
	0.5	116±28	206±52	116±16	
	2	152±13	265±19[a]	127±8	
	4	143±21	285±40[a]	183±57	

Data are mean±s.e.m. values (n=4) expressed as % of control.[a]$p<0.05$; [b]$p<0.01$ *vs* saline (one-way ANOVA followed by Dunnett's *t*-test). n.d.; no detectable.

In summary, these data show that not only *arc* and *c-fos* but also, *homer* and *Zif-268* increase in expression in response to the 5-HT_2 agonist, DOI. Of these IEGs, *arc* appears to be the most sensitive measure of increased 5-HT function.

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136P EFFECTS OF 5-HT, FENFLURAMINE, KETANSERIN AND THE 5-HT AUTORECEPTOR ANTAGONIST, SB-272183, ON BASAL [^{3}H]5-HT RELEASE FROM GUINEA-PIG CORTICAL SLICES IN THE PRESENCE AND ABSENCE OF PAROXETINE

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We have compared the ability of 5-HT, fenfluramine, the 5-HT_{2A} receptor antagonist ketanserin and the 5-HT_{1A}/5-HT_{1B}/5-HT_{1D} receptor antagonist SB-272183 (Roberts *et al.*, 2001) to increase basal release of [^{3}H]5-HT from guinea-pig cortical slices. We have also examined the effects of paroxetine (Px, 1μM) on the release of [^{3}H]5-HT by these agents. Paroxetine is a competitive inhibitor of the serotonin transporter (SerT) but, unlike fenfluramine, is not a substrate (Thomas *et al.*, 1987; Fuller *et al.*, 1988).

Release experiments were carried out as described by Roberts *et al.* (1996) in the presence or absence of paroxetine (1μM), but in the absence of any electrical stimulation, in order to measure the effects of the drugs on basal release of [^{3}H]5-HT. The effects of 5-HT, fenfluramine, ketanserin and SB-272183 on [^{3}H]5-HT release in the presence and absence of paroxetine are summarised in Table 1.

Control	5-HT (30nM)	Fenfluramine (10μM)	Ketanserin 100nM	Ketanserin 1μM	SB-272183 100nM	SB-272183 1μM
Compound alone	303 ± 12*	348 ± 21*	129 ± 18	319 ± 65*	102 ± 7	109 ± 12
+ 1μM Px	103 ± 2**	198 ± 53	142 ± 19	263 ± 63*	100 ± 7	125 ± 8

Table 1. Data shown are peak effects (mean ± s.e.m.; n = 3) expressed as % of relative control values (* significant increase cf. basal, ** significant reversal with 1μM Px; $p < 0.01$, 2-way ANOVA).

5-HT (30nM) potently stimulated [^{3}H]5-HT release, an effect which was fully reversed by paroxetine. Fenfluramine (10μM) and ketanserin (1μM) both stimulated [^{3}H]5-HT release to similar extents. Paroxetine partially reversed the effect of fenfluramine, but had no effect on ketanserin-stimulated release. SB-272183 had little or no effect on basal [^{3}H]5-HT release. These data indicate that 5-HT stimulation of its own release occurs by homo-exchange of [^{3}H]5-HT for 5-HT via the SerT, since the effect is fully reversed by the SerT inhibitor paroxetine. Fenfluramine is also a substrate for the SerT; however, hetero-exchange appears only partially responsible for fenfluramine-induced [^{3}H]5-HT release, since there is a residual component of release that is insensitive to paroxetine. Since fenfluramine-induced 5-HT release can be partially inhibited by KN-62, an inhibitor of depolarisation-induced Ca^{2+} influx through voltage-gated Ca^{2+} channels (Cinquanta *et al.*, 1997), the paroxetine-insensitive component of release might involve a Ca^{2+} dependent, exocytotic-like mechanism.

Ketanserin can bind to non-serotonergic sites in mammalian striatum and increase 5-HT, dopamine and noradrenaline efflux from striatal slices (Leysen *et al.*, 1988). Here we demonstrate this effect on 5-HT in guinea-pig cortex and show that it is insensitive to paroxetine. Ketanserin also has affinity for the 5-HT_{1D} receptor (K_i = 79 nM; Gothert *et al.*, 1996), a purported 5-HT autoreceptor; however, the autoreceptor antagonist SB-272183 has no effect on basal [^{3}H]5-HT release, suggesting that ketanserin is not producing its effect via this pathway. In conclusion, 5-HT, fenfluramine and ketanserin all stimulate [^{3}H]5-HT efflux from guinea-pig cortical slices. These effects are respectively, fully reversed, partially reversed and unaffected by paroxetine.

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137P EFFECT OF CHRONIC FLUOXETINE IN COMBINATION WITH WAY 100635 ON 5-HT$_{1A}$ RECEPTORS: AN AUTORADIOGRAPHIC AND ELECTROPHYSIOLOGICAL STUDY IN RAT BRAIN

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Combined treatment with 5-HT$_{1A}$ antagonists and fluoxetine may prove useful to shorten onset of antidepressant effect of selective serotonin (5-HT) re-uptake inhibitors (SSRIs). Recently, it has also been shown using microdialysis that concurrent administration of WAY100635 with fluoxetine prevents desensitization of dorsal raphe nucleus (DRN) 5-HT$_{1A}$ autoreceptors (Hervás et al., 2001) but there are not electrophysiological studies addressing this matter. Thus, this study was aimed to analyse the effect of a 14 days treatment (osmotic minipumps) with fluoxetine (10 mg/kg/day), WAY100635 (0.1 mg/kg/day) or the combined treatment in male Wistar rats (250-300 g).

Cryostat-cut brain sections (20 µM) were preincubated in 170 mM Tris-HCl buffer (4 mM $CaCl_2$, ascorbic acid 0.01%, pH 7.6) for 30 min at room temperature. The sections were then incubated in the same buffer with 2 nM [^{3}H]8-OH-DPAT in the presence of 10 µM pargyline for 1 h at room temperature. Non-specific binding was defined by 10 µM 5-HT. Following incubation, sections were dipped, rinsed twice 5 min in Tris-HCl (4°C, pH 7.4), dipped in cold water and then dried under a cold air stream. Sections were exposed to [^{3}H]-Hyperfilm (4 weeks) before quantification by image analysis.

Serotonergic cell firing in DRN was measured in chloral-hydrate anaesthetised rats (400 mg/kg i.p.) using standard extracellular recording methods (Aghajanian et al., 1970). Single-barrelled glass micropipettes (3-8 MΩ) were implanted in DRN (ML 0 mm and AP 1 mm to lambda). 5-HT neurons (0.5-2.5 Hz and 0.8-1.2 ms) were encountered over –5-6 mm DV). The effect of cumulative doses of (±) 8-OH-DPAT (0.5 to 10 µg/kg, i.v.) was assessed.

Density of 5-HT$_{1A}$ receptors was not altered after chronic fluoxetine, WAY 100635 or their combination (table 1).

Table 1. Densities of ^{3}H-8-OH-DPAT (fmol/mg tissue)

Area	Vehicle	WAY 100635	Fluoxetine	Fluoxetine + WAY100635
CA1	177.7±15.8	192.8 ± 22.5	166.9±17.0	167.4±15.7
CA3	79.5±7.6	81.8 ± 8.3	67.1±5.9	77.3±8.3
DG	247.0±16.2	297.7 ± 27.1	274.3±24.8	249.9±30.1
DRN	315.9 ± 39.4	333.1 ± 51.0	383.6±61.4	305.4±31.4

Data are mean ± s.e.m. (n = 6) and were analysed by one-way ANOVA. DG, dentate gyrus.

In control animals, 8-OH-DPAT suppressed the firing rate of DRN neurons with a ED_{50} of 2.1±0.3 µg/kg (n=10) whereas in rats treated with fluoxetine the ED_{50} was increased two-fold (ED_{50}=4.7±0.3 µg/kg, n=6, p<0.01, ANOVA-Newman-Keuls). Chronic WAY100635 did not alter the responsiveness of 5-HT neurons (ED_{50}=2.5±0.3 µg/kg, n=5). Interestingly, after the concurrent administration with WAY100635, the ED_{50} was significantly reduced towards control values (ED_{50}=2.1±0.6 µg/kg, n=5, p< 0.01 vs. fluoxetine-group). Thus, concurrent administration of fluoxetine and WAY100635 prevents the desensitization of DRN neurons with no parallel modification in 5-HT$_{1A}$ receptor density.

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Supported by CICYT (SAF98-064-CO2-01 and 1FD97-1597).

138P SPECIES DIFFERENCES IN THE APPARENT POTENCY OF SEROTONIN RE-UPTAKE INHIBITORS (SRIs) AT THE HUMAN, DOG AND RAT SEROTONIN TRANSPORTERS

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The aim of this study was to investigate whether there is a species difference in the potency of selected SRIs at the human (cloned and endogenous) and endogenous rat and dog serotonin transporters (SERTs). If such differences were present, the use of animal models to predict the clinical efficacy of an SRI in human may no longer be an accurate basis for such predictions. The potency of some SRIs against the cloned human SERT (Agnel *et al* 1996, Owens *et al* 1997), and the endogenous SERT in rat brain synaptosomes (Johnson *et al* 1985, Bolden-Watson *et al* 1993) has previously been investigated. However, no single study comparing all eight of the SRIs tested here (Table 1) against human, rat and dog transporters has been published.

Platelets were isolated from blood samples collected into sodium citrate (0.38% w/v) from CD rats (200-300g), Beagle dogs (10-12kg) and human volunteers in accordance with the protocol described by Wheeler-Jones *et al* (1992). HEK-293 cells expressing recombinant human SERT were provided by Dr R. Blakely (Blakely *et al* 1991). Inhibitor potencies were determined by performing transport assays (22°C, 100rpm, 5 min pre-incubation with inhibitor) in 96-well filter plates with 25nM [^{3}H]-5-HT and 25nM 5-HT. Substrate incubation times were 5 min for the rat and human platelets, and HEK-293 cells, and 15 min for the dog platelets. Cell concentrations were $1x10^7$ platelets/well and $7.5x10^4$ HEK-293 cells/well. Incubations were terminated upon rapid filtration using a Millipore vacuum manifold followed by sequential washing with ice-cold Bradford buffer (containing 26mM Tris, 5.6mM glucose, 1.3mM $MgCl_2$, 1.1mM ascorbic acid, 0.1mM pargyline, 2.8mM$CaCl_2$, pH 7.4). Plates were then dried in a microwave, allowed to cool to room temperature, and 50µl of Microscint O™ was added to each well. After 30 min, plates were placed on a Packard Topcount NXT™ counter and the amount of radioactivity in each well counted and expressed as the % of specific uptake. Individual concentration-effect data were fitted to a sigmoid curve using GraphPad Prism 2.01 to determine IC_{50} values. One-way ANOVA tests were performed for each compound on the individual IC_{50} values, followed by individual pair-wise comparison of geometric means using the Bonferroni adjustment.

Comparison of our data to previously published data (Agnel *et al* 1996), on the whole, demonstrated close similarity. Similarly, within our data set, few compounds exhibited any consistent species differences when comparing the endogenous SERTs. For example, fluvoxamine, sertraline, citalopram, litoxetine and dapoxetine showed no significant differences between their human, dog or rat SERT IC_{50} values (p>0.05). However, the exceptions were clomipramine and fluoxetine, which were both significantly more potent against the endogenous human than the rat SERT (9.7 fold, p<0.001 and 3.5 fold, p<0.01). Interestingly, although clomipramine was also significantly more potent against the endogenous human than dog SERT (p<0.01), fluoxetine did not show this effect (p>0.05). Comparison of the endogenous versus cloned human SERT indicated no significant differences for fluoxetine, sertraline, citalopram dapoxetine, fluvoxamine and litoxetine (p>0.05). However, both clomipramine and paroxetine were significantly more potent against the endogenous versus cloned human SERT (3.6 and 11 fold, both p<0.001).

Table 1 Geometric mean IC_{50} values (nM) with 95% CI in brackets, n=3-6.

SRI	human clone	human platelet	dog platelet	rat platelet
Paroxetine	6.6 (3.5-12.6)	0.6 (0.4-0.9)	0.5 (0.1-2.3)	1.6 (1.1-2.4)
Fluoxetine	11.3 (6.8-18.6)	11.7 (8.4-16.3)	9.7 (7.1-13.3)	41.5 (23.9-72.1)
Clomipramine	7.2 (5.4-9.5)	2.0 (0.7-5.8)	5.3 (3.5-8.0)	19.4 (13.8-27.3)
Sertraline	3.0 (1.8-5.1)	3.2 (1.7-6.0)	2.1 (1.0-4.2)	4.3 (3.1-6.0)
Citalopram	15.3 (10.6-22.0)	10.1 (3.6-28.0)	24.6 (11.9-50.7)	23.9 (15.7-36.4)
Dapoxetine	8.7 (1.8-43.3)	3.1 (0.9-10.9)	2.7 (0.9-7.9)	2.1 (0.7-6.1)
Litoxetine	5.9 (4.4-7.8)	3.5 (1.4-9.1)	1.9 (0.5-6.9)	4.2 (2.7-6.5)
Fluvoxamine	18.9 (14.6-24.3)	16.1 (11.3-22.8)	6.5 (1.4-29.8)	14.4 (11.2-18.4)

In conclusion, this study has demonstrated few significant differences in the pharmacology of the human, dog and rat SERTs. However, some differences were apparent from our study, indicating that neither the cloned human, or endogenous rat and dog SERT can be relied upon in all cases to predict SRI activity against the endogenous human SERT. Therefore, it cannot be assumed that animal models or human recombinant assays can always be used to accurately predict clinically efficacious doses of SRIs in man.

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139P INVESTIGATION OF HIGH AND LOW AGONIST AFFINITY STATES OF 5-HT1_A RECEPTORS IN RAT HIPPOCAMPUS

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It has previously been reported that human (h) recombinant 5-HT$_{1A}$ receptors exist in both high and low agonist affinity states (Watson *et al.*, 2000). In this study we have investigated the differential binding affinities of 5-HT$_{1A}$ receptor ligands in rat hippocampus using the agonist radioligand [^{3}H]8-OH-DPAT, and the antagonist radioligand [^{3}H]WAY100635 in the presence or absence of the GTP analogue, Gpp(NH)p. We have also assessed the functional potency of selected ligands at rat native tissue 5-HT$_{1A}$ receptors by measurement of cell firing from rat dorsal raphe nucleus (DRN). Rat hippocampal membranes were prepared and assayed according to Watson et *al.* (2000). Studies measuring cell firing rate from rat DRN were carried out according to Corradetti *et al.* (1998).

From radioligand binding studies using [^{3}H]8-OH-DPAT, all of the test ligands showed moderate to high affinity for rat native tissue 5-HT$_{1A}$ receptors (Table 1). From similar studies using [^{3}H]WAY100635, all ligands which have previously been reported to show agonist / partial agonist activity at h5-HT$_{1A}$ receptors, 5-HT, 8-OH-DPAT, pindolol, and SB-272183 (Watson *et al.*, 2001), showed a decrease of up to 1 log unit in affinity for rat 5-HT$_{1A}$ receptors. The affinity of the 5-HT$_{1A}$ receptor antagonists, WAY100635 and spiperone were similar in each study. Agonists displayed even lower affinity against [^{3}H]WAY100635 binding in the presence of Gpp(NH)p (100 μM), whereas antagonist affinities were unchanged. From cell firing studies, the potencies of WAY100635 and spiperone, and the partial agonists pindolol and SB-272183, were comparable to the affinity of these ligands for 5-HT$_{1A}$ receptors determined by [^{3}H]WAY100635 in the presence of Gpp(NH)p (Table 1).

Table 1. Summary of Radioligand Binding Data in Rat Hippocampal Membranes and Cell Firing Studies in Rat DRN.

	pKi [^{3}H]8-OH-DPAT	pKi [^{3}H]WAY100635 (no Gpp(NH)p)	pKi [^{3}H]WAY100635 (+ Gpp(NH)p)	pK_b (cell firing)
5-HT	8.6 ±0.1	7.7 ± 0.1	6.2 ± 0.0	-
8-OH-DPAT	8.7 ± 0.0	8.4 ± 0.0	6.7 ± 0.0	-
WAY100635	9.4 ± 0.1	9.0 ± 0.1	9.1 ± 0.1	8.6 ± 0.1
Pindolol	7.7 ± 0.3	6.8 ± 0.1	6.3 ± 0.1	6.7 ± 0.1
Spiperone	7.2 ± 0.1	6.9 ± 0.1	7.2 ± 0.1	7.3 ± 0.2
SB-272183	8.7 ± 0.1	8.1 ± 0.0	7.2 ± 0.0	7.1 ± 0.2

Data are mean ± SEM from 3 independent experiments

These studies suggest that, *in vitro*, rat hippocampus expresses both the high and low agonist affinity states of 5-HT$_{1A}$ receptors. When the system is manipulated such that all of the receptors are switched to the low agonist affinity state, that is, in the presence of Gpp(NH)p, full agonists show a substantial decrease in affinity for the receptors, partial agonists show a lesser degree of difference and antagonist affinity is unaltered. Indeed, it has been reported that the difference in affinity of a compound for high and low agonist affinity states of h5-HT$_{1A}$ receptors correlates well to its intrinsic activity (Watson *et al.*, 2000). The apparent pK_b values of selected ligands, generated from cell firing studies in rat DRN, were comparable to their affinity for 5-HT$_{1A}$ receptors measured by [^{3}H]WAY100635 in the presence of Gpp(NH)p. These data suggest that functional 5-HT$_{1A}$ receptors in rat DRN are in the low agonist affinity state. Further studies are required to investigate this finding.

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140P THE AFFINITY AND EFFICACY OF A SERIES OF DIPROPYLAMINOTETRALINS AT THE h5-HT1_A RECEPTOR

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Although several models of the mechanisms of action of G protein coupled receptors (GPCRs) have been proposed, there is little detailed knowledge as to how the interaction of ligands with receptors leads to their activation. One experimental approach to further our understanding is to study the pharmacology of structurally related compounds with defined changes in their functional groups. In the present report a series of homologous 2-(dipropylamino)tetralins which differ in the position of the hydroxyl group and the orientation of the amino-dipropyl bond (*R* or *S*), have been tested at the h5-HT$_{1A}$ receptor expressed in CHO cells. Ligand affinity was determined in competition binding experiments against the 5-HT$_{1A}$ receptor antagonist [^{3}H]NAD-199, (Jerning *et al.*, 1998) and ligand potency and efficacy were determined using [^{35}S]GTPγS binding.

All ligands displayed biphasic competition curves that could be resolved into two sites. Affinity constants for the high affinity (K_h) and low affinity (K_l) sites were determined, see Table 1. For most ligands K_h values corresponded well to previously reported K_i values for competition against [^{3}H]8-OH-DPAT, (Alder *et al.*, 2000). The percentage of high affinity binding sites was similar for the different ligands (40-50 %) except for *S*-6-OH-DPAT (22 %). The non-hydroxylated *R*- and *S*- DPAT both exhibit similar binding affinities (K_l) of about 1 μM. 8-OH-DPAT has the highest affinity with the *S*- having a 4 fold greater affinity than the *R*- isomer. Addition of 5-OH, 6-OH and 7-OH groups was unfavourable for binding to the lower affinity state.

A similar pattern is seen for [^{35}S]GTPγS binding, the rank order of binding affinities (K_l) and potencies being similar (Pearson r = 0.97, p<0.01). All of the compounds tested were agonists at the h5-HT$_{1A}$ receptor demonstrating a range of efficacies, see Table 1. Efficacy was expressed as the percentage of the response given by 5-hydroxytryptamine (5-HT). The non-hydroxylated *R*- and *S*-DPAT and *R*- and *S*-8-OH-DPAT all behaved as full agonists, however, addition of the 8-OH group significantly increased the potency of the ligand (student's *t*-test, p<0.01). The hydroxyl group in the other positions decreased the potency and efficacy of the ligands. Interestingly there is no stereoselectivity in the effect of *R*- and *S*-8-OH-DPAT suggesting that the receptor can be activated by two different binding orientations. The ternary complex model (De Lean *et al.*, 1980) predicts that K_l/K_h should correlate with ligand efficacy. However analysis of the log K_l/K_h ratio and efficacy in this study does not show a significant correlation. A significant correlation is observed if *S*-6-OH-DPAT is excluded, (Pearson r = 0.72, p<0.05).

Table 1. Ligand affinity, efficacy and potency at the 5-HT$_{1A}$ receptor.

		[^{35}S]GTPγS binding		[^{3}H]NAD-199 binding	
Compound		% 5-HT	pEC50	pK_h	pK_l
DPAT	*R*	96.3 ± 3.8	7.14 ± 0.07	8.22 ± 0.05	6.09 ± 0.01
	S	83.4 ± 5.8	6.69 ± 0.15	7.74 ± 0.19	5.96 ± 0.03
5-OH-DPAT	*R*	67.8 ± 4.5	5.57 ± 0.16	6.53 ± 0.23	5.07 ± 0.13
	S	65.9 ± 6.7	5.85 ± 0.23	7.48 ± 0.18	5.54 ± 0.13
6-OH-DPAT	*R*	57.0 ± 10	5.44 ± 0.19	6.32 ± 0.09	4.61 ± 0.12
	S	25.6 ± 8.9	5.31 ± 0.39	7.04 ± 0.36	5.22 ± 0.06
7-OH-DPAT	*R*	80.7 ± 6.5	6.36 ± 0.11	7.45 ± 0.13	5.57 ± 0.04
	S	64.3 ± 6.1	5.69 ± 0.10	6.84 ± 0.20	5.42 ± 0.09
8-OH-DPAT	*R*	93.0 ± 3.8	8.27 ± 0.05	9.26 ± 0.12	7.24 ± 0.05
	S	92.1 ± 4.0	8.13 ± 0.08	9.76 ± 0.33	7.81 ± 0.10

Data are mean ± s.e.mean from at least three independent experiments.

This work was supported by the BBSRC.

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141P CHRONIC ANTIDEPRESSANT DRUG TREATMENT DOES NOT ALTER NEUROKININ-1 RECEPTOR EXPRESSION IN THE RAT BRAIN REGIONS

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Increasing evidence indicates an important role for substance P in the treatment of mood disorder. Specifically, neurokinin-1 (NK1) receptor antagonists have anxiolytic and antidepressant effects in animal models (File 2000; Papp *et al.*, 2000). Moreover, the NK1 receptor antagonist, MK-869, demonstrated antidepressant efficacy in clinical trial (Kramer *et al.*, 1998). The NK1 receptor may also play a role in the action of current antidepressant therapies. Indeed, we recently reported that electroconvulsive shock (ECS) induces adaptive increases in NK1 receptor expression in rat cortical areas (Burnet *et al.*, 2001). Here we tested whether antidepressant drugs also influence NK1 receptor expression.

Male Sprague-Dawley rats (220-230 g) were injected i.p. twice daily for 13-14 days, saline vehicle, desipramine (DMI; 10 mg/kg), paroxetine (PAR; 5 mg/kg) or venlafaxine (VEN; 10 mg/kg). About 18 h after the last injection animals were killed, and brains were removed and frozen prior to sectioning. The density of NK1 binding sites was determined by quantitative receptor autoradiography using [^{125}I]-Bolton-Hunter-substance P (Burnet *et al.*, 2001). Abundance of NK1 mRNA was determined by in situ hybridisation histochemistry (Burnet *et al.*, 2001). Image analysis was performed using densitometric quantification of autoradiograms. Data were analysed with one-way ANOVA.

Compared to vehicle controls, repeated treatment with DMI, PAR or VEN had no effect on the density of NK1 binding sites in any of 24 brain areas examined including regions of the cortex and others listed in Table 1. Relative to vehicle controls, DMI, PAR and VEN did not alter the abundance of NK1 receptor mRNA in the cortex or other areas.

Table 1. Effect of antidepressant treatment on NK1 binding site densities (fmol/mg protein) in rat brain.

Brain area	Saline	DMI	PAR	VEN
Cingulate cortex	31.8±1.5	31.9±3.3	32.6±3.9	35.3±2.2
Frontal cortex	32.7±1.8	32.9±2.2	33.7±2.7	32.5±1.7
Parietal cortex	33.4±1.1	33.4±2.2	31.9±2.1	32.2±2.0
Dentate gyrus	51.2±1.5	48.6±1.2	52.0±2.1	56.4±1.5
Medial habenula	56.5±2.0	51.1±2.5	57.4±2.6	57.7±2.5
Medial amygdala	50.4±2.5	50.4±2.5	51.7±2.3	52.4±1.6
Dorsal raphe	36.9±3.1	36.3±1.9	38.7±1.9	40.1±1.6

Data expressed as mean±s.e.m. N=5-6 per group.

In summary, chronic treatment with the antidepressant drugs tested had no significant effect on the expression of NK1 receptors in rat forebrain. This result contrasts with the finding that ECS increases the density of NK1 binding sites in cortical areas (Burnet *et al.*, 2001). Therefore, altered NK1 receptor expression is not an effect common to all antidepressants but may be of relevance for the antidepressant efficacy of ECS.

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142P STUDIES ON THE MECHANISMS INVOLVED IN THE DEGENERATION OF DOPAMINE NERVE ENDINGS IN MOUSE BRAIN FOLLOWING ADMINISTRATION OF MDMA ('ECSTASY')

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Administration of 3,4-methylenedioxymethamphetamine, (MDMA, ecstasy) to rats produces selective neurodegeneration of 5-HT nerve terminals in the forebrain, leaving dopamine (DA) neurones intact (see Green et al., 1995). In contrast, MDMA administration to mice produces sustained depletion of DA but not 5-HT (Stone et al., 1987; O'Shea et al., 2001). We have now examined this difference further by investigating the ability of several compounds to protect against MDMA-induced dopaminergic damage in mouse brain.

Adult male NIHS mice (Harlan Olac, Bicester) were injected 3 times at 3 h intervals with MDMA (25 mg kg^{-1} i.p.). Rectal temperature was measured regularly over 7 h using a rectal probe. Putative neuroprotective agents were injected 30 min prior to each dose of MDMA. Mice were killed 7 days later and striatal DA concentrations measured by h.p.l.c. with electrochemical detection.

Neither of the NMDA antagonists MK-801 (0.5 mg kg^{-1}) and AR-R15896AR (20 mg kg^{-1}) produced significant protection against the MDMA-induced 40% loss in striatial dopamine. Similarly, neither drug altered MDMA-induced hyperthermia following the first 2 injections, but attenuated the hyperthermia following the 3rd dose. Clomethiazole (50 mg kg^{-1}) had little effect on the MDMA-induced hyperthermia and was not neuroprotective. In contrast the selective nNOS inhibitor AR-R17477 (5 mg kg^{-1}) was strongly protective, preventing the dopamine loss induced by MDMA without altering the acute hyperthermic response (Fig. 1).

Early reports suggested that NMDA antagonists protected against methamphetamine-induced dopamine loss in mice and MDMA-induced 5-HT loss in rats (e.g. Sonsalla et al., 1989) but recent data indicate that NMDA antagonists only protect because they attenuate the MDMA-induced hyperthermia (Albers & Sonsalla, 1995; Colado et al., 1998). The current study shows that NMDA antagonists also failed to protect against MDMA-induced DA loss in mice. Clomethiazole protected against MDMA-induced serotonergic loss in rats (Colado et al., 1999), but was without effect against MDMA-induced dopaminergic loss in mice. The results suggest a role for nNOS in the production of MDMA-induced dopaminergic damage in mice since AR-R17477 was neuroprotective and had little effect on MDMA-induced hyperthermia.

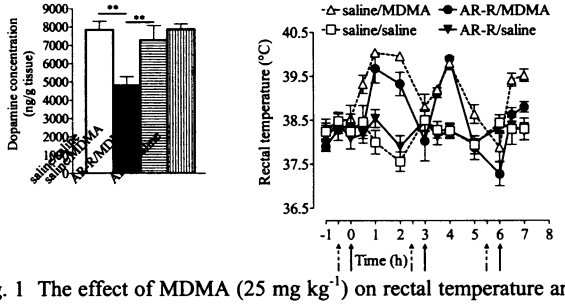

Fig. 1 The effect of MDMA (25 mg kg^{-1}) on rectal temperature and DA content of mouse striatum and effect of AR-R17477 (5 mg kg^{-1}) pretreatment. Results shown as mean ± s.e. mean (n = 4-5), ** different $p < 0.01$.

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143P THE ROLE OF TYROSINE RESIDUES IN THE EXTRACELLULAR DOMAIN OF THE MOUSE 5-HT$_3$ RECEPTOR

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The 5-HT$_3$ receptor is a member of the cys-loop family of ligand gated ion channels. Receptor subunits of this family are composed of a large N-terminal extracellular domain (ECD) thought to be wholly responsible for ligand binding, and four transmembrane domains, one of which (M2) lines the channel pore. Studies suggest that the ligand binding domain is constituted of 3-6 distinct regions of the ECD, and as a considerable proportion of these residues are aromatic, we have explored in this study the role of tyrosine residues in the ECD.

Tyrosine (Y) residues in the mouse 5-HT$_{3A(b)}$ receptor subunit subunit were converted to alanine (A) and serine (S) using the Kunkel method (Kunkel, 1985) in the expression vector pcDNA3 (Clontech); this was then transiently transfected into HEK 293 cells using calcium phosphate precipitation (Chen & Okayama, 1988). Receptor characteristics were examined using radioligand binding assays on transfected cell membranes (Spier & Lummis, 2000). Briefly, membrane samples were incubated with [^{3}H]granisetron, a 5-HT$_3$ receptor antagonist, with concentrations ranging from 0.05 to 20nM in 10mM HEPES buffer for 1h at 0°C. Non-specific binding was determined using 1μM d-tubocurarine. Data were analysed using PRISM (Graphpad) software.

For those receptors for which specific binding was detected, K_d values are shown in Table 1. No specific binding was detected in cells transfected with Y50, Y91, Y141, Y153S, Y234 or Y240A mutants (n=3-8).

Table 1.

[^{3}H]granisetron binding affinities for 5-HT$_3$ receptor mutants

Receptor	K_d	Receptor	K_d
Wild type	0.34 ± 0.09	Y143A	1.52 ± 0.15*
Y73A	0.43 ± 0.06	Y143S	1.77 ± 0.36*
Y73S	0.52 ± 0.11	Y153A	2.95 ± 0.60*
Y88A	0.28 ± 0.05	Y167A	0.29 ± 0.06
Y88S	0.23 ± 0.03	Y167S	0.31 ± 0.10
Y94A	0.52 ± 0.11	Y240S	0.14 ± 0.02
Y94S	0.41 ± 0.11		

Values are mean ± s.e.m., n=3-10. * indicates significantly different to wild type ($P<0.05$, Student's t test).

Thus mutating Y143 and Y153 changes receptor affinity, and the two Y240 mutants were different, suggesting that these residues may play a role in ligand binding. Conversely, changing Y73, Y88, Y94 or Y167 has no significant effect, suggesting they do not play a critical role in the structure or function of the ECD, whilst mutation of Y50, Y91, Y141 and Y234 result in non functional receptors, suggesting that substitution of these residues may interfere with correct receptor expression, assembly and/or structure.

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144P SITE-DIRECTED MUTAGENESIS OF α3β4 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST BINDING SITES

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Nicotinic acetylcholine receptors (AChRs) from autonomic ganglia are pentameric proteins with the subunit composition $2\alpha_3\ 3\beta_4$. Within this complex are two agonist binding sites formed between the clockwise faces of the α_3 subunits and the anticlockwise faces of their neighbouring β_4 subunits. Based largely on mutagenesis and affinity labelling studies on the prototypical skeletal muscle AChR, our current understanding has lead to a model where three segments of the α_3 subunit (loops A-C) and four segments of the β_4 subunit (loops 1-4) contribute to each agonist binding site.

Previously, (Brown, Comet & Prince, 2001) we tested this model by mutating a conserved tyrosine residue in loop A of the α_3 subunit and its analogous position in β_4. Consistent with our current picture of the agonist binding sites we found that α_3Y93F produced a large increase in the EC_{50} of the receptor for DMPP and nicotine whilst the analogous mutation β_4Y71F was without effect. In the present study we have extended these observations by examining the effect of point mutations in loops C and 4 previously implicated in ligand binding.

Mutations were introduced into the α_3 (Y190D and N166D) and β_4 (Q168D) subunits using the QuikChange site-directed mutagenesis kit (Stratagene). Stage V/VI *Xenopus* oocytes were used to express nicotinic acetylcholine receptors. The two electrode voltage clamp method as described by Gerzanich *et al* (1998) was used to measure responses to DMPP (dimethylphenylpiperazinium). Four different combinations of subunits were expressed: $\alpha_3\beta_4$ wild type, α_3Y190Dβ_4, $\alpha_3\beta_4$Q168D and α_3N166Dβ_4. Prism 3.0 (GraphPad software) was used to fit concentration response curves to the Hill equation. Fitted EC_{50} values expressed as mean ± SEM for each subunit combination are summarised in. Table 1. Statistical comparisons were made on Log EC_{50} values using Student's unpaired t test.

Table 1. Parameters for the activation of wild-type and mutant $\alpha_3\beta_4$ ACh receptors.

	EC_{50} (μM) DMPP
$\alpha_3\beta_4$	14.1 ± 3.8 (n=5)
α_3Y190Dβ_4	No response (n=3)
$\alpha_3\beta_4$Q168D	11.8 ± 0.9 (n=5)
α_3N166Dβ_4	19.3 ± 4.4 (n=4)

Our results show that for α_3N166D and β_4Q168D mutations there was no significant difference in agonist affinity compared to wild type. This data suggests that N166 (loop 4) in α subunits and Q168 (loop C) in β subunits are not part of the binding site. The α_3Y190D (loop C) mutation (analogous to β_4Q168D) did not give any responses with concentrations up to 10mM. This may reflect a dramatic decrease in agonist affinity. Alternatively Y190D may prevent normal assembly of the subunit and receptor.

These and previous results (Brown, Comet and Prince, 2001) support a nicotinic AChR agonist binding site model in which, the clockwise face of α subunits containing loops A-C and the anticlockwise face of β subunits containing loops 1-4, together form the agonist binding sites.

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This work was supported by the Wellcome Trust.

145P PHARMACOLOGICAL CHARACTERIZATION OF NOVEL AMPA RECEPTOR LIGANDS IN RAT BRAIN.

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AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors bind the excitatory neurotransmitter glutamate and may play a role in synaptic plasticity (Bear and Malenka, 1994). To date, very few potent, selective AMPA receptor antagonists have been reported and none are known which are selective for individual subunits. As AMPA receptors are thought to be involved in excitotoxicity and epileptogenesis (Menniti *et al.*, 2000), new antagonists may be of therapeutic use. Structure-activity studies on 5-substituted analogues of the AMPA/ kainate receptor agonist, (S)-willardiine, led to the potent agonist, (S)-5-fluorowillardiine (Patneau *et al.*, 1992). Addition of a substituent to willardiine at N^3, using a similar strategy to that performed with AMPA (Krogsgaard-Larsen *et al.*, 1991), may produce potent, selective antagonists. This study aims to characterize binding of willardiine-based AMPA receptor ligands in rat brain and to investigate their selectivity for AMPA receptors over kainate receptors.

Cerebellum-free brain membrane preparations were made from male Wistar rats (250-300 g). Binding assays were performed using 0.4 mg/ml protein, increasing concentrations of novel compound and either 10 nm [^{3}H](S)-5-fluoro-willardiine or 5 nm [^{3}H](2S,4R)-4-methylglutamate, depending on whether selectivity for AMPA or kainate receptors was being studied. Mixtures were incubated at 4 °C for 40 mins. Non-specific binding was defined in the presence of 1 mM L-glutamate. Unbound radioligand was washed off in assay buffer (50 mM Tris HCl/ 100 mM KCl, pH 7.4) using a Brandell Cell Harvester. Bound ligand was assessed using a Wallac Scintillation counter. Concentration-inhibition curves for each compound were drawn in GraphPad Prism and IC_{50} values derived.

The carboxy-alkyl side chain at the N^3 position of the uracil ring was found to have an optimal length of two carbons for binding to AMPA receptors:- (S)-3-(2-carboxyethyl)-willardiine ((S)-3-CEW) IC_{50} 15.59 ± 1.20 μM, (S)-3-(3-carboxypropyl)-willardiine ((S)-3-CPW) IC_{50} 21.58 ± 1.48 μM. In both cases (R) isomers were far less potent than the (S) forms (IC_{50} > 110 μM). Addition of a benzene ring to the carboxy-alkyl side chain produced (S)-3-(4-carboxybenzyl)-willardiine ((S)-3-(4-CBW) with a potency intermediate to the above (S) isomers (IC_{50} 16.22 ± 1.23 μM). Addition of a tetrazole ring in (S)-3-tetrazolylmethyl willardiine ((S)-3-TMW), IC_{50} 25.20 ± 1.19 μM, conferred a potency similar to that of (S)-3-CPW. (All determinations n=3). None of the (S) isomers of the above compounds had a high affinity for the kainate receptor (IC_{50} > 127 μM).

Thus, ligands with increased AMPA receptor selectivity can be created from willardiine. These compounds are likely to be useful tools in distinguishing between AMPA and kainate receptors.

Supported by the EPSRC and Tocris Cookson Ltd.

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146P EFFECT OF CHRONIC ELECTROCONVULSIVE SHOCK AND ANTIDEPRESSANT DRUGS ON mRNA EXPRESSION OF AMPA RECEPTOR SUBUNITS IN THE RAT BRAIN

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Current antidepressant therapies suffer from a delay of onset of therapeutic effect of several weeks. To understand this problem, the molecular basis of the neuroadaptive changes that accompany long-term treatment with antidepressants is being established. Increasing preclinical evidence indicates that antidepressant treatment induces adaptive changes in brain glutamate receptor expression. In particular, several studies show altered expression of NMDA receptor subunits and binding sites following chronic treatment with electroconvulsive shock (ECS) and antidepressant drugs (Watkin *et al.*, 1998; Skolnick, 1999). Here we tested whether chronic administration of ECS and different antidepressant drugs alter the expression of the AMPA receptor subunits, GluR1, GluR2, GluR3 and GluR4.

Groups of male Sprague-Dawley rats (220-250g) were administered ECS or antidepressant drugs as follows: i) ECS 5 times over 10 days under halothane anaesthesia or halothane alone (sham control), ii) saline vehicle, paroxetine (5 mg/kg, i.p.), desipramine (5 mg/kg, i.p.) or venlafaxine (5 mg/kg, i.p.) injected twice daily for 14 days. Rats were killed about 18 h after the last treatment, and brains were frozen prior to being processed for *in situ* hybridization for GluR1, GluR2, GluR3 and GluR4 mRNA using ^{35}S-labeled oligonucleotides (Pei *et al.*, 1997). Autoradiograms were quantified by computer-aided densitometry.

As shown in Table 1, ECS significantly reduced GluR1 and GluR2 mRNA abundance in the CA1 region of hippocampus, and increased GluR3 in the dentate gyrus, when compared to sham controls. In the frontal and parietal cortex, GluR3 was reduced but GluR1, GluR2 and GluR4 were unchanged. In contrast to ECS, paroxetine, desipramine and venlafaxine had no statistically significant effect on mRNA abundance of any AMPA receptor subunits in these regions, when compared to saline-injected controls.

Table 1. Effect of ECS on the mRNA abundance of GluR1, GluR2, GluR3 and GluR4 in the rat brain.

	DG	CA1	CA3	PTX	Fr	Pir
GluR1	96±6	79±7*	89±9	96±3	117±16	101±17
GluR2	103±5	88±3*	90±6	94±7	101±7	112±8
GluR3	128±2*	96±1	101±8	77±3*	91±1*	98±4
GluR4	101±6	89±5	96±3	97±3	98±3	98±3

Data are mean ±s.e.m. values (n=5) expressed as % of sham control (100 %). *p<0.05 versus sham (unpaired Student t-test following one way ANOVA). DG dentate gyrus, PTX parietal cortex, Fr frontal cortex, Pir piriform cortex.

In summary, these results show that chronic ECS alter mRNA expression of AMPA receptors in a subunit and brain region specific manner. In contrast, chronic administration of the antidepressant drugs tested had no effect. Since the properties of the ionotropic glutamate receptors are determined by subunit composition, differential changes in AMPA subunit expression may result in changes in glutamate receptor function following chronic ECS.

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147P THE EFFECT OF THE CO-ADMINISTRATION OF ELIPRODIL WITH MEMANTINE IN NAÏVE AND RESERPINISED MICE

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There is ample evidence to suggest that NMDA receptor activation plays a crucial part in glutamate overactivity within the basal ganglia of the Parkinsonian brain (Starr & Starr, 1994: Dansyz *et al.*, 1997). In a previous report, we have shown that the NR2B antagonist, eliprodil (10 and 20mg/kg), mildly stimulates locomotor activity in reserpine-treated male TO mice, without adverse effects (Devadasan *et al.*, 1999). Furthermore, combination of eliprodil with MK801 produces a synergistic locomotor stimulation (Brooks *et al.*, 1996), although pronounced ataxia associated with MK801was apparent. In the present study, the potential of eliprodil to ameliorate a model of Parkinsonism was further investigated. Memantine, which is used in the clinic as an antiparkinsonian agent (Dansyz *et al.*, 1997), is a less potent open channel blocker than MK801, and has a less pronounced side effect profile.

Eliprodil (5, 10 or 20mg/kg, s.c.; n=6/dose) was co-administered with memantine (5, 10 or 20 mg/kg, s.c.; n=6/dose) in naïve or reserpinised male TO mice (30 – 40 g). Locomotor count was recorded every 10 minutes over a period of 2 hours using a Panlab Actisystem, which detects horizontal movement. Significance of data was assessed using 2-way ANOVA and post-hoc analysis, and results were expressed as mean ± s.e.m. Reserpine (5 mg/kg) was administered subcutaneously 24 hours prior to the study. Reserpinised mice displayed classical akinesia and tremors. Memantine (5, 10 or 20mg/kg, s.c.; n=6/dose) was administered alone in naïve and reserpinised mice.

In naïve mice, memantine had a significant dose-dependent stimulatory effect on locomotor activity compared to control mice, with $P<0.0001$ for all doses (mean locomotor count at t=120 min for each of the above doses:365±75, 515±55, 810±22 respectively). At the 2 hour time point, the locomotor count in reserpinised control animals was 10±0. Memantine 10 and 20 mg/kg, but not 5 mg/kg caused a significant increase in locomotor activity (count of 110±11 ($P< 0.0001$) and 215±24 ($P< 0.0001$) with 10 and 20 mg/kg dose respectively). Combination of memantine and eliprodil in naïve mice had a significant dose-dependent stimulatory effect on locomotor activity that mimicked the effect of memantine alone.

In reserpinised mice, co-administration of memantine (5 mg/kg) with eliprodil (5 mg/kg) caused a significant stimulatory effect on locomotor activity (locomotor count of 68±12 at t = 120 min; $P< 0.05$), which was not apparent when these doses were administered individually. Combination of eliprodil (10 or 20 mg/kg) with memantine (5, 10 or 20 mg/kg) failed to exhibit any additive effects. However, a tight circling behaviour seen with memantine treatment alone was not observed when combined with eliprodil, therefore the combination counteracted this adverse effect. In summary, the combined administration of memantine and eliprodil in reserpinised mice showed some additive effects at the lowest dose tested, although the synergism was lost with the higher doses. However, interestingly, the tight circling adverse effect caused by memantine was abolished by co-administration with eliprodil. The potential of NR2B antagonists to treat Parkinson's disease is worthy of further study.

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148P EFFECTS OF EXTRACELLULAR CALCIUM REMOVAL, NPPB AND DL-TBOA ON GLUTAMATE EFFLUX FROM RAT CORTICAL PRISMS IN RESPONSE TO SIMULATED ISCHAEMIA *IN VITRO*

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Elevated extracellular glutamate in response to acute cerebral ischaemia has been ascribed to four sources: extracellular Ca^{2+}-dependent exocytosis from nerve terminals, flux through swelling-activated anion channels, reversed operation of uptake transporters, and cell lysis (Szatkowski and Attwell 1994). We demonstrated that rat cortical prisms subjected to simulated ischaemia (hypoxia-hypoglycaemia) exhibited time-dependent glutamate efflux that was not accompanied by widespread cell lysis (Nelson *et al.*, 2000). The origin of this glutamate efflux has now been investigated further using removal of extracellular Ca^{2+}, the volume-activated anion channel inhibitor NPPB, and DL-TBOA, a competitive, non-transported inhibitor of Na^+-dependent excitatory amino acid transporters EAAT1-3 (Shimamoto *et al.*,1998).

Prisms (350 μm^2) of cerebral cortex from adult female rats (Wistar 200-250 g) were incubated for 30-45 min at 37°C either in a normoxic HEPES-buffered saline containing glucose (10mM) or in an equivalent saline equilibrated with N_2 and with glucose omitted. Glutamate in the perfusate was assayed fluorimetrically using glutamate dehydrogenase (Nelson *et al.*, 2000). Statistical analysis was performed using 2-way ANOVA with Bonferroni's correction.

Omitting Ca^{2+} (2.5 mM) from the extracellular solution with equimolar replacement by Mg^{2+} and 2mM EGTA reduced ischaemia-evoked efflux over the first 25 min of incubation by 60 ± 8% (n=5). NPPB (100 μM) did not affect ischaemia-evoked efflux over this early period but reduced efflux by 52 ± 8 % thereafter (n=5). DL-TBOA (100 μM) augmented basal efflux and inhibited ischaemia-induced glutamate efflux throughout the 45 min incubation period (Figure 1).

We conclude that Ca^{2+} dependent pathways contribute to ischaemia-induced glutamate efflux in this model in the first 25 min, volume-activated anion channels thereafter, and reversed uptake throughout.

We thank Dr K Shimamoto, SUNBOR, Osaka, for DL-TBOA and AstraZeneca R&D Södertäle, for financial support.

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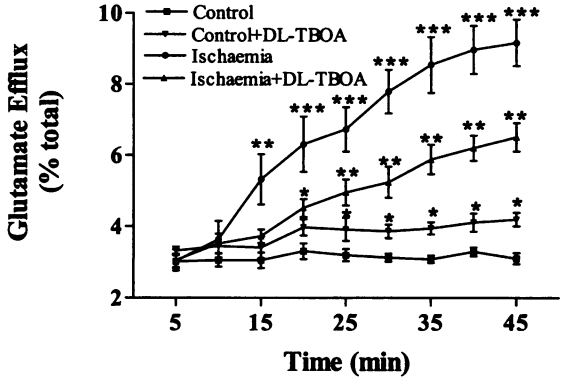

Figure 1. Inhibition of ischaemia-evoked glutamate efflux by DL-TBOA. Glutamate efflux expressed as % of total tissue glutamate content, mean ± s.e. mean, n=5. Different from Control: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

149P A PRESYNAPTIC GluR5 KAINATE RECEPTOR MODULATES EXCITATORY SYNAPTIC TRANSMISSION IN THE MEDIAL PERFORANT PATH-GRANULE CELL SYNAPSE IN THE RAT DENTATE GYRUS

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Five subunits of the kainate receptor have been cloned, GluR5, GluR6, GluR7, KA1 and KA2. In the present study we have investigated the effects of activation of kainate receptors on excitatory synaptic transmission in slices of the rat dentate gyrus. Field EPSPs were recorded from the middle molecular layer in response to stimulation of the medial perforant path. Significance of data was assessed using Student t-test and results were expressed as mean±s.e.m. Perfusion of kainate or the GluR5 selective agonist ATPA [(RS)-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid] resulted in a non-desensitizing concentration-dependent inhibition of the AMPA receptor-mediated EPSP, with threshold values of inhibition of ~0.02 μM and ~0.2 μM respectively and IC50 values of 0.64±0.15 μM and 3.9±0.55 μM (n=5), respectively. The inhibition by kainate and ATPA was accompanied by a reduction in paired pulse depression and also of short-term depression evoked by brief high frequency trains, demonstrating a presynaptic site of action of the kainate receptor agonists. ATPA produced a similar inhibition of the isolated NMDA receptor mediated EPSPs recorded in the presence of the selective AMPA receptor antagonist GYK 52466 (100 μM).

Perfusion of the glutamate agonist SYM 2081 [2S,4R-4-methylglutamate] at a concentration of 20 μM caused a small (~10%) non-desensitizing enhancement of the EPSP ($P<0.05$), although a lower (1 μM) or higher concentration (40 μM) did not result in a significant change of the EPSP. In order to determine whether SYM 2081 produced desensitization of kainate receptors, as has been previously observed at native GluR5 and homomers of GluR6 (Jones *et al.*, 1997), SYM 2081 (20-40 μM) was applied prior to perfusion of ATPA (6μM). SYM 2081 did not cause an inhibition of subsequent ATPA-evoked inhibition, demonstrating a lack of SYM 2081 evoked desensitization of the presynaptic kainate receptors. These studies demonstrate the presence of presynaptic GluR5-containing kainate receptors at the medial perforant path-dentate granule cell synapse.

This work was supported by Enterprise Ireland.

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150P METHOCTRAMINE INDUCES A FAST ONSET, LONG-LASTING POTENTIATION IN THE HIPPOCAMPAL CA1 REGION OF THE ANAESETHETISED RAT

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The mechanisms underlying high frequency stimulation-induced long-term potentiation (LTP) may play a key role in learning and memory processes (Bliss and Collingridge, 1993). M_2 muscarinic receptor antagonists have been reported to improve memory in rats (Quirion *et al.*, 1995; Aura *et al.*, 1997). The present study investigated the effects of an M_2 antagonist on excitatory synaptic transmission in the rat hippocampal CA1 area *in vivo*.

Experiments were performed on urethane-anaesthetised (1.5 g/kg, i.p.) male adult Wistar rats (250-350 g). The animal care and experimental protocol were licensed by the Department of Health. Field excitatory postsynaptic potentials (EPSPs) were recorded from the stratum radiatum in response to stimulation (0.033 Hz, 50% maximum EPSP) of the Schaffer collateral/ commissural pathway. The drugs were injected i.c.v. via a cannula in the lateral cerebral ventricle in a volume of 2.5 μl, delivered over 30 seconds.

The M_2 antagonist methoctramine (17 nmol) induced a rapid onset, persitent increase in synaptic transmission (139.7 ± 13.2%, 161.6±14.6% and 168.2±26.7%, mean ± SEM baseline EPSP slope at 5, 60 and 180 min, respectively, n=8, $P<0.001$, Student t-test). The induction (n=7, $P>0.05$), but not the maintenance (n=6, $P<0.01$), of this LTP-like effect was blocked by scopolamine (13 nmol, n=6), a non-subtype selective muscarinic receptor antagonist. The potentiation was blocked by the M_3 antagonist 4-DAMP (14 nmol, n=7, $P<0.05$). Furthermore, the potentiation induced by methoctramine was not blocked by the NMDA receptor antagonist R,S-carboxypiperazinyl-propyl–phosphonic acid (CPP, 10 mg/kg, i.p., n=4, $P>0.05$). The paired-pulse facilitation ratio, a presynaptic indicator, decreased (n=8, $P<0.05$) when the EPSP was increased after methoctramine injection.

In conclusion, the M_2 receptor antagonist methoctramine triggered a fast onset LTP-like change in synaptic strength. Unlike LTP induced by standard high frequency stimulation protocols, its induction is not NMDA receptor-dependent but is dependent on muscarinic receptor activation, possibly the M_3 subtype. The associated decrease in paired-pulse facilitation is consistent with a persistent increase in glutamate release mediating this effect.

Supported by the Wellcome Trust.

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151P NEUROPLASTIC EVENTS REQUIRED FOR LTP AND AVOIDANCE LEARNING ARE IMPAIRED BY OPIATE ADDICTION

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Persistent use of heroin leads to physical dependence and drug seeking behaviours (Koob & Loal, 1997). These learned behaviours are robust and may account for craving (Koob & Loal, 1997; Di Chiara, 1999). Clearly, the reinforcing potential of the drug frustrates rehabilitation of these learned responses but it is unclear if it perturbs the basic mechanisms of learning. We, therefore, have determined the consequence of opiate dependence on the generation of long-term potentiation (LTP) and passive avoidance conditioning.

Wistar rats self-administered heroin via a cannula chronically implanted in the jugular vein for one hour each day. The rate of self-administration steadily increased over the first 10 days and remained constant thereafter (approx. 5-7 active lever presses; 0.5-0.7mg/kg/session; n = 6 per treatment).

At 24h after the last self-administration of heroin (day 15), a period in which the majority of the last dose would be eliminated, perforant path LTP could not be elicited in urethane-anaesthetised animals. Similarly treated animals, when trained to successfully acquire a passive avoidance response, failed to consolidate memory, as recall 12h post-training was defective (escape latency median 582 sec, inter-quartile range 99 sec vs 121.5 sec, 182.5 sec for control vs heroin-treated animals, respectively, n = 6 per treatment; $p<0.05$, Mann-Whitney U-test). Impaired learning could not be attributed to somatic symptoms associated with withdrawal, as open-field behaviour was normal.

The inability of heroin-addicted rats to generate LTP or consolidate avoidance learning could not be attributed to a non-specific loss of neuroplastic potential, as the basal expression of polysialylated neural cell adhesion molecule (NCAM PSA) at the dentate infragranular zone was normal (60.7 ± 2.4 and 61.26 ± 5.4 cells/unit dentate granule cell area for control and heroin treated animals, respectively, n = 6 per treatment; $p>0.05$, Student's t-test; [Murphy & Reagan, 1998]). Chronic exposure to heroin also failed to alter polysialylated neural cell adhesion molecule expression in the nucleus accumbens or the amygdala, areas associated with drug reward and emotional response, respectively (Koob *et al.*, 1997).

Funded by Enterprise Ireland.

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152P GABA-ERGIC AND GLUTAMATERGIC MECHANISMS IN STRESS MODULATION OF SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS OF ANAESTHETISED RATS

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Changes in both hormone and transmitter levels have been implicated in the regulation by stress of plasticity at excitatory synapses in the hippocampus. Acute inescapable stress can block high frequency stimulation (HFS) induced long-term potentiation (LTP) and enable low frequency stimulation-induced long-term depression (LTD) of glutamatergic transmission. Here the potential involvement of aspects of both GABA and NMDA receptor mechanisms in the modulation of the response to HFS by stress was assessed.

Male Wistar rats (280-350g) were either left in their home cage or exposed to an acute episode of inescapable stress by placement upon a raised platform for 30 min immediately prior to anaesthesia (urethane, 1.5 g/kg, i.p.). Field EPSPs were recorded from the dorsal hippocampal CA1 area. The effectiveness of stress in blocking HFS-induced LTP was assessed before treatment with either picrotoxin (PTX, 2.5mg/kg, i.p.) or CPP (R,S-carboxypiperazinyl-propyl–phosphonic acid, 15mg/kg, i.p.). A second tetanus was then applied. The animal care and experimental protocol were licensed by the Department of Health.

In stressed animals the first tetanus blocked HFS-induced LTP (105.1±10.5%, mean±SEM % pre-HFS baseline measured at 60 min post-HFS, n=5). The second tetanus, applied 20 min after PTX was administered, induced a delayed onset (15 min), but marked decrease in EPSP amplitude (61.3±11.8%, $P<0.05$ compared to baseline, 97.4±7.1%, or vehicle-injected controls, 102±3.9%, t-test, n=5 per group). In non-stressed animals PTX blocked LTP but did not enable a persistent depression (105.9±3.5% versus123.3±12% in vehicle-injected controls, $P<0.05$, n=5 per group, both values at 60 min post-HFS). In further experiments PTX was administered prior to anaesthesia. LTP was blocked in animals that were placed either in their home cage (94.8±7.1%, n=5) or on the elevated platform (83.6±16.1, n=4) for 30 min after the PTX injection. In the case of CPP, the injection was given 60 min before the second HFS in stressed animals, a time when LTP is blocked in non-stressed animals. The second tetanus induced a gradual and persistent reduction of the EPSP amplitude (59.0±18.0% at 120 min post-HFS versus 98.7±2.1% in vehicle injected animals, $P<0.05$, n=5 per group).

These findings provide further evidence for the modulation of synaptic plasticity by stress. They also show that blockade of GABA-mediated transmission or NMDA receptors under stressed conditions can lead to a facilitation in the induction of an LTD-like persistent use-dependent decrease in synaptic transmission.

Supported by the Wellcome Trust.

153P INHIBITION OF COLORECTAL DISTENSION INDUCED C-FOS EXPRESSION IN THE SPINAL CORD FOLLOWING MK801 AND GV196771 IN THE ANAESTHETISED RAT.

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Repeated colorectal distension (CRD) evokes Fos-like immunoreactivity (Fos-LI) in discrete areas of the rat spinal cord associated with nociceptive input (Traub *et al.*, 1992). We report the effect of a NMDA antagonist MK801 (Haghighi *et al.*, 1996) and a glycine site (on the NMDA receptors) antagonist GV196771 (E-4,6-dichloro-3-(2-oxo-1-phenyl-pyrrolidin-3-ylidenemethyl)-1H-indole-2-carboxylic acid sodium salt), (Quartaroli *et al.*, 1999) on numbers of Fos-LI nuclei in the spinal cord following CRD.

Male Wistar rats (270-335g) were anaesthetised with urethane (1.5gkg^{-1} i.p.) and a colorectal balloon was inflated to 80mmHg for 30s, every 2min, for 120min. MK801 (0.3mgkg^{-1} i.v.), GV196771 (0.3mgkg^{-1} i.v.) or vehicle were administered 10min before CRD onset. Following cardiac perfusion with phosphate buffer and formalin, the lumbar spinal cord (L6-S1) was removed and post-fixed in formalin. 40µm sections of spinal cord were processed for visualisation of Fos-LI using Avidin-Biotin-peroxidase Complex (ABC). Fos-LI nuclei were manually counted (total in 12 sections; L6-S1). Sections were divided into 4 areas: area 1 (laminae I-II), area 2 (laminae III-IV), area 3 (laminae V-VI) and area 4 (laminae V-X). Statistical significance was assessed using unpaired Students t-test ($P<0.05$ significant).

Pre-treatment with MK801 significantly inhibited total numbers of Fos-LI nuclei in all areas to 879±91 (n=5; $P<0.05$) vs 1252±144 (n=5; control) and in area 4. MK801 did not significantly reduce Fos-LI nuclei in areas 1, 2 and 3. Similarly, GV196771 significantly inhibited total numbers of Fos-LI nuclei in all areas to 820±79 (n=4; $P<0.05$) vs 1163±96 (n=4; control) and in area 4. GV196771 did not significantly inhibit Fos-LI nuclei in areas 1, 2 and 3 (Table 1.).

Table 1. Results expressed as number (± standard error of mean) of Fos-LI nuclei in spinal cord following CRD in vehicle, MK801 and GV196771 treated rats.

Compound	Area 1	Area 2	Area 3	Area 4
Control	345 ±66	136 ±32	392 ±37	379 ±27
MK801	268 ±38	80 ±18	280 ±43	251* ±45
Control	286 ±35	130 ±22	410 ±37	337 ±30
GV196771	229 ±26	87 ±33	311 ±24	192* ±43

* ($P<0.05$) relative to respective control group

In conclusion, MK801 and GV196771 inhibit Fos-LI in area 4, but do not in areas 1, 2 and 3 following activation of visceral nociceptive pathways. NMDA antagonist or antagonists at the glycine site of the NMDA receptor may therefore be useful in the treatment of visceral pain by modulating neuronal activation in the spinal cord.

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154P SECONDARY HYPERALGESIA FOLLOWING INTRAPLANTAR CAPSAICIN INJECTION IN THE ANAESTHETISED RAT

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Intradermal capsaicin injection is used as a model of central sensitisation in human volunteers (Simone et al., 1987) and has been shown to elicit both allodynia and secondary hyperalgesia. Behavioural studies in rats have also demonstrated that capsaicin injection into the hindpaw produces hyperalgesia to both thermal and mechanical stimuli (Gilchrist et al, 1996).

Here we have examined the response of second order dorsal horn (DH) neurones to intraplantar (i.pl.) injection of capsaicin into an area outside the receptive field of the neurone, using in vivo DH recording in the anaesthetised rat.

Male Sprague Dawley rats (300-350g) were anaesthetised (pentobarbitone 30 mgkg^{-1}h^{-1} i.v.) and the spinal cord exposed by laminectomy at vertebral level L1. Single unit wide dynamic range (WDR) neurones were identified in the dorsal spinal cord which responded to noxious pinch of the hindpaw receptive field (HRF). Baseline responses were obtained to mechanical (von Frey filaments ranging from 0.976g to 164.32g) stimulation to the HRF before i.pl. injection of capsaicin (10µg, 10 µl) or control vehicle (10µl, 10% ethanol, 10% Tween 80 in saline) approximately 1.5cm outside the receptive field (RF) of the cell. Thereafter, mechanical stimulation was continued every 15mins for 75mins, during which time the ongoing neuronal activity was continuously recorded. Data were normalised to the maximum response to a mechanical stimulation during the experiment and analysed by Student's paired t-test. A probability (P) value of <0.05 was taken as significant. I.pl vehicle injection outside the RF of the neurone did not alter the spontaneous firing rate of the DH neurones (14.9±7.4 Hz pre-injection, 10.41±5.3 Hz post-injection, n=5). In addition, hindpaw vehicle injection did not alter the DH neuronal response to mechanical stimulation (n=5). In contrast, i.pl. injection of capsaicin into an area outside the RF resulted in both an increased response to the same mechanical stimulus force (e.g. 164.32g elicited 24±9% of the maximum response pre capsaicin and 99±0.5% post capsaicin, demonstrating hyperalgesia, n=5, $P<0.001$) and a decrease in the force required to elicit a response (e.g. 0.976g elicited a negligible response (0.2±0.2% of the maximum) pre capsaicin and 4±1% post capsaicin, demonstrating allodynia, n=5, $P<0.05$). The mean force-ratio to elicit a 10% response was 0.13±0.02 (post:pre capsaicin; n=5). The spontaneous firing rate of the DH neurones was unaltered by capsaicin (1.6±0.5 Hz pre-capsaicin, 1.6±0.8 Hz post-capsaicin, n=5). Supporting evidence that the capsaicin did not diffuse into the neuronal RF was obtained by injection of brilliant cresyl blue (10µg in 10µl capsaicin vehicle) into the plantar surface in separate experiments; diffusion of the dye was <2mm from the injection site in any direction.

These data indicate that i.pl. capsaicin causes hyperexcitability of central neurones resulting in an increase in the size of the receptive field of DH neurones, an increased response to the same stimulus, indicative of hyperalgesia and a decreased threshold for activation indicative of allodynia.

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155P MODULATION OF VOLTAGE-GATED CALCIUM AND POTASSIUM CURRENTS BY SELECTIVE ADENOSINE A1 RECEPTOR AGONISTS IN RAT ISOLATED DORSAL ROOT GANGLION CELLS

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We have previously described the analgesic properties of adenosine A1 receptor agonists in a range of animal models of neuropathic and inflammatory pain (Clayton *et al.,* 2000), but pro-algesic properties have also been noted using localised administration of these agents (Dowd *et al.,* 1998). A1 receptor agonists are thought to mediate the inhibition of calcium flux through voltage-gated Ca^{2+} channels in a range of cell and tissue types including dorsal root ganglion (DRG) cells (Dolphin *et al.,* 1986). In the present study, we compare the intrinsic efficacy of the full A1 receptor agonist, GR79236, and the partial agonist, GR190178, in their modulation of voltage gated Ca^{2+} and K^+ currents in isolated DRG cells.

DRGs were removed from neonatal (7-12 days old) Sprague-Dawley rats (approx. 15 grams), and cells dissociated by mild enzymatic treatment and trituration. Cells were plated onto laminin/poly-ornithine coated coverslips and cultured using standard methods. Whole-cell voltage-gated Ca^{2+} and K^+ currents were isolated using standard recording techniques (external solutions containing tetrodotoxin (TTX) (0.1 μM) and TEACl (50mM) with internal Cs^+ (110mM) for Ca^{2+} currents, and external TTX (0.1 μM) and $CdCl_2$ (500μM) for K^+ current recordings). Inward Ca^{2+} currents were evoked using depolarising ramps from a holding potential of –80mV to +40mV, duration 200msec and frequency 0.05Hz. Non-cumulative concentration-effect curves were determined for GR79236 and GR190178 (10nM-100μM) once reproducible Ca^{2+} currents were attained. Outward K^+ currents were evoked with a depolarising pulse from a holding potential of –90mV to +10mV, duration 500msec, and frequency 0.1Hz. In order to assess the stability of currents, initial ramps and voltage steps were performed in the absence of vehicle/compound. Data are mean inhibition ± s.e.mean. IC_{50} values are geometric means with 95% confidence intervals in parentheses. Statistical analyses were performed using the Student's unpaired *t*-test.

The full agonist, GR79236, inhibited peak Ca^{2+} current, determined at –15mV (maximal effect of 36.1 ± 8.7% at 1μM; n=5) in a concentration-dependent manner with an IC_{50} of 0.13 μM [10.6nM-2.1 μM]; n=5). GR190178 displayed similar efficacy (maximal inhibition 26.5± 7.9%; n=5, not significantly different from GR79236 maximal inhibition) but with lower potency (IC_{50} of 1.24 μM [0.32nM-2.3mM]; n=5). Interestingly, both A1 agonists also exhibited inhibition of voltage-gated K^+ currents. Determined at a test potential of +10mV, GR79236 (1nM) inhibited K^+ currents with a maximal inhibition of 23.8 ± 4.7% (n=10), and GR190178 (0.1μM) produced similar inhibition of 26.8 ± 9.4% (n=5; P>0.05) at a concentration of 0.1 μM.

These results further demonstrate the role of adenosine A1 receptors in the modulation of somatic sensory processing. Importantly, while GR79236 and GR190178 exhibit differences in intrinsic efficacy determined using recombinant systems, it is clear that in native systems both appear equally efficacious. Furthermore, activity at both Ca^{2+} and K^+ channels may underlie analgesic and proalgesic effects of A1 agonists respectively.

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156P ANTINOCICEPTIVE EFFECTS OF THE BISPHOSPHONATE, ZOLEDRONIC ACID, IN A NOVEL RAT MODEL OF BONE CANCER PAIN

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One third of patients with advanced cancer will develop clinically relevant skeletal metastases during the course of their disease (Thurliman & de-Stoutz, 1996). Bone pain is the most common pain syndrome encountered in cancer patients with treatment limited to the use of opioids, often only within a dose range that produces side effects (World Health Organisation, 1990). Here we describe a new model of bone cancer pain in the rat and evaluate the pharmacological effect of the bisphosphate, zoledronate.

Intra-tibial injections of $3x10^3$ or $3x10^4$ MRMT-1 cells produced a rapidly expanding tumour within the boundaries of the tibia, causing severe remodelling of the bone in female Sprague-Dawley rats. Radiography showed severe damage to the compact bone by day 15 after inoculation of $3x10^3$ MRMT-1 cells, and by day 20, the damage was threatening the integrity of the bone. Both mineral content and mineral density decreased significantly in the cancerous bone. The general activity of the MRMT-1-treated group declined during the progress of the disease. Rats displayed the gradual development of mechanical allodynia and mechanical hyperalgesia/hind limb sparing. These symptoms were not observed in rats receiving heat-killed cells or vehicle. The bisphosphonate, zoledronic acid (10-30 μg/kg, s.c.) produced a significant anti-allodynic effect, death of tumour cells and reduction of osteoclast activity after repeated administration for 2 weeks. The bone structure in the zoledronic acid-treated group was retained. Acute or chronic treatment with diclofenac or celebrex did not affect mechanical allodynia or weight bearing.

In summary, the induction of bone cancer in the rat by the MRMT-1 mammary tumour cell line provides a valid preclinical model for pain associated with bone metastases. Zoledronic acid, which has therapeutic potential in bone cancer attenuated allodynia and hyperalgesia in the model. NSAIDs and the selective COX-2 inhibitors have no influence on the pain-related behavioural changes in this model.

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157P THE ANTI-HYPERALGESIC EFFECTS OF THE HIGH AND LOW INTRINSIC EFFICACY ADENOSINE$_1$ RECEPTOR AGONISTS GR79236X AND GR190178X IN A MOUSE MODEL OF INFLAMMATORY HYPERALGESIA

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Activation of spinal adenosine receptors produces anti-hyperalgesic activity in animal models of acute, chronic inflammatory and neuropathic pain states (Keil & Salter, 1996). We have previously reported on the anti-hyperalgesic effects of the high intrinsic efficacy Adenosine$_1$ receptor agonist GR79236X (Clayton., et al., 2000a), and the low intrinsic efficacy Adenosine$_1$ receptor agonist GR190178X (Clayton, et al., 2000b), in rat models of inflammatory hyperalgesia. More recently, we have developed a mouse model of inflammatory hyperalgesia induced by Freund's Complete Adjuvant (FCA, 1mg/ml Mycobacterium tuberculosis, Sigma). Here we describe the anti-hyperalgesic activity of both GR79236X and GR190178X in this model. We also investigated the effect of the Adenosine$_1$ receptor antagonist DPCPX (Halleen, et al. 1987) on the anti-hyperalgesic effects of GR79236X in the same model.

Inflammatory hyperalgesia was induced in male C57BL/6 mice (20-30gms) by intraplantar FCA (50µl). Animals received either GR79236X (0.1-1mg.kg^{-1} sc) or GR190178X (1-10mg.kg^{-1} po) 48 hours post FCA. In the antagonist study, DPCPX (5mg.kg^{-1} po) was administered 15 minutes post GR79236X (0.3mg.kg^{-1} sc). Animals were acclimatised for 30 minutes before withdrawal latencies were assessed on the left inflamed paw using a modified version of the Hargreaves Plantar Test (Hargreaves, et al. 1988). Five readings were taken from each animal (n=6) at five minute intervals. The 5 readings was then totalled for each animal, the resulting 6 totals were averaged to produce a single figure for each dose group.

Results are expressed as mean paw withdrawal latencey in seconds ± s.e.m. Statistical analysis was carried out to determine if there was a significant difference between the vehicle treated group and the drug treated group using unpaired Student t test.

GR79236X (0.1-1mg.kg^{-1} sc) produced a dose related inhibition of the FCA induced decrease in thermal paw withdrawal latency, returning to baseline values at 1mg.kg^{-1} sc. (untreated controls 52(s) ±4, FCA + vehicle 18(s) ±1, FCA + GR79236X; 0.1mg.kg^{-1} 23(s) ±2, 0.3mg.kg^{-1} 42(s) ±2, and 1mg.kg^{-1} 58(s) ±5. GR190178X (1-10mg.kg^{-1} po) also produced a dose inhibition of the FCA induced decrease in thermal paw withdrawal latency, although on this occasion, the withdrawal latencey were not returned to baseline values, (untreated controls 41(s) ± 6, FCA + vehicle 8.5(s) ± 1, FCA + GR190178X; 1mg.kg^{-1} 11(s) ±3, 3mg.kg^{-1} 20(s) ±4, and 10mg.kg^{-1} po 26(s) ±5). In the final study, the Adenosine$_1$ receptor antagonist DPCPX blocked the anti-hyperalgesic activity of GR79236X, (untreated controls 39(s) ±5, FCA + vehicle 10(s) ±1.5, FCA + GR79236X (0.3mg.kg^{-1} sc) 42(s) ±2, FCA + GR79236X (0.3mg.kg^{-1} sc)/ DPCPX (5mg.kg^{-1} po) 16(s) ± 2.5).

In conclusion, GR79236X and GR190178X produced anti-hyperlagesic activity in a mouse model of FCA-induced inflammatory hyperalgesia, as well as rat models of inflammatory hyperalgesia as we have previously reported. The Adenosine$_1$ receptor antagonist DPCPX blocked the anti-hyperalgesic effects of GR79236X. GR190178X produced only a partial reversal of the decrease in paw withdrawal latency, this could be due to the low intrinsic efficacy of the molecule.

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158P THE ROLE OF CENTRAL AND PERIPHERAL BRADYKININ B1 RECEPTORS IN MODELS OF NEUROPATHIC AND INFLAMMATORY PAIN IN THE RAT

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Kinins are important mediators of inflammation and pain (Perkins & Dray 1996). Activation of peripheral B1 receptors produces hyperalgesia following inflammation (Kelly & Perkins 1998). In the rat sensory nervous system, B2 receptors have been localised to sections of dorsal root ganglia and dorsal horn. B1 receptor expression has been more difficult to demonstrate (Davis et al 1996), but a recent study (Winter *et al*, 2000) provided evidence of constitutive expression of B1 receptor protein in dorsal root ganglion neurones and spinal cord. Here we have examined the effect of centrally versus peripherally administered B1 antagonists and agonists in models of inflammation and chronic neuropathic pain.

Chronic neuropathic hyperalgesia was induced by partial ligation of the left sciatic nerve (Seltzer *et al*, 1990). Inflammatory hyperalgesia was induced by intraplantar injection of 25µl Freund's complete adjuvant (FCA), into the left hind paw. 10-21 days following surgery and 24 hours following FCA mechanical hyperalgesia was assessed by measuring paw withdrawal thresholds using an Ugo Basile Analgesymeter. Mechanical thresholds were determined on both hind paws prior to and then up to 6 h following intrathecal or subcutaneous drug or vehicle administration. Drug effects are expressed as D_{50} values (dose producing a 50% reversal of predose hyperalgesia) and efficacy (maximal percentage reversal of hyperalgesia).

Subcutaneous administration of the B1 receptor antagonist, desArg^{10}HOE140 (50 – 1000nMoles/kg) produced up to 63% reversal of inflammatory mechanical hyperalgesia 1 hour post. There was no effect in the neuropathic model. Intrathecal administration of desArg^{10}HOE140 (1-50µg), produced a dose dependent inhibition of both inflammatory and neuropathic mechanical hyperalgesia. In the FCA model a maximal 70% reversal of mechanical hyperalgesia was observed 1 hour following administration. The antagonist was less effective in the neuropathic pain model producing a maximum reversal of approximately 40%. For comparison the B2 receptor antagonist HOE140 (1-50µg), produced a maximum reversal of 50% at 3 hours (D_{50} 30.7µg). Intrathecal administration of a B1 agonist (desArg^{9}BK, 1-50µg) did not affect paw withdrawal thresholds in naïve animals, but produced a significant decrease in contralateral paw withdrawal thresholds 24h following FCA injection into the ipsilateral paw.

In summary, these data show that B1 receptors located in the spinal cord may be involved in the mechanisms of inflammatory and neuropathic hyperalgesia.

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159P PHARMACOLOGICAL EVALUATION OF THE MOUSE MODEL OF COMPOUND 48/80 INDUCED ITCH

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The aim of this study was to evaluate the effect of different classes of drugs in the compound 48/80 induced model of itch in the mouse (Kuraishi, Y., 1995).

Experiments were done in adult female C57BL/6 mice (25-30 g). Mice received the drug/vehicle 30 or 60 minutes prior to the pruritic compound 48/80 (30µg/10µl; s.c. into the dorsal neck region). Mice were placed into a clear Perspex box and continuously observed for 30 minutes following injection. 'Scratching episodes' were defined as scratching focused at the site of injection. The total duration of scratching behaviour was recorded by using a keyboard linked to three stop-clocks. Differences between treatment groups were analysed by ANOVA followed by Tukey's HSD test ($P < 0.05$).

The 5-HT_3 antagonist, Y25130 (p.o.) inhibited the scratching by 51%. The selective mGluR5 antagonist MPEP (p.o.) also significantly shortened the duration of itch at the highest dose (100 $mgkg^{-1}$). Subcutaneously administered bradykinin B_1 and B_2 antagonists, $DesArg^{10}Hoe140$ and Bradyzide did not inhibit the compound 48/80 induced itch. While the histamine H_1 antagonist, terfenadine produced a significant, dose-dependent reduction (max 80%) of the duration of itch, histamine H_2 and H_3 antagonists, ICI162846 and thioperamide or the NSAID diclofenac (all p.o.) did not have any effect. Drug effects were compared to the relevant vehicle. The controls did not show any pruritogenic activity. For doses and summary, see table 1.

Table 1: Effect of different classes of drugs in the mouse model of compound 48/80 induced itch (n=6).

Drug (route)	dose range	Antipruritic effect
Y25130 (5-HT_3) (p.o.)	0.1-3 $mgkg^{-1}$	+
MPEP (mGlu5) (p.o.)	10-100 $mgkg^{-1}$	+
$desArg^{10}Hoe140$ (s.c.)	0.1-1 $\mu mol/kg^{-1}$	-
bradyzide (p.o.)	3-10 $\mu mol/kg^{-1}$	-
terfenadine (H_1) (p.o.)	1-100 $mgkg^{-1}$	+
ICI162846 (H_2) (p.o.)	5-20 $mgkg^{-1}$	-
thioperamide (H_3) (p.o.)	3-30 $mgkg^{-1}$	+
diclofenac (p.o.)	3-30 $mgkg^{-1}$	-

Kuraishi, Y., Eur J Pharmcol 1995; 275:229-233

160P CANNABINOID CB_1 RECEPTORS IN THE PERIAQUEDUCTAL GREY MAY MODULATE FORMALIN-EVOKED PAIN BEHAVIOUR IN RATS

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The midbrain periaqueductal grey (PAG) mediates behavioural responses to nociception (Behbehani, 1995). The PAG contains CB_1 receptors (Tsou *et al.*, 1998) and may be an important component of a central cannabinergic pain-modulatory system (Walker *et al.*, 1999) with CB_1 receptors in this region acting as antinociceptive targets (Martin *et al.*, 1995; Strangman *et al.*, 1998). The present study investigated the effects of intra-PAG administration of the CB_1 agonist HU210 (6aR-*trans*-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetra-hydro-1 hydroxy-6,6-dimethyl-6H dibenzo [b,d]pyran -9-methanol) and the CB_1 antagonist SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H* -pyrazole-3-carboxamide) in the rat formalin pain test.

Male Lister-Hooded rats (250-300 g) were cannulated in the dorsal PAG (AP: –6.7 lat: + 1.7, depth: -4.1 mm relative to bregma and dura, 20° angle) under halothane anaesthesia. Experiment 1 (Exp.1) began 7-10 days post-surgery with rats receiving a single intra-PAG micro-injection of HU210 (1 µg/250 nl), SR141716A (1 µg/250 nl) or vehicle (60% DMSO). Using Ethovision, behaviour was tracked 10 min prior to and 60 min after subcutaneous injection (50 µl) of 5% formalin into the right hind paw. In experiment 2 (Exp.2), rats received vehicle or a higher dose of HU210 (5 µg/250 nl). Pain behaviour, defined as any attention given to the injected paw, was distinguished from normal grooming behaviour. Locomotor activity was also measured. Data were analysed by one-way ANOVA followed by Student-Newman-Keuls test).

Formalin-evoked pain behaviour was biphasic with two peaks of intensity occurring within the first 5 min and at 25-30 min post-formalin in vehicle treated rats. HU210 (5 µg/250 nl) administration significantly reduced pain behaviour in the second phase 25-35 min post-formalin compared with vehicle treated controls (Figure 1). Duration of pain behaviour over a 60 min period post-formalin was not significantly affected by SR141716A (1657.5 ± 183.8 secs) or low dose (1 µg/250 nl) HU210 (1572 ± 205.7 secs) compared with controls (1245.4 ± 149.7 secs). Distance moved post-formalin was not significantly affected by HU210 (Exp.1: 6622 ± 1050 cm; Exp.2: 4554 ± 562 cm) or SR141716A (Exp.1: 7194 ± 195 cm) compared with controls (Exp.1: 8715 ± 2516 cm; Exp.2: 4787 ± 880 cm). Post-formalin grooming was not significantly affected by HU210 (Exp.1: 131.2 ± 40.8 secs; Exp.2: 59.8 ± 11.2 secs) or SR141716A (Exp.1: 52.2 ± 16 secs) compared with controls (Exp.1: 81.2 ± 29 secs; Exp.2: 78 ± 21.9 secs).

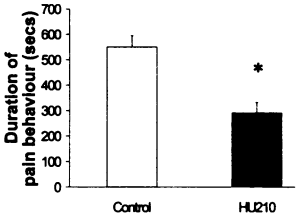

Figure 1. Effect of intra-dorsal PAG micro-injection of the CB_1 agonist HU210 (5 µg/250 nl) on the duration of pain behaviour 25-35 min following formalin injection into the right hind paw of rats. Data represent mean ± s.e. mean (n = 4-5). $^*P < 0.05$ *vs* vehicle treated controls.

These data provide evidence for a specific antinociceptive effect of a CB_1 agonist at the level of the dorsal PAG. The existence of an endogenous cannabinoid antinociceptive system in the PAG requires further investigation.

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161P CANNABINOID RECEPTORS IN POSTMORTEM HUMAN BRAIN: A RADIOMETRIC AND TRANSDUCTIONAL STUDY IN MAJOR DEPRESSION

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Central cannabinoid receptors (CB_1) belong to the superfamily of G protein-coupled receptors (GPCRs), and mediate most of the cannabinoid effects on the central nervous system across different species. These receptors are expressed at high densities in human brain (Herkenham *et al.*, 1991), and negatively coupled to adenylyl cyclase through Gi/o proteins (Howlett *et al.*, 1986). The aim of this study was to evaluate, by radiolabelled agonists binding and autoradiographic techniques, the possible modifications in the properties, distribution and activity of CB_1 receptors in *postmortem* brain samples from suicide victims with a diagnosis of major depression, and matched control subjects without any record of neuropsychiatric disease. Both types of brain samples were obtained immediately after autopsy.

Briefly, the CB_1 protein was labelled in brain sections (n=15) with the cannabinoid agonist [^{3}H]CP55,940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxy-propyl)cyclohexanol), [^{3}H]-). Unfixed sections were incubated for 2 h in 50 mM Tris-HCl, 5% BSA, with 3 nM [^{3}H]CP55,940, in the presence of 10 μM WIN55,212-2. Agonist stimulated [^{35}S]GTPγS binding (n=9) was performed by incubation of frontal cortex membranes for 2 h in 50 mM Tris-HCl, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA, 0.2 mM DTT and 0.5% BSA, in the presence of 0.05 nM [^{35}S] GTPγS, 100 μM GDP, and a range of WIN55,212-2 concentrations (1 nM-100 μM). Functional receptor autoradiography was carried out pre-incubating brain sections (n=7) in the same buffer (but 1mM EGTA and 100 μM GDP) for 30 min, following a 2 h incubation in the presence of 0.04 nM [^{35}S]GTPγS and 100 μM WIN55-212-2. In both cases, non-specific binding was defined with 100 μM GTPγS.

[^{3}H]CP55,940 autoradiography yielded no significant changes in CB_1 receptor density, though a slight tendency to the increase was observed for the major depression cases in some hippocampal regions (68 ± 17 fmol/mg tissue for suicide cases, 36 ± 10 fmol/mg tissue for control cases in the granular layer of the dentate gyrus; p=0.06, Student's paired t test). When [^{35}S]GTPγS assays were carried out, a significant increase in WIN55,212-2 maximal stimulation ability (% Emax) was found for the suicide cases in frontal cortex membranes (282 ± 18% for suicide cases, 230 ± 18% for control cases; p=0.03, Student's paired t test). A tendency to the increase in %Emax was also observed for the major depression cases in frontal cortex sections (371 ± 59% for suicide cases, 283 ± 38% for control cases, in layer IV; p=0.25, Student's paired t test). These data suggest that postreceptorial changes associated to CB_1 receptors may be implicated in major depression.

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162P CANNABINOID ACTIVATION OF RECOMBINANT AND ENDOGENOUS VANILLOID RECEPTORS

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To date only a single functional vanilloid receptor (VR1) has been cloned (Caterina *et al.*, 1997), although there is pharmacological evidence suggesting vanilloid receptor heterogeneity in biological tissues. Therefore in the present study the effects of vanilloids, cannabinoids and the anandamide transport inhibitor *N*-(4-hydroxyphenyl)-arachidonylamine (AM404) (Zygmunt *et al.*, 1999; Jerman *et al.*, 2000) in the rat isolated mesenteric arterial bed, which endogenously expresses vanilloid receptors, were compared to those in HEK293 cells stably expressing rat or human VR1 (rVR1 & hVR1).

HEK293 cells, stably expressing rVR1, were seeded into Costar 96 well black walled plates (25,000 cells per well) and cultured overnight in minimum essential medium containing 10 % foetal calf serum, 2 mM L-glutamine and 400 μg ml^{-1} G418. Cells were loaded with the cytoplasmic calcium indicator Fluo-3AM (4 μM) at 25°C for 2 h. They were then washed with, and finally resuspended in, Tyrode's medium, before being incubated with buffer alone or antagonist for 30 min. Fluorescence was monitored using a FLIPR (λ_{EX}=488 nm, (λ_{EM}=540 nm) before and after the addition of various agonists (10 pM-10 μM). Male Wistar rats (250-300 g) were sacrificed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused via the superior mesenteric artery with oxygenated Krebs' solution at 5 ml min^{-1} (Ralevic *et al.*, 1995). After 30 min, preparations were preconstricted with methoxamine (10-50 μM) and relaxant responses to cumulative concentrations of agonists established. Data reported are means ± s.e.m. of results from at least five experiments. Data were compared by ANOVA.

In rVR1- and hVR1-HEK293 cells anandamide, AM404 and vanilloids were all full agonists, causing concentration-dependent increases in $[Ca^{2+}]_i$ with a rank order of potency of olvanil≥capsaicin>AM404>anandamide (pEC_{50} values 8.09±0.09, 7.97±0.13, 6.53±0.12 and 5.73±0.04, respectively at rVR1 and 7.73±0.05, 7.1±0.06, 6.6±0.12 and 5.6±0.1, respectively at hVR1). Methanandamide was less potent than anandamide, only evoking a 44.6±3.8% response at 10 μM. These responses were inhibited by the VR1 antagonist, capsazepine. In the mesenteric arteries, cannabinoids and vanilloids were full agonists, evoking vasorelaxation with a similar potency order as at rVR1 and hVR1, although the actual potencies were higher. pEC_{50} values were 8.75±0.05, 8.16±0.07, 7.14±0.12, 6.32±0.04 and 5.98±0.05 respectively, for olvanil, capsaicin, AM404, anandamide and methanandamide. Capsaicin pre-treatment (10 μM, 2h) blocked relaxations evoked by AM404 in the mesenteric bed.

In conclusion, the present study has shown that AM404, anandamide and methanandamide are agonists at hVR1. Their potencies, and potency order, at hVR1, and that of olvanil and the archetypal vanilloid capsaicin, were similar to those observed at rVR1. A similar potency order of AM404, cannabinoids and vanilloids was also obtained for vasorelaxation mediated via endogenous vanilloid receptors in the rat mesenteric arterial bed. These data indicate that VR1 mediates vasorelaxation to AM404, cannabinoids (anandamide and methanandamide) and vanilloids in the rat mesenteric arterial bed.

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163P NO INFLUENCE OF CANNABINOIDS ON ELECTRICALLY-STIMULATED RELEASE OF 3H-NORADRENALINE FROM RAT ATRIA

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Cannabinoids can have potent effects on the cardiovascular system including bradycardia, hypotension and vasorelaxation (Randall & Kendall, 1998). Activation of CB_1 receptors by plant cannabinoids or the endogenous ligand, anandamide causes hypotension in anaesthetized rats via a sympathoinhibitory action (Varga *et al.*, 1995). There is also evidence for cannabinoid modulation of sympathetic neurotransmission in rat atria (Ishac *et al.*,1996) although the identity of the receptor(s) involved has not been resolved. The present report investigated the effects of cannabinoids on the electrically-stimulated release of ^{3}H-noradrenaline (^{3}H-NA) from rat atria *in vitro*.

Male Wistar rats (250-300g) were killed by exposure to carbon dioxide and decapitation and both atria were removed. The atria were pre-labelled with 10 µL of ^{3}H-NA (37kBq/µL) in 1 ml of Krebs' Henseleit buffer (KHB). The tissue was pre-incubated for 45 minutes in a shaking water bath at 37° C. and then rinsed 3 times with KHB to remove excess radiolabel. Each atrium was placed in a chamber of a Brandel suprafusion apparatus and washed for 90 minutes with KHB at 0.8 ml/ min. Two samples of suprafusate were collected at 3 min intervals as a baseline reading. The atria were then electrically stimulated for 1 min (S1) (biphasic pulses, 1 ms duration, 100 mA at 4 Hz). This sample and a further two post stimulation samples were collected. The atria were then exposed to drugs for 30 mins followed by a second stimulation (S2) identical to S1. ^{3}H-NA was quantified by liquid scintillation counting. Release data are expressed as the ratios of S2/S1 (means ± s.e.m.) after subtraction of baseline values. One way ANOVA, with Dunnett's Multiple Comparison Test was used for statistical comparisons.

Electrical stimulation caused a substantial (S1) release of ^{3}H-NA (approximately 3-fold basal) from the atria. S2/S1 ratio in the absence of drug exposure was 0.93 ± 0.4 (n=27).

Release was essentially abolished by the removal of Ca^{2+} from the KHB or by the inclusion of tetrodotoxin (1µM), demonstrating that release was exocytotic. The cannabinoid CB_1 receptor antagonists SR141716A and LY320135 (both 1µM) had no effect on stimulated release; S2/S1 = 0.75 ± 0.2 and 0.84 ± 0.1 respectively (n=6, P>0.05) indicating an absence of endogenous cannabinoid tone. The non-selective cannabinoid receptor agonist HU-210, at concentrations of 1µM and 3µM, was also without effect; S2/S1 = 1.02 ± 0.1 (n=6) and 1.24 ± 0.2 (n=8, P>0.05) respectively. Similarly, the endogenous cannabinoid receptor agonist anandamide did not significantly alter electrically-stimulated release of ^{3}H-NA.; S2/S1 = 0.96 ± 0.1 (n=6, P>0.05). The α2-adrenoceptor antagonist idazoxan (0.1µM) enhanced release as expected; S2/S1 = 1.88 ± 0.2 (n=8, P<0.01). The inclusion of idazoxan and atropine to inhibit α2-adrenoceptor and muscarinic heteroceptors failed to reveal any effect of HU210 on the release of ^{3}H-NA.

The data presented contrast those of Ishac *et al.* (1996), who reported inhibitions of noradrenaline release from rat atria due to tetrahydrocannabinol and anandamide. The present results do not, therefore, support the existence of functional pre-synaptic cannabinoid receptors on sympathetic nerves supplying the atria and suggest that the most likely locus of action of the cannabinoids in altering sympathetic drive to the heart is within the central nervous system.

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164P MODULATION OF INTERLEUKIN-8 (IL-8) SECRETION IN THE HUMAN COLON EPITHELIAL CELL LINE HT-29 BY CANNABINOIDS.

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There is evidence that cannabinoids modulate immune cell function (Berdyshev, 2000) but the receptors, which may mediate these effects, are poorly characterized. We have investigated the effects of cannabinoid receptor agonists and antagonists on tumour necrosis factor-α (TNF-α) induced secretion of IL-8 from HT-29 cells.

Confluent monolayers of HT-29 cells were incubated in the presence or absence of cannabinoid agonists for 2 hours prior to stimulation with TNF-α (100ng/ml). Supernatants were harvested 18 hours later and IL-8 release assayed by ELISA as described by Crocker *et al.* (1996). In experiments where the effects of antagonists were studied, a CB_1 or CB_2 receptor antagonist was added 30 minutes before addition of cannabinoid agonists. Data were analysed using Prism (Graph Pad Inc.). pA_2 values were calculated using the Schild equation (Kenakin, 1993). $EC_{1/2max}$ values are represented as geometric mean and 95% confidence limits (C.L.). All other data are arithmetic mean ± S.E.M (n=6 for all data presented).

Confluent monolayers of HT-29 cells (final density=$3x10^6$ ±0.5 cells/ ml) constitutively released small amounts of IL-8 (33.8±3.8pg/ml) and TNF-α (100ng/ml) stimulation evoked a marked increase in IL-8 release (4,187±197pg/ml). The cannabinoid agonists CP55,940 ($EC_{1/2max}$ = $1.2x10^{-7}$M, 95% C.L. = $3.8x10^{-8}$M-$3.6x10^{-7}$M), Δ^9-THC ($EC_{1/2max}$ = $5.3x10^{-8}$M, 95% C.L. = $9.7x10^{-9}$M-$2.9x10^{-7}$M), WIN55212-2 ($EC_{1/2max}$ = $1.7x10^{-7}$M, 95% C.L.= $1.2x10^{-7}$M-$2.5x10^{-7}$M) and 1-propyl-2-methyl-3-(1-naphthyl) indole (JWH 015) ($EC_{1/2max}$=$9.8x10^{-8}$M, 95% C.L. = $6.8x10^{-8}$M-$1.3x10^{-7}$M) inhibited TNF-α induced release of IL-8 in a concentration-dependent manner. The endogenous cannabinoid agonist, anandamide and the cannabinoid CB_1 selective agonist, arachidonyl-2-chloroethylamide (ACEA) had no significant inhibitory effect on TNF-α induced release of IL-8.

The CB_1 receptor antagonist SR141716A (1µM) antagonized the inhibitory effect of CP55,940 (pA_2 = 8.3±0.2), but did not antagonize the effects of WIN55,212-2 (pA_2 = <6) and JWH 015 (pA_2 = <6) respectively. The CB_2 receptor antagonist, SR144528 (1µM) antagonized the inhibitory effects of CP55,940 (pA_2= 8.2±0.3), WIN55,212-2 (pA_2 = 7.1±0.3) and JWH 015 (pA_2 = 7.6±0.4) respectively.

We conclude that in HT-29 cells, IL-8 release is inhibited by cannabinoids mainly through activation of CB_2 receptors. This study provides further evidence that cannabinoids inhibit immune cell function by suppressing cytokine release.

We are grateful to Pfizer U.K. (Ltd.) for the gift of CP55,940.

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165P AGONIST-INDUCED INTERNALIZATION OF METABOTROPIC GLUTAMATE RECEPTOR 1 SPLICE VARIANTS A AND C (mGluR1A AND mGluR1C)

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Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that play important roles in modulating glutamate neurotransmission in the brain. These receptors are regulated by agonist-induced desensitization, often involving receptor phosphorylation and usually followed by receptor internalization. In this study, we investigated the agonist-induced internalization of mGluR1a and mGluR1c.

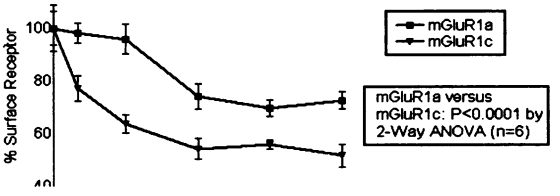
The cDNAs of these receptors were cloned in the pcDNA3 expression vector, tagged with the haemagglutinin (HA) epitope between Pro57 and Pro58 in the extracellular N-terminus, and transiently expressed in human embryonic kidney cells (HEK293). The receptor expression in HEK293 cell membrane was analysed by ELISA assay as described previously by Daunt et al. (1997) using a mouse anti-HA monoclonal antibody and an anti-mouse IgG antibody conjugated to alkaline phosphatase. The same mouse anti-HA monoclonal antibody was used with a rhodamine conjugated anti-mouse IgG antibody to investigate the receptor trafficking by immunofluorescence.

Incubation of cells with the agonists glutamate, quisqualic acid or (S)-3,5-DHPG for 30 min, induced a concentration-dependent internalization of mGluR1a and c (Table 1; maximum extent of internalization 25-50%). The internalization induced by 200 μM glutamate of both mGluR1a and c was completely inhibited by 100μM of the mGluR1 antagonists CPCCOEt, MCPG and LY367385. The time course of receptor internalization induced by 200 μM glutamate on mGluR1a and c is shown in figure 1.

Table 1. Potency of mGluR1 agonists in inducing receptor internalization (EC_{50} ± SEM, in μM).

	Glutamate	Quisqualic acid	(S)-3,5-DHPG
mGluR1a	4.6 ± 0.9	0.44 ± 0.16	6.3 ± 2.2
mGluR1c	6.1 ± 1.4	0.62 ± 0.25	7.8 ± 3.1

Figure 1. Time course of agonist–induced mGluR1a and c internalization.



In conclusion, the potencies of tested agonists in inducing receptor internalization were similar for mGluR1a and c and broadly in agreement with values for mGluR1a activation (Pin et al., 1999). mGluR1a, which is characterized by a long intracellular C-terminus, internalized more slowly and less extensively than mGluR1c. This indicates that the C-terminus of mGluR1a contains domains able to interfere with agonist-induced internalization, or perhaps domains able to promote more rapid recycling of mGluR1a as compared to mGluR1c.

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166P ACTIONS OF THE METABOTROPIC GLUTAMATE RECEPTOR SUBTYPE 5 LIGANDS, DHPG AND MPEP, ON NEURONAL GLUTAMATE RELEASE *IN VITRO* AND *IN VIVO*

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It has previously been reported that glutamate release from rat cerebrocortical synaptosomes is positively modulated by (1S, 3R)-ACPD, a non-selective metabotropic glutamate (mGlu) receptor agonist (Herrero et al., 1992). We now investigate the actions of the potent mGlu5 receptor-subtype ligands, 3,5-dihydroxyphenylglycine (DHPG; agonist) and 2-methyl-6-(phenlyethynyl)-pyridine (MPEP; non-competitive antagonist), on neuronal glutamate release both in vitro and in vivo.

Glutamate release in vitro was monitored using electrically-evoked [^{3}H]D-aspartate ([^{3}H]D-asp) overflow from rat forebrain slices (male Wistars; 240±10g), as previously described (Patel & Croucher, 1997). Responses to electrical stimulation (S_1 & S_2) were calculated as the total levels of stimulated efflux minus mean basal levels and ratios of basal (B_2/B_1) and electrically-evoked (S_2/S_1) efflux of label before and after drug application ((S)-DHPG, 0.1-3.0μM; MPEP, 0.1-10μM) were calculated to assess the influence of the drugs on basal and stimulated release. Actions of the ligands in vivo were studied using intrastriatal microdialysis of preloaded [^{3}H]L-glutamate ([^{3}H]L-glu) in conscious, freely moving male Wistar rats (275-300g; for details see Patel et al., 2001). (RS)-DHPG (0.1-100μM) was applied to the striatum by reverse dialysis for 15min. MPEP (10μM) was applied for 30min commencing 15min prior to agonist introduction. Responses were expressed as maximum percentage change with respect to the preceding baseline sample. All results are mean ± s.e.m. of 5-8 independent observations. Significance of differences in responses was determined using ANOVA followed by Student's post hoc t-test for independent groups.

Electrically-stimulated efflux of [^{3}H]D-asp from rat forebrain slices was significantly enhanced, in a concentration-dependent manner, by (S)-DHPG (max. response 337% of control at 3μM; $P<0.01$). The EC_{50} for this response was 1.10μM (GraphPad, Prism software). MPEP potently inhibited (S)-DHPG, 1μM-evoked responses, with complete abolition observed following MPEP, 10μM (IC_{50}=240nM). Administration of MPEP (10μM) alone showed no effect on basal or electrically-stimulated release of radiolabel. Following reverse dialysis into the corpus striatum in vivo, (RS)-DHPG evoked a concentration-dependent increase in [^{3}H]L-glu release into the dialysis stream (max. response 121.0% of control at 100μM; $P<0.01$; EC_{50}=1.67μM in the dialysate). Co-administration of MPEP, 10μM with (RS)-DHPG, 10μM resulted in full inhibition of the agonist-evoked response. No marked behavioural changes were evident following intrastriatal administration of the mGlu5 receptor ligands, with all animals remaining alert but generally inactive throughout the experiments.

These data provide compelling evidence for a role of mGlu5-subtype glutamate autoreceptors in the regulation of neuronal glutamate release in the mammalian central nervous system and demonstrate, for the first time, functional presynaptic mGlu5 receptors in conscious, freely moving animals.

We thank The Sir Jules Thorn Charitable Trust and the Wellcome Trust (Grant No. 040256/Z/93/Z) for generous financial support. MPEP was a kind gift from Dr. F. Gasparini (Novartis Pharma AG, Basle, Switzerland).

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167P ACTIVATION OF Gαi2 BY D_2(ser^{311}→cys), A NATURALLY OCCURRING POLYMORPHISM OF THE DOPAMINE D_{2L} RECEPTOR

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The naturally occurring polymorphism of the D_{2L} dopamine receptor Ser^{311} → Cys (Cys^{311}) occurs in 3% of the Caucasian population and has been shown to exhibit decreased inhibition of adenylate cyclase compared to the Ser^{311} variant of D_{2L} (Cravchik *et al.*, 1996). Gαi2 has been shown to be important in D_2 inhibition of adenylate cyclase (Senogles, 1994), consequently we have investigated the ability of the two variants to activate this G protein.

Human D_{2L} dopamine Ser^{311} and Cys^{311} receptors were FLAG tagged at the N-terminus by PCR, subcloned into the baculovirus expression vector pVL1392 and recombined into Baculogold® DNA. Baculoviruses encoding the G protein subunits αi2, β1, γ2 were donated by T Kozasa (University of Texas, Dallas). Receptor and G protein subunits were expressed in *Spodoptera Frugiperda* (*Sf*9) cells at the multiplicity of infection 3:6:6:6 (R:α:β:γ). Cell membranes were prepared 48 hours after infection. Receptor levels were determined by saturation analysis with [^{3}H]spiperone as described by Gardner *et al.*, (1996). The number of G proteins was estimated by isotopic dilution GTPγS saturation analysis. Total numbers of αi2 G proteins were measured by comparing the binding of GTPγS in membranes co-expressing R:α:β:γ to membranes co-expressing only R:β:γ. Agonist efficacy and potency for stimulating [^{35}S]GTPγS binding was determined using a variation of the method described by Gardner *et al* (1996) (100µl final volume, 5µg membranes, 200pM [^{35}S]GTPγS). [^{35}S]GTPγS reactions were terminated and radioactivity determined as for saturation studies.

Saturation analysis of [^{3}H]spiperone binding to Ser^{311} and Cys^{311} receptors revealed that [^{3}H]spiperone exhibited the same affinity (Kd) for both receptors (Table 1), this was unaffected by the co-expression of G protein subunits. Receptor and G protein levels in the two preparations used for comparison were not significantly different ($p>0.05$ Student's *t* test) (Table 1).

Dopamine was unable to stimulate [^{35}S]GTPγS binding through either Ser^{311} or Cys^{311} receptors in membranes expressing only receptor whereas clear stimulation was observed when αi2 subunits were co-expressed. Maximal specific stimulation with dopamine at Ser^{311} and Cys^{311} was 41±5 and 40±4 fmol [^{35}S]GTPγS bound per mg of protein respectively (n=4). No variation in agonist efficacy or potency was observed between the two receptors ($p>0.05$ Students *t* test) (Table 2). Therefore it is concluded that the reduced ability of Cys^{311} to inhibit adenylate cyclase is not mediated by the αi2 G protein subunit.

Table 1. Receptor and G protein levels as measured by [^{3}H]spiperone saturation and isotopic dilution GTPγS saturation respectively (n=4)

	Ser^{311}αi2β1γ2	Cys^{311}αi2β1γ2
Spiperone pKd±s.e.m (Kd pM)	10.22±0.09 (60)	10.21±0.07 (62)
Bmax [^{3}H]spiperone (pmol/mg)	0.74±0.09	0.80±0.08
GTPγS Apparent pKd±s.e.m (Kd nM)	8.37±0.21 (4.27)	8.39±0.07 (4.07)
Apparent Bmax GTPγS (pmol/mg)	9.2±2.1	8.4±2.6

Table 2. Potency and efficacy values of agonists at Ser^{311} and Cys^{311} D_{2L} receptors co-expressed with αi2β1γ2 G protein subunits (n=3-4). Efficacy values are expressed as percentage of the maximal dopamine response.

	Ser^{311}αi2β1γ2		Cys^{311}αi2β1γ2	
	pEC_{50}± s.e.m (EC_{50} µM)	Efficacy ±s.e.m	pEC_{50}± s.e.m (EC_{50} µM)	Efficacy ±s.e.m
Dopamine	5.91±0.16 (1.23)	100	5.81±0.18 (1.55)	100
(+)-3PPP	5.48±0.21 (3.31)	59±7	5.37±0.20 (4.27)	52±7
Quinpirole	5.89±0.09 (1.29)	66±10	5.94±0.11 (1.15)	69±5

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168P HUMAN DOPAMINE $D_{2L(long)}$ RECEPTOR COUPLING TO $G_{αi2}$ AND $G_{αo}$ G PROTEINS IN Sf9 INSECT CELLS

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The two isoforms of dopamine D2 ($D_{2S(short)}$ and $D_{2L(long)}$) receptors may have different functions in the brain (Usiello et al., 2000). The D_{2S} is believed to function as a pre-synaptic receptor, whereas D_{2L} functions as a post-synaptic receptor. Interestingly, mice lacking the dopamine D_{2L} receptors do not exhibit catalepsy induced by haloperidol, suggesting a selective targetting of that isoform by drugs such as haloperidol. Different functions may arise from different combination of receptor and G protein subtypes in the brain. Therefore, analysing how the different receptors signal via different types of G proteins may bring further light in our understanding of the mechanism of action of some drugs. We recently reported a preferential coupling of human dopamine D_{2S} to $G_{αo}$ compared to $G_{αi2}$ G proteins in Sf9 insect cells (Nickolls & Strange, 2000). These findings prompted us to investigate the functional coupling of the long isoform of the receptor (D_{2L}) in the same system.

The human dopamine D_{2L} receptor was co-expressed with $G_{αo}$ or $G_{αi2}$ along with $G_{β1}$ and $G_{γ2}$ G protein subunits, using the baculovirus expression vector system. Sf9 insect cells co-expressing the receptor and the different G proteins were harvested 48h post-infection and membranes were prepared as described previously (Gardner et al., 1996). Receptor density (B_{max}) was determined by [^{3}H]spiperone saturation binding. Stimulation of [^{35}S]GTPγS binding (Gardner et al., 1996) was used to determine the functional coupling of the receptor to G protein.

[^{3}H]spiperone was found to label a homogenous population of receptors in membranes prepared from Sf9 insect cells expressing the human dopamine D_{2L} receptor alone (B_{max}=2.03±0.22 pmol.mg^{-1} of protein, K_D 95±18 pM). When the receptor was co-expressed with $G_{αoβ1γ2}$ or $G_{αi2β1γ2}$ the B_{max} amounted 2.88±0.16 pmol.mg^{-1}and 2.80±0.27 pmol.mg^{-1} of protein, respectively. The K_D of [^{3}H]spiperone was not significantly changed. The G protein expression levels determined by isotopic dilution of [^{35}S]GTPγS binding were similar in the two preparations.

The efficacies (relative to that of dopamine) and the potencies (pEC_{50}) of several dopamine D2 receptor ligands for stimulation of [^{35}S]GTPγS binding are summarised in Table1.

Table 1. Agonist stimulation of [^{35}S]GTPγS binding to Sf9 insect cells co-expressing the dopamine D_{2L} receptor and Gi/o G proteins

	$D_{2L}G_{αoβ1γ2}$		$D_{2L}G_{αi2β1γ2}$	
Ligands	*Efficacy*	*pEC_{50}*	*Efficacy*	*pEC_{50}*
dopamine	100	6.35±0.27	100	5.60±0.13
bromocriptine	106±14	9.83±0.91	105±21	8.70±0.92
NPA	125±8	8.90±0.27	99±2	8.29±0.15
quinpirole	82±10	6.58±0.46	78±8	5.38±0.48
R-(+)-3-PPP	75±6	6.12±0.30	46±13	5.97±0.70
m-tyramine	85±4	6.08±0.80	57±16	4.88±0.10

Efficacies are relative to that of dopamine. Data are mean ± s.e.mean. n 3.

Dopamine stimulated in a concentration-dependent manner the binding of [^{35}S]GTPγS to both membrane preparations (E_{max} 44±6 and 90±11% over basal levels for $D_{2L}G_{αoβ1γ2}$ and $D_{2L}G_{αi2β1γ2}$, respectively). No agonist effect was found in Sf9 insect cells expressing the dopamine D_{2L} receptor alone. The dopamine D2 receptor ligands show the following rank order of potencies at $D_{2L}G_{αoβ1γ2}$: bromocriptine > NPA >> quinpirole ≥ dopamine > R-(+)-3-PPP ≥ m-tyramine. When tested at $D_{2L}G_{αi2β1γ2}$ the rank order of potencies for the same ligands was bromocriptine > NPA >> R-(+)-3-PPP > dopamine > quinpirole > m-tyramine. As for relative efficacies, bromocriptine and NPA behaved as full agonists, and all the other ligands tested behaved as partial agonists in both preparations.

These results show a differential coupling of human dopamine D_{2L} receptor to $G_{αi2}$ and $G_{αo}$ G proteins. Most of the compounds tested showed higher efficacies and higher potencies at $D_{2L}G_{αoβ1γ2}$, demonstrating a preferential coupling of the dopamine D_{2L} receptor to $G_{αo}$. Comparison of the ratio of pEC_{50}s shows different degrees of selectivity for the different agonists. This suggests that the coupling of the receptor to a subtype of G protein may depend on the receptor conformation stabilised by the ligand used.

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This work was supported by the BBSRC.

169P SIMULTANEOUS DETECTION OF AGONIST-STIMULATED ACTIVATION OF cAMP RESPONSE ELEMENT- (CRE) AND SERUM RESPONSE ELEMENT- (SRE) MEDIATED GENE TRANSCRIPTION IN HEK-293 CELLS

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β_2-Adrenergic receptors (β_2-AR) can stimulate gene transcription via both the mitogen activated protein kinase (MAPK) and cyclic AMP/protein kinase A pathways (McDonnell *et al.*, 1998). In the present study we report the development of a dual reporter gene assay in HEK-293 cells that allows both pathways be studied simultaneously.

Human Embryonic kidney 293 (HEK293) cells stably transfected with the human β_2-AR (specific ^{125}I-cyanopindolol binding: B_{MAX} = 959±142 fmol mg protein^{-1} and K_D value of 38±13pM; n=3), were then stably transfected with both Secreted Placental Alkaline Phosphatase (SPAP) and firefly luciferase reporter genes. The SPAP reporter gene is regulated by six cyclic AMP response elements (CREs) as described previously (McDonnell *et al.*, 1998), while the luciferase reporter is regulated by a single serum response element (SRE) and can respond to activation of MAP kinase (Hill & Treisman., 1995; Whitmarsh *et al.*, 1995). Cyclic AMP accumulation, SPAP secretion and ^{125}I-cyoanopindolol binding studies were performed as described previously (McDonnell *et al.*, 1998). Media was harvested to measure SPAP secretion, the cells were then lysed and cytosolic luciferase activity was measured using the Packard Luclite *Plus*™ assay kit following the manufacturer's instructions.

The *β_2-AR* agonist isoprenaline produced concentration dependent increases in cyclic AMP (log EC_{50} -7.17±0.04; n=3) which was antagonised by ICI 118551 (-log K_D=8.61±0.13; n=3). Isoprenaline also produced concentration-dependent increases in the expression of both CRE-SPAP (log EC_{50}= -9.37 ± 0.30; E_{MAX} =7.10 ± 0.50 fold over basal; n=4) and SRE-luciferase (log EC_{50} = -8.89 ± 0.16; E_{MAX} 1.60 ± 0.03 fold over basal; n=4) reporters. The β_2-AR selective antagonist ICI118551 (100nM; apparent log K_D value -7.93±0.13, SPAP secretion and -7.67±0.32, luciferase; n=4) competitively inhibited these responses to isoprenaline.

Endogenous adenosine A_{2B} receptors have been shown previously to activate MAP kinase and cyclic AMP accumulation in HEK-293 cells (Zhenhai *et al.*, 1999). Here we show that 5'-N-ethylcarboxyamidoadenosine (NECA) can also stimulate both CRE-SPAP (log EC_{50} = -5.35 +0.09; E_{MAX} = 2.39 ± 0.04 fold over basal; n=4) and SRE-luciferase (log EC_{50} = -5.96 +0.27; E_{MAX} = 1.35 ± 0.02 fold over basal; n=4).

These data therefore suggest that this dual CRE-SPAP/SRE-luciferase reporter gene system may be very useful in monitoring the extent to which different signalling pathways are utilised by different G-protein-coupled receptors in signalling to the nucleus.

We thank GlaxoSmithKline for financial support

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170P THE ROLE OF CAPACITATIVE CALCIUM ENTRY IN MUSCARINIC RECEPTOR-MEDIATED ENHANCEMENT OF A_{2B} ADENOSINE RECEPTOR-INDUCED CYCLIC AMP GENERATION IN HEK 293 CELLS

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It has previously been suggested that Ca^{2+}-stimulatable adenylyl cyclase (AC) isoforms (over-expressed in HEK 293 cells) are stimulated by elevations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) only as a result of capacitative Ca^{2+} entry (Fagan *et al.*1996). We have previously demonstrated a muscarinic receptor-mediated enhancement of A_{2B} adenosine receptor-induced cAMP generation in HEK 293 cells, which appeared to occur largely through elevation of $[Ca^{2+}]_i$ (Jackson *et al.*, 2000). We have, therefore, investigated the role of capacitative Ca^{2+} entry in this cross-talk phenomenon.

HEK 293 cells (passage 26-40), stably transfected with recombinant human H_1 histamine receptor DNA (Presland & Hill, 1998), were grown as described previously (Cooper *et al.*, 1997). Cyclic AMP generation and changes in intracellular Ca^{2+} were measured as described previously (Jackson *et al.*, 2000). For cAMP time-course experiments, time-points were at 2 min intervals. "Calcium-free conditions" refers to experiments in which the assay buffer contained no added Ca^{2+} and 0.1 mM EGTA. Statistical significance was determined using one-way ANOVA with a Newman-Keuls *post-hoc* test in all cases unless otherwise stated.

Ionomycin (66 nM), elicited an increase in $[Ca^{2+}]_i$ which was not significantly different from that elicited by 300 μM carbachol (CCh) (n=3, unpaired t-test). Ionomycin (66 nM) failed to alter cAMP levels in either the absence or presence of NECA (10 μM) (n=3).

Under Ca^{2+}-free conditions, CCh (300 μM) elicited a substantial increase in $[Ca^{2+}]_i$. Subsequent application of atropine (1 μM) after ~200 s caused an immediate reduction of $[Ca^{2+}]_i$ to baseline levels. Application of 15 mM Ca^{2+} after a further ~150 s produced an increase in $[Ca^{2+}]_i$ to levels not significantly different from those elicited by 300 μM CCh (n=3, unpaired t-test).

In a parallel examination of the cAMP generation time-course, cells were stimulated (Ca^{2+}-free conditions) with NECA (10 μM) or NECA (10 μM) and CCh (300 μM). Atropine (1 μM) was added immediately after the 10 min time-point, and Ca^{2+} (15 mM) was added immediately after the 16 min time-point. After 10 min, NECA produced a substantial increase in cAMP generation (99±5 % of 10 μM NECA control in Ca^{2+} containing buffer), while NECA/CCh elicited a significantly greater cAMP generation (194±12 %, n=3, P<0.001). Atropine did not significantly affect NECA- or NECA/CCh-mediated cAMP generation. Ca^{2+} did not affect the NECA/CCh-mediated cAMP generation significantly, but did increase NECA-mediated cAMP generation (P<0.05, n=3, 26 min).

The lack of effect of ionomycin on NECA-mediated cAMP generation is consistent with the hypothesis that a localized, rather than a generalized increase in $[Ca^{2+}]_i$ is required for the manifestation of this potentiation. The failure of atropine to attenuate CCh-mediated potentiation of the NECA cAMP signal indicates that continued muscarinic receptor activation is not required for continued potentiation of cAMP generation. The observation that addition of Ca^{2+} failed to stimulate an additional significant increase in NECA/CCh-mediated cAMP generation suggests that non-receptor-mediated Ca^{2+} entry does not play a role in this potentiation, or that NECA/CCh have already effected a maximal activation of AC.

AMJ holds an MRC studentship.

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171P COMPARISON OF [^{3}H]ZM-241385 BINDING TO ADENOSINE A_{2A} RECEPTORS FROM RAT STRIATUM AND CORTEX

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The neuromodulatory effects of adenosine are mediated in part by the adenosine A_{2A} receptor. The adenosine A_{2A} receptor is enriched in striatal tissue. These receptors can be specifically labelled by the agonist [^{3}H]CGS-21680 (Jarvis et al., 1989) and more recently by the antagonist [^{3}H]ZM-241385 (Alexander, 1999). Adenosine A_{2A} receptors are also present in the rat cortex. However they have low abundance in this tissue. This, coupled to the low affinity for [^{3}H]CGS-21680 has hampered the pharmacological characterisation of this receptor population. In the present study we have identified and investigated the pharmacology of the binding site for [^{3}H]ZM-241385 in rat cortex and compared this to the binding site for [^{3}H]ZM-241385 in rat striatum.

Binding assays were carried out by the method of Jarvis et al. (1989). Rat striatal and cortical membranes were prepared as described by Ji and Jacobson (1993). For displacement studies receptors were labelled with 3nM [^{3}H]ZM-241385 in striatal membranes and 1nM [^{3}H]ZM-241385 in cortical membranes to preferentially label the high affinity site.

[^{3}H]ZM-241385 bound to a single saturable site in the rat striatum with an affinity (Kd) of 2.7 ± 1.0nM and a maximum number of binding sites (Bmax) of 4.3 ± 0.3 pmol/mg protein. [^{3}H]ZM-241385 revealed two binding sites in the rat cortical membranes with affinities of 0.32 ± 0.03 nM and 54 ± 0.1 nM, which had Bmax values of 141±12.8 and 849± 46.5 fmol/mg protein respectively.

Table 1 shows the results of displacement of [^{3}H]ZM-241385 in both rat cortex and striatum by ZM-241385, DPCPX , CGS-21680 and NECA. In the striatum compounds showed a rank order of potency of ZM-241385>CGS-21680>NECA>DPCPX which is consistent with adenosine A_{2A} pharmaology. [^{3}H]ZM-241385 labelled two sites in the cortex, the high affinity site had the rank order of potency of ZM-241395>CGS-21680=NECA>DPCPX. This is similar to the rank order of potency of these compounds in the striatum although the compounds have a higher potency in the cortex. This suggests that [^{3}H]ZM-241385 labels two populations of receptors in the rat cortex, one of which is an adenosine A_{2A} receptor.

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

Compound	Ki (nM) in Striatum	Ki (nM) in Cortex
ZM-241385	1.3 ± 0.6	0.34 ± 0.05
DPCPX	599 ± 48	123 ± 16.5
CGS-21680	206 ± 31	58.5 ± 13.6
NECA	306 ± 61.0	48.9 ± 4.0

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172P STRUCTURE-FUNCTION RELATIONSHIPS OF NECA ANALOGUES AT A1 ADENOSINE RECEPTORS

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We have recently shown that the stimulation of adenosine A1 receptors leads to the activation of both pertussis toxin-sensitive (Gi/o) and pertussis toxin-insensitive (Gs and Gq/11) G-proteins, in an agonist-dependent manner. The agonists 5'-(N-ethyl)-carboxyamidoadenosine (NECA) and N^6-cyclopentyladenosine (CPA) were equally efficacious in activating pertussis toxin-sensitive G-proteins, whilst NECA was twice as efficacious as CPA in activating pertussis toxin-insensitive G-proteins (Cordeaux *et al.*, 2000). In the present study we investigated the ability of a series of NECA analogues to stimulate inositol phosphate accumulation, in the presence or absence of pertussis toxin, and to stimulate [^{35}S]GTPγS binding, compared to CPA.

5'-N-cyclobutylcarboxyamidoadenosine and 5'-N-cyclopentyl carboxyamidoadenosine (CBuCA and CPeCA) were synthesized as previously described (de Zwart *et al.* 1999). [^{3}H]Inositol phosphate (IP) accumulation was measured in CHO cells, expressing the human adenosine A1 receptor at approximately 3 pmols/mg protein, as previously described (Cordeaux *et al.*, 2000). Where stated, cells were pre-incubated overnight in the presence of 100ng/ml pertussis toxin (PTX). Stimulation of [^{35}S]GTPγS binding was measured as previously described (Cordeaux *et al.*, 2000).

Data obtained from dose response curves with 5'-N-cyclopropylcarboxyamidoadenosine (CPCA), CBuCA and CPeCA in each of the three assays are shown in Table 1.

Table 1. Stimulation of [^{35}S]GTPγS binding and inositol phosphate accumulation by NECA analogues in CHO cells. Maximal responses (Emax) are expressed as the percentage stimulation over basal, relative to 100μM CPA (100%). Data represent mean values ± standard error, obtained from 3 independent experiments, perfomed in triplicate.

	[^{35}S]GTPγS		IP		IP + PTX	
	pEC_{50}	Emax	pEC_{50}	Emax	pEC_{50}	Emax
CPCA	6.72 ± 0.31	92.0 ± 3.7	6.01 ± 0.06	171.4 ± 1.1	5.76 ± 0.12	232.5 ± 22.5
CBuCA	6.82 ± 0.14	99.7 ± 4.3	6.18 ± 0.18	124.5 ± 7.8	5.52 ± 0.03	208.6 ± 31.8
CPeCA	5.18 ± 0.28	95.9 ± 11.3	5.32 ± 0.42	61.4 ± 5.5	4.78 ± 0.13	21.6 ± 3.4

For [^{35}S]GTPγS binding, the Emax values of all three NECA analogues were not significantly different from that of CPA ($p>0.05$). For IP accumulation, CPCA was significantly more efficacious than CPA ($p<0.05$). The efficacy of CBuCA however, was not significantly higher than that of CPA ($p>0.05$) and the efficacy of CPeCA was significantly lower ($p<0.05$). In the presence of pertussis toxin, the relative efficacy of CPCA and CBuCA was exacerbated, both being significantly higher than that of CPA ($p<0.01$). These results show that analogues of NECA are also more efficacious than CPA, particularly in activating PTX-insensitive G-proteins. In addition, with increasing substituent size, their efficacy relative to CPA is decreased.

We thank the Wellcome Trust for financial support.

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173P [^{3}H]-INOSITOL PHOSPHATE RESPONSES TO HISTAMINE MEDIATED BY A HISTAMINE-H_1RECEPTOR COUPLED TO G_{11} BUT NOT G_Q PROTEIN IN PRIMARY HUMAN PROSTATIC STROMAL CELLS

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Histamine receptors can be divided into H_1, H_2, H_3 and H_4 receptor subtypes and belong to the G-protein coupled family of receptors (GPCR) (Hill et.al.,1997, Nguyen et.al., 2001). Histamine H_1 receptors are thought to couple to a pertussis toxin insensitive $G_{q/11}$ protein which activates phospholipase C and subsequently hydrolyses inositol phosphates (Smit et.al,1999). The aim of this study was to functionally characterise the receptor subtype responsible for inositol phosphate responses to histamine in a primary cell culture model of prostatic stromal cells.

Benign hyperplastic tissue was obtained from transurethral resection of prostate chips. Primary human prostatic stromal cells (HPSC) were established using a technique previously described (Harriss et.al.,1995). Immunohistochemistry with anti-smooth muscle α-actin, anti-prolyl-5B-hydroxylase and anti-smooth muscle myosin confirmed a cell population of smooth muscle, myofibroblasts and fibroblasts consistent with prostatic stromal cells. Total [^{3}H]-inositol phosphate accumulation in response to histamine was measured as previously described (Harriss *et.al.*,1995) in the presence and absence of selective histamine antagonists and apparent pA_2 values were calculated. Chinese hamster ovary (CHO) cells transfected with the human H_1 receptor were used as control. Western blot was used to investigate G-protein subtypes in HPSC and compared to CHO and human embryonic kidney cells (HEK).

Histamine produced a significant and concentration-dependent rise in total [^{3}H]-inositol phosphate accumulation (EC_{50} = 2.1± 0.2 x 10^{-5} M). This response was competitively antagonized by mepyramine (pA_2=7.5 ± 0.2 ;n=10), chlorpheniramine (+) isomer (pA_2=7.45 ± 0.2; n=6), chlorpheniramine (-) isomer (pA_2=<6; n=3), triprolidine Promethazine (pA_2=8.3 ± 0.2; n=4). Doxepin (50nM) was a potent non-competitive antagonist. Thioperamide (1μM), tiotidine (1μM) and 18-hour pre-incubation with pertussis toxin (100ng/ml) to uncouple $G_{i/o}$-mediated responses, did not have a significant effect (n=3). In CHO cells, [^{3}H]-inositol phosphate responses were competitively antagonised by mepyramine (pA_2=8.2 ± 0.1;n=3), chlorpheniramine (+) isomer (pA_2=8.3 ± 0.1;n=3) and triprolidine (pA_2=9.2 ± 0.2 ;n=3). It is notable, that the pA_2 values for antagonism of histamine responses in stromal cells were approximately an order of magnitude lower than those obtained in CHO cells.

Western blotting using antibodies probing for $G\alpha_{i-2}$, $G\alpha_{i-3}$, $G\alpha_o$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{q/11}$ and $G\alpha_s$, demonstrated the absence of $G\alpha_q$ in HPSC in comparison to CHO cells and HEK cells transfected with the human histamine H_1 receptor. All other G protein subtypes were present in all three cell types.

These data confirm the presence of a histamine H_1 receptor subtype mediating inositol phosphate responses in primary human prostate stromal cells. However, classical histamine antagonists appear to demonstrate reduced potency in comparison to transfected cell lines. We suggest that this may be due to the histamine H_1 receptor coupling to $G\alpha_{11}$ alone in HPSC in comparison to dual coupling to $G\alpha_{q/11}$ as has been previously assumed.

MAA-H holds a Royal College of Surgeons/BUF fellowship

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174P CHARACTERISATION OF A GFP TAGGED HUMAN HISTAMINE H_1 RECEPTOR EXPRESSED IN CHOK1 CELLS

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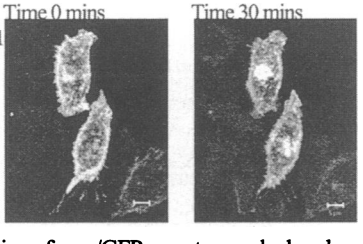
Green fluorescent protein (GFP) has enabled the visualisation of a number of G-protein coupled receptors (Milligan, 1997). We have constructed stable CHO-K1 clones expressing wild type (WT), N-terminal myc (N-myc) or N-myc and C-terminus GFP tagged (GFP/myc) human histamine H_1 (hH_1) receptors. The aim of the current study was to compare and contrast the functional response of the cell lines and to visualise hH_1 receptor localisation and trafficking.

The WT and N-myc hH1 cell lines have been described previously (Scott & Hill, 1999). For the GFP/myc-hH1 receptor, the N-myc-hH_1 receptor cDNA (minus the stop codon) was ligated in frame with GFP-topaz (Packard). ^{3}H-inositol phosphate responses were measured as described previously (Cordeaux *et al.*, 2000). Values represent means ± SEM of 5-7 experiments. Immunocytochemistry was performed using monoclonal mouse anti-myc and polyclonal rabbit anti-GFP antibodies. Secondary antibodies used were goat anti-mouse rhodamine red-X and goat anti-rabbit Alexa fluor 488. Cells were visualised by confocal microscopy.

Histamine stimulated [^{3}H]inositol accumulation in all cell lines. There was a greater level of stimulation cells expressing the GFP/myc hH1 compared to either the WT or N-myc hH_1 receptor cell lines (9.06±1.13, 6.58±0.42 and 2.37±0.42 fold over basal respectively). There was also no significant difference in the potency of histamine in WT, N-myc or GFP/myc hH1 receptors ($LogEC_{50}$ of –5.47±0.10, -5.79±0.03 and -5.41±0.09 respectively). Pretreatment of the cells with mepyramine (100nM for 30 mins) caused a parallel shift to the right of the histamine concentration response curves in all cell lines (apparent pK_b 8.11±0.08, 8.00±0.11 and 7.97±0.09 for WT, N-myc- and GFP/myc- hH1 receptors respectively). Immunocytochemistry demonstrated that both the myc and GFP/myc tagged receptors were localised on the cell surface and internally. Real time imaging demonstrated that GFP tagged receptor underwent internalisation following histamine (0.1mM) stimulation. Under basal conditions receptors were evenly distributed on the cell surface. After 5 mins histamine exposure receptors were seen to coalesce on the cell membrane. Receptors were seen to internalise into large focal spots within the cell over the subsequent 25 mins (Fig 1).

Fig. 1



Visualisation of myc/GFP receptors under basal conditions and following histamine exposure (0.1mM, 30mins).

In conclusion we have constructed a functionally active GFP tagged human H_1 receptor which is expressed on the cell surface and is internalised following histamine stimulation. This cell line provides a useful tool for further characterisation of agonist induced trafficking of the human H_1 receptor.

We thank the MRC and Wellcome Trust for financial support

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175P TRANSIENT HISTAMINE H_1-RECEPTOR MEDIATED AGONIST RESPONSES MAY YIELD ERRONEOUS pK_A ESTIMATES: APPLICATION OF EQUILIBRIUM AND KINETICS MODELS OF AGONIST ACTION

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In this study we determined the pK_A value of histamine (HA) at H_1-receptors in two functional *in vitro* guinea pig (male Dunken Hartley, 250-500g) bioassays: gallbladder (gb) and gastric muscle (gm) (Watt *et al.*, 1995). In both assays HA responses faded, albeit with different characteristics. The responses to a ~90% maximally effective HA concentration (1μM) in gm reached a peak at 16.6±3.1 min (n=6), which faded slowly towards baseline (% fade (n=6): 60 min 13.7±2.9; 120 min, 30.4±6.3). However, in the gb, the responses reached a peak within 6.7±1.2 min ([HA]=10μM, n=6) and faded faster initially achieving a steady state level (% fade (n=6): 60 min, 59.2±5.7; 120 min, 57.8±6.8). Over the same time period the muscarinic receptor agonist, 5-methylfurmethide, produced stable responses in both assays. We interpret the HA response fade to be due to receptor desensitisation rather than non-specific tissue fatigue.

In both assays cumulative HA concentration response (E/[A]) curves were constructed by incremental rapid dosing at the peak response (E/[A] curve duration: gb, ~12 min & gm, ~40 min). Additionally it was also possible to construct a more prolonged (~280 min) cumulative E/[A] curve in the gb by incremental dosing at the steady state response level. These E/[A] curves were generated following progressive degrees of receptor alkylation with PBZ (0.1-10μM with 5 min tissue exposure) and the data analysed using an equilibrium model (Black & Leff, 1983) to obtain *apparent* pK_A values ($pK_{A(app.)}$). Replicate experiments using the rapid E/[A] curve approach gave similar results (gb; 5.09±0.48 & 4.89±0.27: gm; 5.66±0.44, 5.76±0.34 & 5.71±0.41) and thus were pooled. The $pK_{A(app)}$ values in the gb were ~0.7 log units lower than the gm ($P<0.01$). Furthermore, for the gb the $pK_{A(app)}$ estimate was ~1.5 log units higher when using the prolonged rather than rapid E/[A] curve and so does not lie within the 95% confidence interval for the rapid E/[A] curve $pK_{A(app)}$ value. Although ambiguity surrounds pK_A values, they have proven useful in differentiating receptor subtypes. Superficially, these $pK_{A(app)}$ differences may indicate different HA receptor subtypes. However, the phenomena of receptor inactivation, manifested as fade, has been reported to result in an underestimation of pK_A values (Leff, 1986) but this model could not account for the fade characteristics in this study. Therefore the simplest model (Lew *et al.*, 2000), which incorporates both receptor inactivation and reactivation, was employed to analyse the time-response profiles and re-interpret the receptor alkylation data sets. The resulting simulations provided good qualitative and quantitative description of the experimental data without the need to invoke differences in the pK_A values (i.e. 5.5, derived from ratio of k_{-1} to k_1) between assays and experimental designs. However, the rates of receptor inactivation and reactivation differed between assays. This pK_A value is similar to the $pK_{A(app)}$ estimate obtained from the equilibrium model fit of the gm data (i.e. 5.7) and is probably an indication that the extent of fade reflects a small degree of receptor inactivation.

These approaches demonstrate how a kinetic model of receptor inactivation with reactivation can be used to avoid potential pitfalls of erroneous agonist pK_A determinations that can arise when equilibrium models are employed to analyse data with underlying fade.

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176P COMPARISON OF TNF-α AND IL-1β MEDIATED IL-6 PRODUCTION IN A NOVEL HUMAN RENAL CO-CULTURE PERFUSION SYSTEM

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Current clinically utilised cytokines are species specific. Animal based models are not ideal for testing species specific effects, while alternative *in vitro* cell culture models fail to closely mimic the *in vivo* state. In order to address these limitations a novel perfusion system was developed to allow for testing of potential side effects of human recombinant cytokines. A new cell culture apparatus allowing for permanent and continuous administration of nutrients, continuous removal of metabolic wastes and continuous administration of test compounds was designed. The apparatus allows for different cell types to be cultured in separate compartments within the same chamber allowing for non-contact cell-cell interaction while exposed to perfusion conditions.

Microporous membranes (0.2μm pore size) were placed in the test chambers and coated with rat tail collagen (2μg/cm^2). Human proximal tubular epithelial cells (HK-2) were cultured in the apical compartment, while human microvascular endothelial cells (5A32) were cultured in the basolateral compartment either in monoculture or non-contact coculture. Cells were cultured in 1% FCS, MCDB 131, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Culture conditions were maintained at 37°C with 95% Air/ 5% CO_2.

Cells were allowed to achieve confluence and acclimatise to perfusion conditions (perfusion rate 1ml / hour) for 24 hours. Cells were treated on both the apical and basolateral side with TNF-α (1 ng/ml) or IL-1β (1 ng/ml) for up to 48 hours. Cell culture medium was removed at 4, 24 and 48 hours and assayed for IL-6 production by ELISA. Morphological examination of the cells was also observed by phase contrast microscopy.

Basal production of IL-6 in HK-2 cells at 4, 24, 48 hours (apical release 81 ± 28, 136 ± 65, 163 ± 63 pg/ml), (basolateral release 68 ± 27, 78 ± 51, 68 ± 25 pg/ml) was high in comparison to 5A32 cells (levels undetectable). Co-cultured HK_2 and 5A32 cells showed basal IL-6 production at 4, 24, 48 hours (apical release 168 ± 19, 183 ± 29, 197 ± 30 pg/ml), (basolateral release 83 ± 5, 87 ± 2, 75 ± 16 pg/ml). TNF-α (1ng/ml) stimulated production of IL-6 in HK-2 and 5A32 co-culture at 4, 24, 48 hours (apical release 450 ± 142, 460 ± 47, 394 ± 33 pg/ml), (basolateral release 633 ± 199, 811 ± 61, 808 ± 151 pg/ml). IL-1β (1ng/ml) stimulated production of IL-6 from HK-2 cells at 4, 24, 48 hours (apical release 1706 ± 446, 4171 ± 599, 3991 ± 740 pg/ml). IL-1β stimulated co-cultures of HK_2 and 5A32 cells at 4, 24, 48 hours also produced IL-6 (apical 1492 ± 21, 2295 ± 591, 3024 ± 667 pg/ml), (basolateral 2097 ± 348, 1986 ± 623, 1449 ± 337 pg/ml).

TNF-α and IL-1β stimulated Il-6 release from HK-2 and 5A32 cells. A novel perfusion system that facilitates co-culture of different renal cell types and continuous monitoring of cellular responses was developed.