1P ADENOSINE A_1 RECEPTOR DUAL-COUPLING: PERTUSSIS TOXIN (PTX)-SENSITIVE AND -INSENSITIVE MECHANISMS

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Adenosine A_1 receptor-mediated responses in central and peripheral nervous tissues are classically attributed to inhibition of adenylyl cyclase mediated via a $G_{i\alpha}$-protein, although other effector mechanisms exist (e.g. $K^+/Ca^{2+}/Cl^-$ channels or guanylyl cyclase stimulation) (reviewed in Linden, 1991). Recent data indicate transfected A_1 receptors can couple simultaneously to PTX-sensitive cAMP inhibition and PLC activation (Freund *et al.*, 1994). Here we investigate 5'-N-ethylcarboxamidoadenosine (NECA)-induced, A_1 receptor-mediated responses using a microphysiometer (Molecular Devices Corp., USA), an apparatus which measures proton extrusion when a cell excretes acidic metabolites to the external environment.

CHO cells, stably transfected with the human adenosine A_1 receptor, were seeded (10^6 cells.ml^{-1}) in specialized chambers 14-18 h prior to experimentation. Chambers were maintained at 37°C and perfused (100 µl.min^{-1}; lag time ~1 min) with minimum essential medium Eagle (pH=7.4) in the presence and absence of drug solutions. NECA-induced responses (cumulative dosing) were calculated as a percentage of the basal acidification rate and EC_{50} values were calculated using ALLFIT. Data represent mean ± s.e.mean of 3-4 experiments.

In these cells, basal acidification rates were between 90-230 µV.s^{-1} (corresponding to ~90-230 milli-pH units.min^{-1}). NECA caused an increase in acidification rate which clearly exhibited a biphasic, concentration-response profile; cells lacking the transfected A_1 receptor were unresponsive. The high-potency response (EC_{50}=2.0 ± 0.3 nM) produced a maximal 23.1 ± 1.5% increase in acidification rate (n=12 chambers); the low-potency component (EC_{50}=1.1 ± 0.2 µM) further elevated acidification rate by 11.4 ± 1.2% (n=7). Pre-incubation with PTX (200 ng.ml^{-1}, 16-17 h, n=8) abolished the high, but not the low-potency response to NECA (EC_{50}=1.5 ± 0.2 µM, 30.1 ± 2.6% maximal increase). When co-applied with NECA (n=6), the non-selective adenosine receptor antagonist 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-*c*]quinazoline (CGS15943; 300 nM, 1 h pre-treatment) antagonized the high-potency component (EC_{50}=265 ± 69 nM, pA_2=8.4 ± 0.1) without changing the maximal response (26.7 ± 2.0%). CGS15943 also antagonized the low-potency NECA, and NECA + PTX responses (dose ratio 40-70; determined at 5-8% increase over basal), but high NECA solvent concentrations prohibited EC_{50} calculation.

The high-potency, PTX-sensitive response is consistent with A_1 receptor-coupling to $G_{i\alpha}$ (Freund *et al.*, 1994). The low-potency, PTX-insensitive response suggests that signalling pathways other than $G_{i\beta\gamma}$ activation of PLC (possibly through $G_{q/11}$ or ion channels) are available to the A_1 receptor. These data provide further support for the existence of A_1 receptor dual-coupling.

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2P REGULATION OF PHOSPHOINOSITIDE TURNOVER IN NEONATAL CEREBRAL CORTEX BY GROUP I- AND II-SELECTIVE METABOTROPIC GLUTAMATE RECEPTOR AGONISTS

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Recent studies suggest that in tissues expressing mixed populations of group I and II metabotropic glutamate receptors (mGluRs) activation of the latter may positively modulate phosphoinositide turnover activated by group I mGluR agonists (Genazzani *et al.*, 1994; Schoepp *et al.*, 1996). Here we use highly selective group I and II mGluR agonists to investigate further the nature of this phenomenon.

Neonatal rat cerebral cortex slices were prepared and incubated as described previously (Mistry & Challiss, 1996). Slices (25 µl) were dispensed into oxygenated Krebs-Henseleit buffer (KHB (250 µl) ± [^{3}H]-inositol) and incubated for 60 min at which time LiCl (5 mM) was added if appropriate. After 15 min agonists or vehicle were added and slices incubated at 37°C for the time indicated and experiments terminated with an equal volume of 1 M trichloroacetic acid. [^{3}H]-InsP_x, Ins(1,4,5)P_3 or cyclic AMP were measured in neutral cell extracts (Mistry & Challiss, 1996).

Exposure of [^{3}H]-inositol labelled slices to the group I-selective mGluR agonist (**S**)-3,5-dihydroxyphenylglycine (DHPG; 100 µM), in the presence of 5 mM LiCl for 15 min, stimulated a 5-fold increase in [^{3}H]-InsP_x accumulation (-log EC_{50} (M), 5.56 ± 0.05). Although addition of the group II-selective mGluR agonist 2**R**,4**R**-4-aminopyrrolidine-2,4-dicarboxylate (APDC; 100 µM) alone failed to stimulate a [^{3}H]-InsP_x response, this agent substantially increased (by 81 ± 6%) the maximum response evoked by DHPG, without affecting agonist potency (-log EC_{50} (M), 5.51 ± 0.03). A marked enhancement of the DHPG response by group II mGluR activation was also observed in the absence of LiCl, in the absence of $[Ca^{2+}]_e$, and irrespective of whether APDC was added prior to (-10 min), simultaneous with, or after (+5 min) the addition of DHPG.

APDC inhibited forskolin-stimulated cyclic AMP accumulation by approx. 50% in neonatal cerebral cortex (-log EC_{50} (M), 5.87 ± 0.09). In comparison, the concentration-dependent enhancement of the DHPG-stimulated [^{3}H]-InsP_x response by APDC was 3-4 fold right-shifted (-log EC_{50} (M), 5.33 ± 0.03).

The effects of APDC on the DHPG-stimulated Ins(1,4,5)P_3 mass response were less marked. Exposure to APDC (100 µM) caused a small (19.8 ± 3.7%), but highly reproducible decrease in basal levels of Ins(1,4,5)P_3. In contrast, co-activation of group I and II mGluRs caused an increase in Ins(1,4,5)P_3 accumulation, over that caused by DHPG *per se*, throughout a 0-300 s time-course. Increasing tissue cyclic AMP levels 10-fold (by addition of forskolin, 10 µM) or 100-fold (by addition of forskolin + the PDE4 inhibitor rolipram (10 µM)) did not significantly alter the modulatory effect of APDC on DHPG-stimulated phosphoinositide responses.

These data confirm previous findings made in adult rat hippocampal slices, that the phosphoinositide response caused by activation of mGluRs by a group I-selective agonist can be positively modulated by co-activation of group II mGluRs. This effect is seen at the level of both [^{3}H]-InsP_x and Ins(1,4,5)P_3 responses and does not appear to be dependent upon suppression of cyclic AMP levels. Further work is needed to determine the nature of the crosstalk between these two groups of mGluRs and whether the phenomenon is specific to mGluR subtypes, or splice-variants within each group.

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3P OVEREXPRESSION OF G PROTEIN-COUPLED RECEPTOR KINASE 2 ENHANCES THE SENSITIVITY OF ADENOSINE A_{2a} RECEPTORS TO DESENSITISATION

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In a previous study using NG108-15 neuroblastoma x glioma hybrid cells stably transfected with the dominant negative mutant form of G protein-coupled receptor kinase 2 (DNM GRK2), we showed that the desensitization of A_{2a} receptors is probably mediated by endogenous GRK2 or a closely related kinase (Mundell *et al.*, 1997). In this study we have investigated the effect of stable overexpression of wild type GRK2 on A_{2a} receptor desensitization in NG108-15 cells. Cells were stably transfected with either the pMEP4 plasmid containing the cDNA for wild type GRK2, or with plasmid alone. Cells were then cultured in DMEM containing 6% fetal bovine serum and 200 μg/ml hygromycin, and surviving colonies isolated and expanded. Following drug treatment (if any), cells were harvested, washed and frozen at -70°C until required. Adenylyl cyclase activity was then assessed in cell homogenates using a binding protein assay (Mundell *et al.*, 1997).

A plasmid-transfected cell clone (P1) and a GRK2-overexpressing clone (B7) were selected for further study. It is estimated by Western blotting that B7 expressed GRK2 >20-fold over that in P1 or wild-type cells. Adenosine A_{2a} receptor-stimulated adenylyl cyclase activity, as assessed by 3 μM CGS21680, was much less in B7 than P1 cells (16.5 ± 1.6 and 6.7 ± 1.7 pmol cAMP/min/mg protein in P1 and B7, respectively, n=4, $P<0.05$ by Student's t test). This reduction in A_{2a} receptor-stimulated activity could be largely reversed by preincubation of cells with adenosine deaminase (AD; 0.5 units/ml for 24 h; 3 μM CGS21680-stimulated adenylyl cyclase activity in P1 cells was 13.9 ± 1.1, in P1 cells following AD 14.7 ± 1.9, in B7 cells 5.9 ± 1.4 and in B7 cells following AD 12.0 ± 0.8 pmol cAMP/min/mg protein; n=4). This indicates that a low concentration of adenosine in the culture medium is sufficient to desensitize A_{2a} responsiveness in B7 but not P1 cells. Further experiments were therefore carried out only on cells pretreated with adenosine deaminase.

P1 and B7 cells were exposed to the adenosine agonist NECA (0.1 or 10.0 μM) for 20 min periods. Following this, 3 μM CGS21680-stimulated adenylyl cyclase activity was assessed in homogenates. After 20 min pretreatment with 10.0 μM NECA, the desensitization of the A_{2a} response was the same in P1 and B7 cells (64.8 ± 2.4 and 65.0 ± 1.3% in P1 and B7, respectively, n=4). However, following pretreatment with 0.1 μM NECA for 20 min, desensitization was much greater in B7 than P7 cells (12.5 ± 6.1 and 50.3 ± 8.0% in P1 and B7, respectively, n=4, $P<0.05$, Student's t test). In conclusion, these data indicate that overexpression of GRK2 in NG108-15 cells markedly enhances the sensitivity of A_{2a} receptors to agonist-induced desensitization.

Mundell,S.J. *et al.* (1997) Mol. Pharmacol., *in press.*

4P MECHANISMS OF SOMATOSTATIN RECEPTOR DESENSITIZATION IN NG108-15 CELLS

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We have investigated two possible mechanisms which could underlie the desensitization of somatostatin (SRIF)-induced calcium channel current inhibition in NG108-15 cells. First, we have studied the roles of the G-protein receptor kinases, GRK2 and GRK3, which are endogenously expressed in NG108-15 cells, using stably transfected cells incorporating a dominant negative mutant construct of GRK2 (DNM cells) (Mundell *et al*, 1997) or intracellular dialysis with peptides which block GRK2 and GRK3 activity (Koch *et al*, 1993). Second, in order to investigate the involvement of receptor sequestration, we have disrupted this pathway using concanavalin A and phenylarsine oxide, functionally distinct inhibitors of internalization.

Cell culture, cell differentiation and electrophysiological recording were as previously described (Hepworth and Henderson, 1996). Calcium channel currents were recorded using the amphotericin B-perforated patch technique (access resistance = 15 ± 1 MΩ (n=108)). When dialysis of cell contents with the pipette solution was required, as with the blocking peptides, whole cell patch clamp was employed (access resistance = 10 ± 1 MΩ (n=46)). Data are expressed as mean ± s.e.mean of at least six cells.

In wild type cells, SRIF (1-1000 nM) inhibited the N-type calcium channel current with an IC_{50} of 25 nM (95% confidence limits 10 to 57 nM). This inhibition rapidly desensitized. Both the amount and rate of desensitization were concentration-dependent. 50% maximal desensitizaton occurred at 30 nM (95% confidence limits 27 to 42 nM), and the time taken to reach 50 % maximal desensitization ($t_{1/2}$) with 300 nM SRIF was 36 ± 6 s, and with 30 nM SRIF was 68 ± 13 s ($p<0.05$). The desensitization of calcium channel current inhibition in cells transfected with plasmid alone was not significantly different from wild type cells ($p>0.05$).

In the DNM cells, desensitization was not reduced, in fact there was a 3-fold shift to the left of the desensitization-concentration dependence curve. The rate of desensitization was not significantly altered from wild type cells ($p>0.05$). Dialysis of wild type cells with the blocking peptides against GRK2 or GRK3 (both at 100 μM) did not alter the amount of desensitization to either 100 nM SRIF (anti-GRK2, 22 ± 8 % and anti-GRK3, 28 ± 6 %) compared to control (28 ± 5 %; $p>0.05$); or 300 nM SRIF (anti-GRK2, 59 ± 9 % and anti-GRK3, 43 ± 9 %) compared to control (53 ± 9 %; $p>0.05$). These data indicate that desensitization of somatostatin receptors in NG108-15 cells does not involve GRK2 or GRK3.

A five minute pre-treatment of wild type cells with concanavalin A (0.3 $mg.ml^{-1}$), or phenylarsine oxide (80 μM), reduced the amount of desensitization to SRIF (300 nM) by 73 ± 14% and 60 ± 5% respectively. The rate of desensitization was also slowed, compared to control cells, in both cases ($p<0.05$). Thus, internalization of somatostatin receptors by a GRK-independent, endocytotic mechanism contributes to rapid somatostatin receptor desensitization in this cell line.

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5P IDENTIFICATION OF AMINO ACIDS IN THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR NR2 SUBUNIT THAT CONTRIBUTE TO THE GLUTAMATE BINDING SITE

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Two regions of ionotropic glutamate receptors, termed S1 and S2, are known to be important for channel activation by agonists (Stern-Bach *et al.*, 1994). Extensive mutagenesis of these regions in the NMDA receptor subunit NR1 revealed residues important for the binding of glycine, but not glutamate (Kuryatov *et al.*, 1994; Wafford *et al.*, 1995). We therefore investigated whether mutations in the NR2 subunit would affect glutamate binding. We mutated the rat NR2A subunit at a series of residues, chosen either because homologous residues in other glutamate receptors were involved in activation by glutamate, or because the residue was conserved in all ionotropic glutamate receptor subunits except NR1.

Mutations on the rat NMDA receptor NR2A subunit were performed by site directed mutagenesis on a fragment of the sequence inserted into pBluescript. The complete sequence, and also the sequence for the rat NMDA receptor NR1 subunit, were then inserted into the expression vector pcDNA3, and transiently transfected into HEK293 cells using calcium phosphate precipitation. Function was assessed using glutamate-induced intracellular calcium ($[Ca^{2+}]_i$) increases using fura-2 ratiometric imaging. Cells were loaded with fura-2-AM (2μM) and changes in $[Ca^{2+}]_i$ in response to application of glutamate and glycine obtained in Mg^{2+}-free buffer (115mM NaCl, 10mM KCl, 5mM Ca_2Cl, 15mM glucose, 25mM HEPES pH7.4).

Glutamate EC_{50} values, obtained in the presence of 10μM glycine, were significantly different from control (EC_{50}= 1.1 ± 0.1μM, n=13) for E394Q (17 ± 0.2μM, 10), K465D (10 ± 0.7μM, 15), R485Q (1.9 ± 0.2μM, 10),V666A (2.5 ± 0.2μM, 11) and G669S (870 ± 94μM, 5). Mutation to I399Y, K465Q and G664A were not significantly different to control. Relative EC_{50} values for glycine were not significantly different to control for any of the mutants. Data from whole cell patch clamp electrophysiology for some mutants showed similar glutamate EC_{50} value changes (n=5): 1.4 ± 0.2μM (control), 23.8 ± 2.4μM (E394Q), 9.3 ± 1.0μM (K465D) and 1.3 ± 0.2μM (K465Q).

Alone, such analyses of mutants cannot distinguish between changes in the measured affinity due to direct changes to the agonist binding site, or changes elsewhere in the structure which indirectly influence channel activation. These data do suggest, however, that NR2 and NR1 play equivalent roles in the activation of NMDA receptors by glutamate and glycine respectively, and emphasise the central role played by the NR2 subunit in NMDA receptor function.

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6P DEPLETION OF INTRACELLULAR CALCIUM POOLS WITH THAPSIGARGIN INHIBITS THE MIGRATION OF A7r5 CELLS, *IN VITRO*

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Thapsigargin (TG) blocks Ca^{2+} uptake into intracellular Ca^{2+} ($[Ca^{2+}]_i$) pools resulting in a depletion of these stores.TG potently inhibits (10 nM) neointima (NI) formation in cultured human saphenous vein (George et al, 1996). Pre-exposure to 10 nM TG for 60 min also significantly inhibited NI formation assessed 14 days later. Since NI formation involves the migration of vascular smooth muscle cell (VSMC), the effect of TG on the migration of VSMCs (rat A7r5 cells) was investigated using a Boyden chamber and $[Ca^{2+}]_i$ pools studied using FURA-2 loaded cells (McArdle et al, 1992).

A7r5 cells were placed in a Boyden chamber containing 0-10 nM TG and migration stimulated with platelet derived growth factor-BB (5ng/ml). Following incubation for 4h, cells were fixed, counted and % cells migrated relative to controls calculated. In pre-exposure studies, cells were treated with 10 nM TG for 0 to 120 min, the cells washed and migration assessed as above. Data are expressed as mean ± s.e.mean and differences between mean values calculated using student's t test (*$p<0.01$). In parallel experiments, cells were preincubated with 10 nM TG for up to 240 min, washed and loaded with FURA-2. Cells were then stimulated with 1μM ionomycin and $[Ca^{2+}]_i$ release detected using dynamic video imaging.

Continuous exposure of cells to TG for 4h significantly inhibited migration (table 1) and prevented ionomycin-stimulated $[Ca^{2+}]_i$ release (figure 1). Pre-exposure to 10 nM TG for 2hr also inhibited migration (table 2) and markedly inhibited the ionomycin response (figure 1).

Table 1. Effect of TG on A7r5 cell migration (4h continuous)

TG concetration (nM):	0	1	5	10
% cells migrated (mean ±SEM):	100±0	109±21	77±10	40±8*

Table 2. Effect of pre-exposure to TG on A7r5 cell migration

exposure time (min):	0	30	60	120
% cells migrated(mean ±SEM):	100±0	115±20	121±31	26±13*

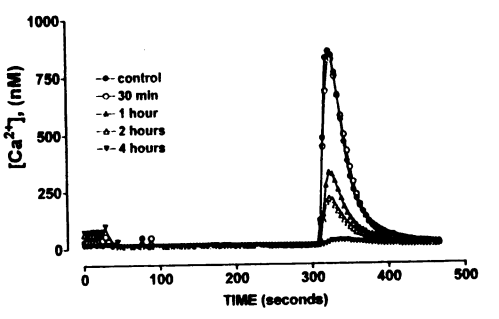

Figure 1: Effect of pre-exposure to 10 nM TG on $[Ca^{2+}]_i$ elevation in A7r5 cells in Ca^{2+} free buffer (mean; n = 6).

These observations suggest that depletion of $[Ca^{2+}]_i$ pools inhibits VSMC migration. Given its potency and long acting effects, TG could be used therapeutically to block NI formation through *ex vivo* exposure of vein grafts prior to implantation or by external application in pluronic gels.

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7P IMIDAZOLE ANTIMYCOTICS INHIBIT BK_{Ca} CHANNELS STABLY EXPRESSED IN HEK 293 CELLS

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The imidazole antimycotics (IA), classically used for their inhibitory effect on cytochrome P-450 mediated reactions, are also potent blockers of a variety of channel types including voltage-dependent Ca^{2+} and K^+ channels and the small conductance, calcium-activated K^+ channel (Hatton et al, 1996., Alvarez et al, 1992). The present study was carried out to investigate the action of these agents on the activity of cloned human brain α and β large conductance, calcium-activated K^+ (BK_{Ca}) channel subunits stably expressed in HEK 293 cells (graciously provided by Dr. S-P Olesen, Neurosearch, Denmark).

The extracellular (pipette) solution contained (mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2 and the intracellular (bath) solution contained (mM): 140KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES, pH 7.2 with either 4 or 4.9 $CaCl_2$ (1μM and 10μM free Ca^{2+} respectively). Under these ionic conditions, the effects of the IA's econazole (ECZ), miconazole (MCZ), clotrimazole (CTZ) and ketoconazole (KTZ) applied to the intracellular aspect of excised inside-out macropatches were examined on steady-state currents elicited by voltage steps from -100 to +100mV (Vh = 0mV, 10mV increments). Fractional open probability (FPo) versus voltage curves were constructed and used to determine $V_{1/2}$ values. All results are expressed as mean ± SEM.

In the presence of 1μM and 10μM free intracellular calcium, an increase in channel activity was observed as membrane voltage became more depolarised with estimated $V_{1/2}$ values of 57 ± 8mV and -46 ± 3mV respectively (n=25 for each).

Application of ECZ, MCZ and CTZ dose dependently (200nM-100μM) and reversibly inhibited the macropatch steady-state currents. In the presence of 10μM of each agent, maximally attainable channel activity (FPo_{max}) was 26 ± 6% (n=7), 26 ± 3% (n=6) and 76 ± 8% (n=3) respectively, compared to control values. Plotting FPo_{max} (normalized to control) versus ECZ, MCZ and CTZ concentrations gave estimated IC_{50} values of 3.1, 3.9 and 90μM respectively. In the presence of 100μM KTZ, FPo_{max} was 80 ± 4% compared to control values (n=5). These results give the potency profile ECZ>MCZ>CTZ>KTZ for inhibition of BK_{Ca}. This profile is similar to that obtained for inhibition of voltage-dependent calcium channels and may indicate a common modulatory site or pathway shared by the two channel types.

F.M. is a Glaxo-Wellcome student.
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8P SINGLE CHANNEL ACTIVITY IN EXCISED INSIDE-OUT PATCHES FROM RAT HIPPOCAMPAL NEURONES

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We have previously reported the presence of a small conductance K^+ channel active at the resting membrane potential in cell-attached patches from rat hippocampal neurones (Richards and Wann,1993). Here we describe a channel of similar conductance which is present in 41.9% of excised inside-out patches from these same neurones. Our culture and recording methods have been described previously (Wann and Richards,1994). In experiments where the bath and the pipette solution was in mM: 140 KCl, 5 NaCl, 1 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 10 HEPES, pH 7.2 this channel was active over a wide range of negative potentials, there being up to four active channels in the patch. The mean unitary current at -100 mV was -2.07 ± 0.06 pA (mean ± s.e.m., n=10), the mean maximum number of active channels was 2.18 ± 0.26 (n=10), the slope conductance was 22.34 ± 1.39 pS (n=7) over the voltage range -40 to -120 mV, the mean reversal potential was -3.19 ± 0.89 mV (n=5). The channel showed rectification both in the presence and absence of Mg^{2+} in the "intracellular" solution, no clear openings being present at positive potentials.

In recordings with 20 mM TEA in the pipette a channel with a mean unitary current of -2.16 ± 0.04 pA was present (n=3). In one of these patches the slope conductance of the channel was 20.29 pS. A small conductance channel was also present in recordings where the patch pipette contained d-tubocurarine (10 μM, n=5). The mean unitary current at -100 mV was -2.04 ± 0.06 pA (n=5), the mean maximum number of active channels was 3.00 ± 0.41 (n=4) and the slope conductance was 25.60 ± 2.11 pS (n=4).

When the Cl^- in the bath and pipette solution was replaced by aspartate, channel activity was present in most excised inside-out patches (15/22). In three patches a small conductance (25-35 pS) channel was active but in only one patch was the level of activity comparable to that observed in Cl^- containing solutions. The unitary current was -1.93 pA at -100 mV, there were two active channels and the slope conductance was 26.00 pS.

The channel reported here has a unitary conductance similar for example to both the inward rectifier and SK class of K^+ channels. Further investigation is required however to assess which category this channel belongs to.

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9P NOVEL REGULATION OF HUMAN 5-HT$_{2A}$ RECEPTORS BY MESULERGINE *IN VITRO*

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The 5-HT$_{2A}$ receptor is unusual in that no supersensitivity or increase in receptor levels is observed following pre-synaptic denervation *in vivo* (Eison, 1989). In addition, chronic treatment with antagonists causes either no change, or a paradoxical decrease in receptor number and function rather than up-regulation, as confirmed in SH-SY5Y cells (Mitchell, 1997). Here we report the ability of mesulergine to cause an increase in 5-HT$_{2A}$ receptor levels and functional response in human neuroblastoma cells stably transfected with human 5-HT$_{2A}$ receptor cDNA.

SH-SY5Y cells expressing the human 5-HT$_{2A}$ receptor and C6 rat glioma cells endogenously expressing 5-HT$_{2A}$ receptor, were grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM) with 5% dialysed foetal calf serum (dFCS), and transfered into either DMEM (control) or DMEM plus drug (treated) for 24 hours. Cells were washed and incubated in DMEM for 30 minutes, then harvested by scraping. Membranes were prepared by homogenisation in 5mMTris/EDTA (pH7.4) and washed three times by high speed centrifugation including a 30 minute incubation at 37°C. Following re-suspension in incubation buffer (50mM Tris, 5mM $MgCl_2$, 1mM EGTA and 1mg/ml ascorbic acid, pH 7.4), membranes were incubated with [^{3}H]ketanserin (0.2-6nM) for 60 minutes at 37°C. Reactions were terminated by rapid filtration through Whatman GF/C filters. Non-specific binding was determined using 10μM mianserin. For functional studies, cells were grown in 24 well plates (vol. 1ml), labelled with [^{3}H]inositol (1μCi/ml) for 48 hours in inositol-free DMEM and 5% dFCS at 37°C. Vehicle or drug (10μl) was added 24 hours prior to assay. Following three washes in inositol free DMEM (1ml), cells were incubated for 30 minutes with 10mM LiCl, then stimulated with 5-HT (1nM-10μM) for 15 minutes at 37°C. Reactions were terminated with ice cold methanol, and [^{3}H]inositol phosphates extracted using Dowex AG1-X8 anion exchange columns.

Cells were originally treated with mesulergine at 900nM, ten times it's Ki value to ensure a high level of receptor occupancy (approximately 90%). A significant increase in specific [^{3}H]ketanserin binding was observed following 24 hour treatment (Bmax=144±10% of control, n=5, $p<0.01$). A slight but significant increase in [^{3}H]ketanserin affinity was observed (control pKd=9.08±0.04, treated pKd=9.15±0.04, n=5, $p<0.01$). Up-regulation was reversed following co-incubation with 30nM ketanserin (Bmax=113±8% of control, n=3). Treatment for 24 hours with 2μM and 10μM mesulergine also increased [^{3}H]ketanserin binding levels (Bmax=46±5% and 61±4% of control respectively, n=3, $p<0.05$). Neither basal PI hydrolysis levels nor Emax in these cells were significantly altered by 24 hour 900nM mesulergine treatment. However, a significant shift in pEC50 was observed (control pEC50=6.86±0.06, treated pEC50=7.11±0.06, $p<0.05$, n=4). Treatment of rat C6 glioma cells for 24 hours with 200nM mesulergine also caused an increase in 5-HT$_{2A}$ receptor levels (Bmax=144±7% of control, n=4, $p<0.05$).

Our results demonstrate that 5-HT$_{2A}$ receptors expressed in SH-SY5Y cells are up-regulated following treatment with mesulergine. This effect appears concentration-dependent and can be reversed following co-incubation with ketanserin. Functional up-regulation is also observed since mesulergine treatment causes a leftward shift in the PI dose-response curve. None of the antagonists we have studied previously caused up-regulation of receptor number (Mitchell 1997). The mechanisms underlying the action of mesulergine may be due to the unique manner in which it interacts with the 5-HT$_{2A}$ receptor (Kao, 1992). Since up-regulation of receptor levels is also seen in rat C6 glioma cells, the effect of mesulergine is not species specific.

Alex Mitchell is an MRC Research Student.

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10P *IN VITRO* BINDING CHARACTERISTICS OF A POTENT, SELECTIVE AMPA RECEPTOR ANTAGONIST, [^{3}H]Ro 48-8587, IN RAT BRAIN

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Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors are ligand-gated cation channels for L-glutamate, the major excitatory transmitter in the mammalian CNS. These receptors, hetero-oligomeric proteins composed of the subunits GluR1-4 with flip and flop forms (Bettler and Mulle, 1995), play key roles in CNS physiology and pathology. To date, several radioligands have been used to characterize AMPA receptors and their ligands, namely [^{3}H]AMPA, [^{3}H]NBQX, [^{3}H]CNQX and [^{3}H]FW (see Dev et al., 1996). We now describe a new radiolabelled antagonist with superior specific binding, potency and selectivity. Ro 48-8587 (9-imidazol-1-yl-8-nitro-2,3,5,6-tetrahydro[1,2,4]-triazolo[1,5-c]quinazoline-2,5-dione) is, to-date, the most potent and selective AMPA receptor antagonist.

[^{3}H]Ro 48-8587 (s.a. 40Ci/mmole) was synthesized, as a triethylammonium salt, at Amersham International. Male Füllinsdorf albino rats (120-180g) were used for the following experiments. Membranes were prepared by homogenization of the whole brain (less cerebellum and medulla) in cold Tris-HCl 50mM, EDTA 10mM, pH 7.1 buffer. They were washed three times before freezing at -80°C. After thawing, the homogenate was washed again three times in cold Tris-HCl 50mM, pH 7 (binding buffer). The final membrane concentration was 200μg of protein/ml. For competition experiments, using 5nM [^{3}H]Ro 48-8587, the incubation time was 1hour at 4°C and the homogenate was filtered onto Whatman GF/B filters followed by 5 washes with cold binding buffer; nonspecific binding was defined in the presence of 10μM quisqualate. Saturation analyses were performed with a 1hour incubation. For film or phosphor imaging radioautography, cryostat sections of fresh-frozen rat brain and spinal cord were pre-washed in buffer (2x 10min) then incubated for 1hour at 4°C in 1nM [^{3}H]Ro 48-8587, rinsed in buffer (2x 30sec + 1min), dried and exposed to tritium-sensitive Ultrofilm (Amersham) or Fuji plates for 2wks or 2 days, respectively. These were subjected to image analysis (MCID, Imaging Res., Ontario).

In rat whole brain homogenates, [^{3}H]Ro 48-8587 bound with a high affinity and capacity (KD= 3±1nM; Bmax= 1±0.2 pmoles/mg prot); non-specific binding was ~20% of total binding. In competition binding experiments, the rank order of affinities (Ki) of antagonists (nM) was: Ro 48-8587 (4), NBQX (14), YM90K (50) and CNQX(83); whereas for agonists (μM) it was: quisqualate (0.4), AMPA (3.7), glutamate (9.5), kainate (11) and NMDA (190). Quantitative receptor radioautography and image analysis of horizontal rat brain sections revealed a high density of high-affinity specific binding to rat cerebral cortex (layers 1,2>>3-6), hippocampal formation, striatum, lat. septum, retic. thalamic nucleus, cerebellar molecular layer and spinal cord dorsal horn. The distribution of binding sites and GluR 1-4 transcripts (revealed by in situ hybridization histochemistry) were compared.

We conclude that Ro 48-8587, due to its potency, high specificity and pharmacological selectivity, is currently the radioligand of choice for future studies on the selectivity of AMPA antagonists.

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11P ISOPRENALINE ACTIVATES ADENYLYL CYCLASE IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS THROUGH SELECTIVE STIMULATION OF β_2-ADRENOCEPTORS

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Cultured human umbilical vein endothelial cells (HUVEC) are extensively used in investigations of human endothelial biochemistry, physiology and pharmacology. They possess β-adrenoceptors (AR), but it is not established which subtypes are present and what their physiological function may be. β-AR stimulation elsewhere generates an increase in adenylyl cyclase (AC) activity, giving rise to an increase in intracellular adenosine 3',5'-cyclic monophosphate (cAMP). The purpose of this study was to determine which β-AR subtypes can stimulate AC in HUVEC.

Fresh umbilical cords were obtained after delivery of healthy babies from healthy normotensive mothers, HUVEC were isolated by collagenase dispersion, and grown in monolayer culture. At confluence, they were passaged at a split ratio of 1:3, and confluent cells at passage 3 were used for preparation of homogenates, as follows. Cells were harvested using a rubber policeman, pelleted at 400*g* for 5 minutes at 4°C, and homogenised on ice in 25mM Tris-HCl pH 7.4, 0.29M sucrose and 0.25mM EDTA. A portion of homogenate was used for protein assay (Bio-Rad DC Protein Assay), and the remainder stored at -80°C.

AC activity was measured in thawed HUVEC homogenates by a modification of the method of Salomon *et al.* (1974), examining the conversion over 15 minutes of [α-^{32}P] adenosine 5'-triphosphate to [^{32}P] cAMP. These were separated by sequential chromatography on columns of Dowex cation exchange resin and aluminium oxide, and [^{32}P] counts in the eluate (representing cAMP) were corrected for column losses by measuring ^{3}H counts in the eluate following addition of a known amount of [^{3}H] cAMP to the column. Concentration-effect curves were determined with addition of isoprenaline (10^{-9} - 10^{-5} M) to the reaction mixture, and AC responses were compared to those after addition of forskolin (10^{-5}M) or bradykinin (10^{-5}M). Measurements were performed in triplicate, and results from experiments on cells from 6 different umbilical cords are given as mean ± s.e.mean.

Basal AC activity was 24.3 ± 4.3 pmol cAMP/min/mg protein. There was no significant response to 10^{-5}M bradykinin, whereas 10^{-5}M forskolin increased AC activity to 143.4 ± 25.5 pmol cAMP/min/mg protein ($P < 0.005$, paired Student's *t* test). Isoprenaline caused a concentration-dependent increase in AC activity, with an EC_{50} of 2.3 x 10^{-8}M and an E_{max} of 76.3 ± 13.0 pmol cAMP/min/mg protein ($P < 0.01$, repeated measures ANOVA). This response was not affected by the selective β_1-AR antagonist CGP 20712A (300nM), but was inhibited by the selective β_2-AR antagonist ICI 118551 (50nM), with EC_{50} values of 6.7 x 10^{-8}M and 3.4 x 10^{-6}M respectively.

We conclude that cultured HUVEC possess functional β-AR, stimulation of which elicits an increase in AC activity. Furthermore, the β-AR mediated AC response in this cell type appears to be mediated selectively by the β_2-AR subtype. The physiological consequences of this response remain to be determined, but endothelial β_2-AR may contribute to the vasodilatation elicited by stimulation of β-AR in blood vessels, possibly through cAMP mediated release of endothelium-derived relaxing factors. This hypothesis is consistent with our recent finding that infusion of β_2-AR agonists into the brachial artery of healthy human subjects increases forearm blood flow, which is inhibited by co-infusion of the nitric oxide synthase antagonist N^G-monomethyl-L-arginine (Dawes *et al.*, 1997).

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12P CHARACTERISATION USING THE CYTOSENSOR MICROPHYSIOMETER OF RECOMBINANT HUMAN CORTICOTROPHIN-RELEASING FACTOR (CRF) RECEPTOR PHARMACOLOGY

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Two human CRF receptors, CRF_1 and CRF_2, with distinct anatomical and pharmacological profiles have been cloned (DeSouza 1995), with the human CRF_2 receptor corresponding to the rat $CRF_{2\alpha}$ splice variant (Liaw *et al.*, 1996). Although the CRF_2 receptor stimulates cAMP formation, it is poorly coupled compared to the CRF_1 receptor (Nabhan *et al.*, 1995), suggesting that the CRF_2 receptor may also couple to other signal transduction mechanisms. The Cytosensor microphysiometer measures the cellular acidification rate, which is a reliable index of the integrated functional response to receptor activation regardless of the type of signalling pathway activated (Smart *et al.*, 1997). Therefore, we have used the Cytosensor to compare the pharmacology of the human CRF_1, and CRF_2, as well as the rat $CRF_{2\alpha}$, receptors stably expressed in Chinese Hamster Ovary (CHO) cells.

The cells were seeded into Cytosensor capsule cups (~$0.6x10^6$ cells per cup) as described previously (Smart *et al.*,1997), and perfused in the Cytosensor at 120µl min^{-1} with bicarbonate-free Hams F-12 containing 0.2% BSA (pH 7.4), with the acidification rate taken every 2min by stopping the perfusion for 15s. CRF, sauvagine (SV), urocortin (UC) or urotensin (UT) (0.1pM-300nM) were introduced serially in the perfusate for 10min at 45min intervals. In some studies the antagonist α-helical CRF (9-41) (αCRF) was included in the perfusate throughout. Data are presented as mean± s.e.mean unless otherwise stated.

In CHO-$hCRF_1$ cells the rank order of potency (ROP) was CRF=SV=UC=UT, with pEC_{50} values of 11.16±0.17, 11.37±0.14, 11.43±0.09 & 11.46±0.13 respectively (n=4). However, in CHO-$hCRF_2$ cells the ROP was UC>SV>>UT>>CRF, with pEC_{50} values of 10.88±0.12, 10.44±0.05, 9.36±0.12 & 8.53±0.07 respectively (n=7-9). Similarly, in CHO-$rCRF_{2\alpha}$ cells the ROP was UC=SV>>UT>>CRF, with pEC_{50} values of 10.67±0.09, 10.64±0.03, 9.85±0.05 & 8.70±0.12 respectively (n=3). Furthermore, αCRF (300nM) was a competitive antagonist at the $hCRF_{2\alpha}$, receptor, with an apparent pK_B of 6.99±0.08 (n=4). However, at the $hCRF_1$ receptor, αCRF (30-300nM) displayed a concentration-related partial agonism, as well as antagonising the CRF-induced response with an apparent pK_B of 7.97±0.15 (n=6).

The ROPs demonstrated in the present study are consistent with the established pharmacology of these receptors (DeSouza 1995, Liaw *et al.*, 1996). However, the actual potencies obtained were considerably higher than those previously reported from studies utilising cAMP measurements (Nabhan *et al.*, 1995). Nevertheless, this was not an artefact of the system, as the pK_B values for the antagonist αCRF were consistent with those generated previously using the cAMP assay (Kishimoto *et al*, 1995; Liaw *et al.*, 1996). In conclusion, these data clearly demonstrate the utility of the Cytosensor for studying the pharmacology of CRF receptor agonists and antagonists.

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13P POTENT INHIBITION OF P2Z ($P2X_7$) RECEPTOR-MEDIATED EFFECTS BY COPPER, ZINC AND NICKEL IONS

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The group IIB metals can affect the function of a number of ligand-gated cation channels. Thus, copper ions potently block glutamate receptors (Weiser & Weinrich, 1996) while zinc ions block glutamate and GABA receptors (Westbrook & Mayer, 1987) and potentiate responses to ATP at $P2X_2$ (Brake et al., 1994) and $P2X_4$ (Soto et al., 1996) receptors. In the present study we have examined the effects of several group IIB metals on the P2Z ($P2X_7$) receptor.

Studies on endogenous P2Z ($P2X_7$) receptors in CHO-K1 cells were performed as described (Michel et al., this meeting). Briefly, dibenzoyl-ATP (DbATP) stimulated ^{45}calcium ($^{45}Ca^{2+}$) influx was measured over 4min in 10mM Hepes, 10mM glucose, 5mM KCl, 0.5mM $CaCl_2$, 280mM sucrose buffer (pH 7.4 at 22°C). Electrophysiological studies were performed in a similar buffer containing 140mM NaCl in place of sucrose. Data are the mean ± s.e.mean of 3-5 experiments. Metal ions were added as chloride salts.

Cu^{2+}, Zn^{2+} and Ni^{2+} were potent, non competitive, inhibitors of the P2Z ($P2X_7$) receptor in CHO-K1 cells. pIC_{50} values for Cu^{2+}, Zn^{2+} and Ni^{2+} in blocking $^{45}Ca^{2+}$ influx stimulated by a supra-maximal concentration of DbATP (30μM) were 6.1±0.1, 5.7±0.1 and 5.5±0.1, respectively. Cu^{2+} and Ni^{2+}, at concentrations up to 10μM, did not affect the EC_{50} for DbATP yet reduced the maximum response to 100μM DbATP by >90%. This would suggest that the effects of Cu^{2+} and Ni^{2+} were not due to chelation of DbATP. Cu^{2+}, Zn^{2+} and Ni^{2+} also blocked responses produced by 100μM ATP with pIC_{50} values of 6.2±0.2, 5.6±0.1 and 5.3±0.1, respectively. The effects of Cu^{2+}, Zn^{2+} and Ni^{2+} on the P2Z ($P2X_7$) receptor contrast with their actions on the $P2X_2$ receptor. Thus, in HEK293 cells expressing rat recombinant $P2X_2$ receptors, Cu^{2+}, Ni^{2+}, and Zn^{2+} potentiated, rather than inhibited, ATP-stimulated $^{45}Ca^{2+}$ influx. The pEC_{50} value for both Cu^{2+} and Ni^{2+} to potentiate $^{45}Ca^{2+}$ influx evoked by 3μM ATP was 5.7±0.1. Zn^{2+}, at 1 and 3μM, also potentiated ATP-stimulated $^{45}Ca^{2+}$ influx. The increase in ATP-stimulated $^{45}Ca^{2+}$ influx was due to an increase in the potency of ATP. Thus, pEC_{50} values for ATP in the absence and presence of 10μM Cu^{2+}, 3μM Zn^{2+} or 10μM Ni^{2+} were 5.4±0.1, 6.0±0.1, 5.7±0.1 and 6.0±0.1, respectively.

DbATP evoked a non-desensitising inward current in CHO-K1 cells. Cu^{2+}, the most potent cation for blocking DbATP stimulated $^{45}Ca^{2+}$ influx, blocked the depolorising response to 300μM DbATP (pIC_{50} 6.8±0.1). The inhibitory effect of 1μM Cu^{2+} was reversible following a 10min washout period.

These results suggest that Cu^{2+}, Ni^{2+} and Zn^{2+} are potent inhibitors of P2Z ($P2X_7$) receptor function. Their effects on DbATP-stimulated $^{45}Ca^{2+}$ influx are unlikely to be non-specific given their differential actions on the P2Z ($P2X_7$) and $P2X_2$ receptors. Furthermore, the inhibitory effect off Cu^{2+} on the P2Z ($P2X_7$) receptor was confirmed in electrophysiological studies. These data further suggest that the group IIB metal ions can be used to differentiate between some of the P2X receptor subtypes since they block P2Z ($P2X_7$) receptor-mediated effects yet potentiate the actions of ATP at the $P2X_2$ and $P2X_4$ receptors.

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14P A ROLE FOR THROMBIN AND PROTEINASE-ACTIVATED RECEPTOR 1 (PAR1) IN GUINEA-PIG BRONCHOSPASM

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In vitro studies have shown that thrombin receptor activating peptides (called TRAPs), which stimulate the thrombin receptor PAR 1 on the cell surface, but are devoid of catalytic activity, are able to reproduce several of the cellular responses evoked by thrombin (Bizios et al., 1986; Hoffmann & Church, 1993). We have recently shown that thrombin induces local inflammation when injected in the rat paw and that this action is mimicked by the TRAP-9, giving direct evidence of the involvement of thrombin in inflammation, through a mechanism that require mast-cell degranulation (Cirino et al., 1996). Here, we have evaluated the effect of in vivo administration to guinea pig of thrombin and TRAP-9 on the arterial blood pressure and bronchial reactivity.

Guinea pigs (400-500g, Charles River, Milan), anaesthetized with sodium pentobarbitone (Sagatal, 40 mg/kg i.p.) and Hypnorm (0.5 ml/kg i.m), were artificially ventilated by a respiration pump, connected to a transducer of bronchospasm. Spontaneous breathing was abolished by administration of pancuronium (2 mg/kg i.v.). Both cervical vagi nerves were transected at the neck level. The jugular vein and the left carotid artery were cannulated for drug administration and monitoring of arterial blood pressure respectively. Histamine (10 μg/kg i.v.) was administered to evaluate animal responsiveness to a bronchoconstrictor agent. Human α-thrombin (THR, 50 and 100 u/kg i.v.), TRAP-9 (SFLLRNPND, 0.1, 0.3 and 1 mg/kg i.v.) or the control peptide (SFLLANPND, 1 mg/kg i.v.) were administered each 20 min, for three consecutive times. A group of animals was pretreated with the thrombin inhibitor hirulog (10 mg/kg i.v.). At the end of the experiment, in some cases, lungs were removed, fixed in formalin histological analysis (haematoxylin/eosin staining).

Injection of THR (100 u/kg) produced a bronchoconstriction of 33.14±8.61% which was significantly reduced at the second (12.41±6.11% $p<0.01$) and third administration (4.3±1.7% $p<0.01$. Two ways ANOVA followed by Bonferroni's test n=7). TRAP-9 (1 mg/kg) mimicked thrombin's bronchoconstrictor effect (30.8±11.42%; 18.4±8.18%, $p>0.05$ and 4.7±1.61%,$p<0.01$; n=7). Thrombin administration caused a biphasic change in blood pressure characterized by a fall (8.5±1.9 mmHg n=7, $p<0.05$ one sample Student's t-test) followed by a rapid increase (11.86±1.86 mmHg n=7$p<0.05$). Thrombin hypotensive phase was unaffected by repeated administrations, while the hypertensive phase was subjected to desensitisation. TRAP-9 induced a similar hypotensive phase (6.57±0.7mmHg n=7$p<0.01$) followed by a more sustained hypertension (23±4.8 mmHg n=7 $p<0.01$). TRAP-9 effect was not subjected to desensitisation. The control peptide caused no bronchoconstriction, or any change in blood pressure. Hirulog pretreatment abolished the THR-induced bronchoconstriction and hypotension, while it did not affect bronchoconstriction or changes in blood pressure induced by TRAP-9. Histological analysis showed a large area of alveolar destruction and stenosis on lungs obtained from both THR and TRAP-9 treated animals. Following these observations, we suggest that thrombin might play a role in bronchial asthma through a mechanism independent of its catalytic activity and that involves the activation of PAR 1 receptors.

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15P IMPORTANCE OF CONSTRICTOR ET_A RECEPTORS IN PIAL ARTERY FROM HUMAN BRAIN REVEALED BY POTENT NONPEPTIDE ENDOTHELIN ANTAGONISTS

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Animal studies suggest that endothelin (ET) may play a significant role in the vasospasm associated with stroke and subarachnoid haemorrhage (SAH). Human pial arteries supplying the cerebral tissue may be an important therapeutic target in these conditions. We have shown that the current drug therapy for SAH, the calcium channel blocker nimodipine, will reduce the maximal effect of ET-1 in human pial arteries without changing its potency (Pierre & Davenport, 1997; Figure).

Our aims were to visualise, using microautoradiography, the ET receptor subtypes present in small pial arteries and to study the effect of selective ET receptor antagonists on responses to ET-1. The ability of these compounds to block ET-1 constriction was compared to data previously obtained for nimodipine.

Cortical tissue and pial arteries were obtained from 27 patients undergoing lobectomy for the treatment of tumours or epilepsy.

Microautoradiography: Slide mounted cryostat sections (10μm) of human cortex were incubated for 2h in buffer containing either [^{125}I]PD151242 (0.1nM) or [^{125}]BQ3020 (0.3nM), both ~ 2000 Ci.mmol^{-1}, to specifically label ET_A and ET_B receptors respectively (Davenport *et al.*, 1994). To visualise binding, slides were dipped in radiation sensitive emulsion, developed after 4 days and viewed under a microscope under darkfield.

In vitro pharmacology: Pial arteries were dissected from the cortical surface and 1-2 mm arterial rings (mean internal diameter 368.0±17.3μm) were set up in a wire-myograph containing oxygenated modified Krebs solution (37°C). PD156707 (ET_A selective; 3-30 nM) or BQ788 (ET_B selective; 1μM), were added 30 min before construction of cumulative concentration response curves (CRC) to ET-1. Antagonist potency (pA_2) was determined by Schild regression or Gaddum equation.

Specific, high density binding of [^{125}I]PD151242 was localised to the vascular smooth muscle of pial and intracerebral arteries. In contrast, little specific binding of [^{125}]BQ3020 was detected in vessels, though intense binding was observed on neuronal tissue.

PD156707 caused parallel rightward shift of the CRC to ET-1 (Figure), yielding a pA_2 of 9.17 ± 0.07 (n=2-6). This is consistent with the presence of an ET_A receptor population. BQ788 also caused a small rightward shift of the CRC with a pA_2 of 6.30 ± 0.28 (n=5). However, this value is ~100 times lower than that expected for ET_B receptor blockade (Ishikawa *et al.*, 1994). Whilst PD156707 reversed an established ET-1 constriction, BQ788 was with little effect.

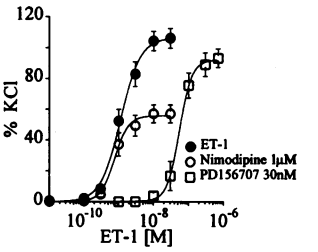

Figure. Effect of PD156707 and nimodipine on responses to ET-1 in human pial artery (n=5-24)

These data confirm the important contribution of ET_A receptors to ET-1 induced constriction in human pial artery. Responses to low concentrations of ET-1 are essentially unaffected by nimodipine, but potently blocked by PD156707. ET_A receptor antagonists may therefore represent an additional and novel target for the treatment of cerebral vascular disorders in man.

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16P PRESERVATION OF FUNCTIONAL RESPONSES OF HUMAN CORONARY ARTERIES TO ET-1 AND 5-HT AFTER 14 DAYS IN ORGAN CULTURE

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Endothelin (ET) receptor antagonists have novel therapeutic potential in coronary artery (CA) disease. Species differences in the ET receptors which contribute to vasoconstriction have made it important to determine which receptor subtypes must be targeted in humans. To maximise the use of the limited human CA tissue which is available, we have determined whether rings of CA can be maintained in culture without loss of their contractile responses to ET-1. This would allow us to investigate the longer term effects of ET antagonists on the ET system in human CA *in vitro*.

Human CA was obtained from 13 male and 2 female patients (28-61 years) transplanted for cardiomyopathy or ischaemic heart disease.

Organ Culture 4mm rings of CA were incubated in supplemented M199 medium for 14 and 28 days, at 37°C, in 5% CO_2. Medium was replaced every 2 days.

In vitro pharmacology Rings of CA were set up for isometric tension recording in organ baths containing oxygenated Krebs solution at 37°C. Cumulative concentration-response curves (CRC) were constructed to ET-1 and 5-HT in control vessels (day 0) and in rings from the same vessels removed from culture after 14 and 28 days. Experiments were terminated by the addition of 50mM KCl to determine the maximum response in mN. Agonist responses were expressed as a percentage of this KCl maximum.

Results Prior to culture (day 0) the maximum force developed to KCl was 57.0±10.3mN. This was reduced by 70% (17.7±2.5mN) after 14 days in culture and by 94% (3.6±0.6mN) after 28 days. This marked attenuation of response at 28 days suggests that culture time should be restricted to a maximum of 14 days.

ET-1 potently constricted all CA rings at each of the three time points with no significant change in potency. Similarly, no change in the potency of 5-HT was detected. However, responses to 5-HT were less consistent, with the number of CA rings which fail to respond increasing from 7% at Day 0, to 25% at Day 14 and to 33% at Day 28. The maximum response to ET-1 compared to the KCl response did not change following organ culture but that of 5-HT appeared more variable (Figure).

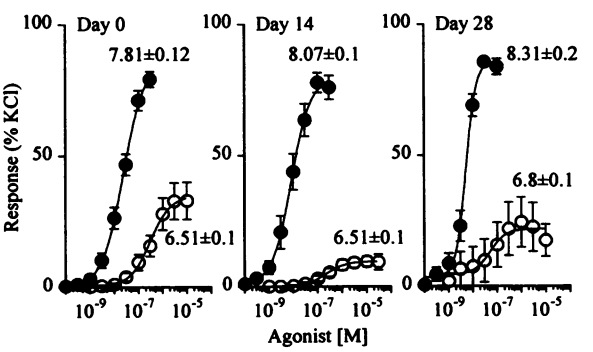

Figure Response of human coronary arteries to ET-1 (●) (n=15) and 5-HT (○) (n=7) after 0, 14 and 28 days in organ culture Values are pD_2 (mean±sem)

These data show that for *in vitro* pharmacological experiments human CA rings remain viable in culture for up to 14 days. During this time additional experiments could be performed to examine the effects of chronic exposure to ET antagonists. However, further characterisation of the ET-1 response is necessary before this technique is routinely employed.

We thank the theatre and surgical staff of Papworth Hospital for permission to collect surgical samples. Supported by the BHF.

17P INTERACTIONS BETWEEN ET_A AND ET_B RECEPTOR-MEDIATED CONTRACTIONS IN THE RABBIT ISOLATED PULMONARY ARTERY

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Although ET_A receptors mediate the contraction of the majority of vascular preparations, ET_B receptors are linked to contraction in some vascular beds (Moreland *et al.*, 1992). ET-evoked contraction in the rabbit pulmonary artery has been shown to be mediated predominantly by ET_B receptors although the presence of ET_A receptors are revealed by radioligand membrane-binding studies (Panek *et al.*, 1992; LaDouceur *et al.*, 1993). Our experiments were designed to investigate the relative functional importance of the ET_A and ET_B receptors in isolated rabbit pulmonary artery.

Female New Zealand White rabbits were anaesthetised (sodium pentobarbitone, 60mg kg^{-1}) and killed by rapid exsanguination. Rings of endothelium-denuded, first order branches of rabbit pulmonary artery (4mm, resting tension 1g) were mounted in siliconised organ baths and maintained at 37°C in oxygenated Krebs buffer containing indomethacin (10μM), N^G-Nitro-L- arginine methyl ester (L-NAME, 100μM) and N^G-Nitro-L- arginine (L-NOARG, 100μM). All data are expressed as mean ± s.e. mean.

ET-1, ET-3 and the ET_B receptor agonist, sarafotoxin S6c, evoked contraction in pulmonary artery rings with similar potency (EC_{50} values 0.5nM, 0.4nM and 0.2nM, respectively). The responses to ET-1 (30pM-300nM) were not inhibited by an ET_A receptor antagonist, BQ123 (10μM, EC_{50}=0.6nM, n=4). In the presence of 10μM BQ788, an ET_B receptor antagonist, the concentration response curve to ET-1 was shifted to the right (EC_{50}=3nM, n=6) and the contractions were potentiated by up to 171 ± 25.7%. SKB209670 (10μM), a non-selective endothelin receptor antagonist, strongly inhibited the ET-1 induced contraction.

Selective desensitization of ET_B receptors by prolonged exposure to sarafotoxin S6c (10nM, 3 hrs) did not significantly affect the potency of ET-1 in the rabbit pulmonary artery (EC_{50}=0.3nM, n=4). After desensitization, the contraction evoked by ET-1 was still antagonised by 1μM BQ123 (EC_{50} approximately 10nM, n=4) and contractions to higher concentrations of ET-1 were potentiated.

Our data show that ET-1 evoked contraction of the first order branch of the rabbit pulmonary artery is mediated by ET_B receptors in agreement with Panek *et al.* (1992). However, ET_A receptors appear also to mediate contraction in response to ET-1. This was revealed after desensitization or pharmacological block of the ET_B receptors. This suggests that cross talk exists between ET_A and ET_B receptors in this tissue. Desensitization of the ET_B receptors was not the same as blockade with BQ788. Desensitization leads to ET-1 induced contractions of control size whereas inhibition of receptors leads to potentiated responses with higher concentrations.

In conclusion, a role for ET_A receptors in the contraction to ET-1 is revealed when ET_B receptors are either inhibited or desensitized. The magnitude of the ET_A receptor mediated contraction to ET-1 seems to depend on whether the ET_B receptors are either desensitised or inhibited by a selective antagonist.

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18P CYTOKINE AND LPS STIMULATION OF ENDOTHELIN-1 RELEASE FROM INTERNAL MAMMARY ARTERY AND SAPHENOUS VEIN SMOOTH MUSCLE CELLS

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Numerous pathological conditions are associated with elevations in the production of ET-1, often within the blood vessel wall. As the cytokines involved in many of these pathological conditions can increase the circulating concentration of ET-1 (Kanse *et al.*, 1991; Klemm *et al.*, 1995) we have examined the effects of a mixture of cytokines and LPS on ET-1 production in human vascular smooth muscle cells (VSMC) derived from the internal mammary artery (IMA) and saphenous vein (SV).

IMA and SV were obtained from 3 patients undergoing coronary artery bypass graft surgery. Explants of VSMC's were grown in DMEM supplemented with 2mM glutamine, penicillin (100 IU.ml^{-1}), streptomycin (0.1 mg.ml^{-1}) and 20% foetal calf serum (37°C; 5% CO_2; 95% air). VSMC's were identified by morphology and α-actin staining. Cells in culture for 48 h were treated with a mixture of tumour necrosis factor-α (10ng.ml^{-1}), interferon γ (1000U.ml^{-1}), interleukin-1β (500U.ml^{-1}) and LPS (10μg.ml^{-1}) for the final 1, 4, 8, 12, 24, 36 or 48h. Medium was removed and ET-1 levels were measured by specific sandwich ELISA (R&D Systems). In addition, total RNA was isolated using a modified method of Chomczynski & Sacchi (1987) and reverse transcriptase coupled with polymerase chain reaction performed using standard methods.

Stimulation of VSMC from IMA and SV with cytokines and LPS markedly elevated both the expression of mRNA for ET-1 (assessed by densitometry) and the release of ET-1 into the culture medium (Figure 1). mRNA for ET-2 or ET-3 was not detectable.

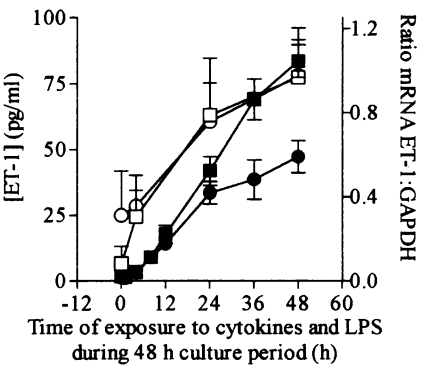

Figure 1. Expression of ET-1 mRNA (open symbols) and ET-1 (filled symbols) in IMA (□, ■) and SV (○, ●) VSMC stimulated with cytokines and LPS. Mean ± s.e.m of data from 3 donor tissues.

Thus, the low levels of ET-1 mRNA expression and peptide production in human VSMC's (Yu and Davenport, 1995) are markedly increased by exposure to cytokines and LPS. This suggests that during inflammatory states the vascular smooth muscle, as well as the endothelium, may be a site of significant ET-1 production within the blood vessel.

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19P DIFFERENTIAL INDUCTION OF CYCLO-OXYGENASE-2 IN HUMAN ARTERIAL AND VENOUS SMOOTH MUSCLE CELLS: ROLE OF ENDOGENOUS PROSTANOIDS

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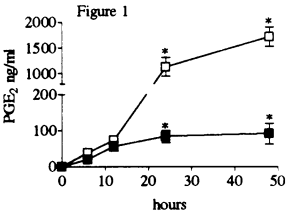
Interleukin-1β (IL-1β) induces cyclo-oxygenase (COX)-2 in segments of human internal mammary artery (IMA) and saphenous vein (SV) in organ culture (Bishop-Bailey et al., 1997a), and IMA smooth muscle cells (SMC) isolated in cell culture (Bishop-Bailey et al., 1997b). Interestingly, SV released more prostanoids than IMA in organ culture (Bishop-Bailey et al., 1997a). Thus, we have compared the ability of isolated vascular SMC from human SV and IMA to express COX-2.

SV and IMA were obtained from patients undergoing coronary artery bypass graft surgery. Vascular SMC were grown by explant, in DMEM supplemented with 2mM glutamine, penicillin (100 IU.ml^{-1}), streptomycin (0.1mg.ml^{-1}), and 20% foetal calf serum (37°C; 5% CO_2; 95% air). COX activity was determined by PGE_2 release by radioimmunoassay, and COX-2 expression by Western blot analysis (Mitchell et al., 1993) using specific antibodies (Chan et al., 1995). Cells were treated with a "cytokine mix" consisting of IL-1β (10ng.ml^{-1}), tumour necrosis factor-α (10ng.ml^{-1}), interferon-γ (1000U.ml^{-1}) and lipopolysaccharide (10μg.ml^{-1}) for 6, 12, 24 and 48h. Cells treated with cytokine mix for 24h were also incubated with the selective COX-2 inhibitor L-745,337 (10 μM, Chan et al., 1995).

COX-2 protein was not detected under control culture conditions, but when SV or IMA SMC were stimulated with cytokine mix, inducible bands at 70 kDa (COX-2) and approximately 60 kDa were recognised by the selective COX-2 antibody. SMC stimulated with cytokine mix, gave a time dependent release of PGE_2 (Figure 1), which was significantly greater than cells cultured with out cytokines (SV 12±3ng.ml^{-1}; and IMA 13±2ng.ml^{-1}). SV released up to 15 times more PGE_2 than IMA and contained significantly more COX-2 protein ($p<0.05$ unpaired t-test; Figure 2). When cytokine mix induced COX-2 was inhibited by L-745-337 (10μM), COX-2 protein was significantly ($p<0.05$ one sample t-test) increased 2.6±0.5 fold in IMA SMC and remained unaltered in SV SMC (decreased 37±16%). Interestingly, the band at 60 kDa was inhibited in the presence of L-745,337 in both SV (by 89±5%) and IMA (by 72±13%) SMC.



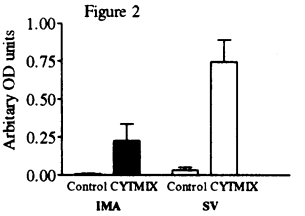


Figure 1. Time dependent release of PGE_2 by cytokine mix treated SV (open squares) and IMA (filled squares) SMC. Figure 2.Comparison of the cytokine mix (24h) induced COX-2 protein expression (densometry OD arbitrary units) in IMA and SV SMC. All the above results represent the mean ± s.e.mean from 12 determinations from 4 patients.(= $p<0.05$ by one-way ANOVA, compared to t=0).*

These results show that COX-2 is differentially induced in human SV and IMA SMC, and suggests that COX-2 can play an important regulatory role in its own expression. Furthermore the identity and regulation by COX-2 of the band at 60 kDa requires further investigation. The difference in regulation of COX-2 in these vessels may play a role in their patency as bypass conduits.

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20P INTERLEUKIN-1β INDUCES ANTI-PROLIFERATIVE PROSTANOIDS VIA CYCLO-OXYGENASE-2 IN HUMAN SAPHENOUS VEIN AND INTERNAL MAMMARY ARTERY SMOOTH MUSCLE CELLS

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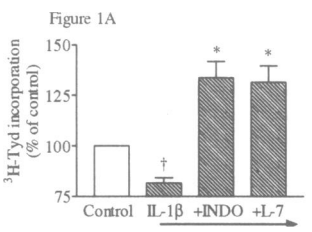
Many of the multi-functional cytokines that induce cyclo-oxygenase (COX)-2 have effects on the proliferation of various cell types. Indeed interleukin (IL)-1β induces the release of anti-proliferative prostanoids in human saphenous vein smooth muscle cells (SMC) (Libby et al., 1988). In this study, we have assessed whether COX-2 regulates IL-1β induced changes in proliferation of human saphenous vein (SV) and internal mammary artery (IMA) SMC.

SMC were grown in 48-well plates in DMEM containing 20% foetal calf serum (FCS) as previously described (Bishop-Bailey et al., 1997). At confluence, cells were serum deprived for 24h. Medium containing optimum concentrations of FCS for proliferation was then added to SV (5%) or IMA (10%) SMC in the presence or absence of IL-1β (10 ng.ml^{-1}). After 25h, 0.5 μCi.ml^{-1} of methy-^{3}H-thymidine (^{3}H-Tyd) was added to the cells for a further 6h. Incorporated ^{3}H-Tyd was extracted and measured by scintillation counting (Dicker and Rozengurt, 1980). In separate experiments, indomethacin (INDO) or its COX-2 selective derivative L-745,337 (L-7; Chan et al., 1995) were added (10μM) in addition to the serum, or IL-1β.

Under serum stimulated conditions, ^{3}H-Tyd (13374±2320 cpm SV, n=6 (3 patients); 9524±1610 cpm IMA; n=10 (5 patients) was incorporated in to the SMC. IL-1β inhibited ^{3}H-Tyd incorporation by approximately 20% in IMA (figure 1A), while having little effect in SV (figure 1B) SMC. However in both IMA and SV stimulated with IL-1β, inhibition of COX by INDO or L-7 greatly increased the ^{3}H-Tyd incorporation (figure 1). In cells cultured with out IL-1β, INDO or L-7 had no effect on ^{3}H-Tyd incorporation in IMA (plus INDO 90±5%; plus L-7 98±6% of control), and small inhibitory effects in SV (plus INDO 90±3%; plus L-7 82±3% of control) SMC; n=6-10 determinations from 3-5 patients.



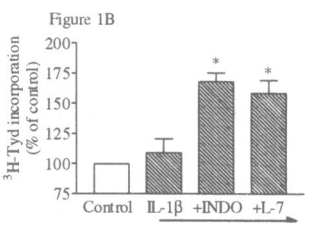


*Figure 1. FCS induced ^{3}H-Tyd incorporation is regulated by IL-1β induced COX-2 in IMA (1A) and SV (1B) SMC. The data represents the mean ± s.e.mean for 6-10 determinations from 3-5 patients. †=$p<0.05$ IL-1β compared to control; *=$p<0.05$ IL-1β alone compared to IL-1β with INDO or L-7 (one sample t-tests).*

This study shows that the relatively low levels of constitutively expressed COX in human vascular SMC have only a minor role in proliferation. However, when COX-2 is induced by IL-1β, anti-proliferative prostanoids are released. Since cytokines which can induce COX-2 are elevated in areas of vascular damage, the induction of COX-2 may represent an endogenous defence mechanism to accelerated cell division associated with atherosclerosis or restenosis. These observations may have particular relevance to coronary artery bypass surgery where SV grafts often fail due to increased intimal thickening and re-occlusion.

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21P INTERLEUKIN (IL)-1β-INDUCED CYCLO-OXYGENASE-2 ACTIVITY INHIBITS IL-1β-INDUCED EXPRESSION OF ICAM-1- AND IL-4-INDUCED EXPRESSION OF VCAM-1 ON SAPHENOUS VEIN SMOOTH MUSCLE CELLS

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Recruitment of inflammatory cells is an important pathological event in the development of vascular diseases. Many of the cytokines that regulate cyclo-oxygenase (COX)-2 also affect adhesion molecule expression on the vascular endothelium. We have assessed whether interleukin (IL)-1β- induced COX-2 activity modulates IL-1β- induced intercellular adhesion molecule (ICAM)-1 expression or IL-4- induced vascular cell adhesion molecule (VCAM)-1 on human saphenous vein (SV) smooth muscle cells (SMC).

SMC were grown to confluence in 96-well plates (Bishop-Bailey et al., 1997). COX-2 activity was measured by the release of PGE_2, and protein expression by Western blot analysis (Mitchell et al., 1993). ICAM-1 and VCAM-1 expression were measured by a specific enzyme linked immunosorbant assay (Burke-Gaffney and Hellewell, 1996), and all results expressed as mean ± s.e.mean $OD_{405}(x10^3)$. Cells were incubated with IL-1β (10ng.ml^{-1}; 24h) to induce COX-2 and ICAM-1 and with IL-4 (10ng.ml^{-1}) to induce VCAM-1. Co-incubation with the selective COX-2 inhibitor L-745,337 (L-7 10μM; Chan et al., 1995) and, or PGE_2 (1μM) were used to assess whether COX-2 or PGE_2 could regulate adhesion molecule expression.

Under control culture conditions COX-2 was undetectable. IL-1β induced COX-2 protein (recognised by a specific antibody; Chan et al., 1995), and PGE_2 release (control 9±2; IL-1β 84±25 ng.ml^{-1}). IL-4 had no effect on PGE_2 release (10±2 ng.ml^{-1}), and inhibited (53±5 ng.ml^{-1}; $p<0.05$ Mann-Whitney) IL-1β induced PGE_2 release. In the same experiments, L-7 (10μM) inhibited the PGE_2 release induced by IL-1β alone to 1±0 ng.ml^{-1} or in the presence of IL-4 to 2±1 ng.ml^{-1}. Constitutive expression of ICAM-1, but not VCAM-1, was detected on SV SMC (Figure 1). In the presence of IL-1β, ICAM-1(Figure 1A) but not VCAM-1 (10±2) was induced. IL-4 induced VCAM-1, which was inhibited by IL-1β (Figure 1B). Inhibition of COX-2 by L-7 increased IL-1β induced ICAM-1 (Figure 1A), and returned IL-1β inhibited VCAM-1 to the levels induced by IL-4 alone (Figure 1B). PGE_2 reversed the increase in ICAM-1 and VCAM-1 when COX-2 was inhibited by L-7 (Figure1). In a subset of experiments, neither L-7, nor PGE_2 had any effect on basal ICAM-1 (control 240±11; L-7, 239±3; PGE_2 219±15) or VCAM-1 (2±1; L-7, 4±1; PGE_2 6±2) expression.

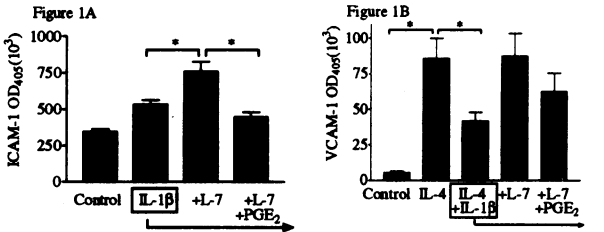

*Figure1. COX-2 products inhibit IL-1β induced ICAM-1 (A) and IL-4 induced VCAM-1 (B). *=p<0.05 one-way ANOVA. Data represents the mean ± s.e.mean for 9-15 determinations from 3-5 patients.*

Thus ICAM-1 and VCAM-1 can both be induced in human SV SMC. Moreover, when COX-2 is co-induced its activity, possibly via PGE_2 formation, suppresses the expression of these adhesion molecules. As inflammatory cell infiltration is an important event in the development of vascular pathologies, the presence of COX-2 may limit the recruitment of cells at the level of adhesion molecule expression.

Supported by the B.H.F. (FS/94075), Wellcome Trust and N.A.C.

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22P INDUCTION OF COX-2 *IN VIVO* LEADS TO GREATLY INCREASED PRODUCTION OF 6-KETO-$PGF_{1\alpha}$ FOLLOWING ADMINISTRATION OF EXOGENOUS ARACHIDONIC ACID OR BRADYKININ

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Inflammation is associated with the induction by cytokines (or bacterial endotoxin, LPS) of cyclo-oxygenase (COX) 2 and inducible nitric oxide synthase (iNOS) (Vane *et al.*, 1994). There is also a release of biologically active peptides, including bradykinin (BK), which activates, via cell surface receptors, phospholipase A_2 (PLA_2) (Conklin *et al.*, 1988), liberating arachidonic acid (AA), so promoting prostanoid generation by COX. Indeed, activation of PLA_2 to supply AA is required if induced COX 2 is to realise its prostanoid generating potential. For example, PGE_2 release by airway epithelial cells expressing COX 2 is greatly increased by BK (Saunders *et al.*, 1997). Here, we have investigated whether *in vivo* the production of prostanoids by COX 2 can be similarly increased by supplying AA.

Male Wistar rats (220-250 g) were anaesthetised with Inactin (120 mg kg^{-1}) and the carotid artery and jugular vein cannulated for the measurement of blood pressure and the administration of compounds or vehicle (saline). After a stabilisation period of 15 min, animals received a bolus of the COX 2 selective inhibitor NS 398 (10 mg kg^{-1}) or vehicle followed 1h later by a continuous 6 h infusion of LPS (0.2 mg kg^{-1} hr^{-1}) or vehicle. At the end of the 6 h infusion period rats were given BK (100 μmol kg^{-1}), AA (3 mg kg^{-1}) or vehicle as an i.v. bolus. Blood samples were taken 6h after the start of LPS infusion and 1 min after BK, AA or vehicle. The plasma content of 6 keto-$PGF_{1\alpha}$ (as a measure of PGI_2 production) and total nitrite/nitrate (NO_2/NO_3) (as a measure of NO production) were then determined by, respectively, radioimmunoassay, and Griess reaction (after NO_3 reduction to NO_2).

LPS infusion for 6h caused marked elevations in the plasma levels of 6 keto-$PGF_{1\alpha}$ and NO_2 (table 1) associated with induction of COX 2 and iNOS (Hamilton *et al.*, 1997). Subsequent AA administration caused within 1 min a further increase in plasma 6 keto-$PGF_{1\alpha}$, to levels 150 times greater than those in non-LPS treated animals (Table 1). Similarly, BK administration increased 6 keto-$PGF_{1\alpha}$ levels by more than 100 fold. NS 398 completely blocked the accumulation of 6 keto-$PGF_{1\alpha}$ over the period of LPS infusion and the increases in 6 keto-$PGF_{1\alpha}$ following subsequent BK or AA challenge. Plasma NO_2/NO_3 was unaffected by AA, BK or NS 398 (Table 1).

In conclusion, increasing the availability of AA for COX 2, either by supplying exogenous AA or by stimulating its release from endogenous sources, reveals the COX 2 system to have the potential to produce very large amounts of prostanoids. Thus induction of COX 2 alone does not *per se* lead to greatly increased prostanoid formation; PLA_2 activation is also required. This contrasts with the activity of iNOS, which appears unaffected by further cell stimulation.

LCH is the recipient of a BHF Ph.D. Studentship (FS/97013). JAM is Wellcome Career Development Fellow and TDW holds a BHF Lectureship (BS/95003)

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	(A) Vehicle + Vehicle		(B) LPS + Vehicle		(C) LPS + NS 398	
Time point	$PGF_{1\alpha}$	NO_2/NO_3	$PGF_{1\alpha}$	NO_2/NO_3	$PGF_{1\alpha}$	NO_2/NO_3
6h (n=8)	0.22 ± 0.09	30.2 ± 3.1	6.5 ± 2.48 *	225 ± 59.0*	0.14 ± 0.07 †	323 ± 49.7
+ AA (n=5)	2.75 ± 1.02	34.5 ± 2.8	404 ± 135 *	213 ± 51.7*	6.76 ± 0.64 †	206 ± 43.6
+ BK (n=4)	0.64 ± 0.31	26.4 ± 6.3	76.4 ± 9.6 *	254 ± 25.0*	0.10 ± 0.06 †	321 ± 55.9

Table 1. * $p < 0.05$ B compared to A; † $p< 0.05$ C compared to B, by ANOVA.

23P COMPARISON OF THE VASORELAXATION CAUSED BY ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR (EDHF) AND ANANDAMIDE IN THE SMALL MESENTERIC ARTERY OF THE RAT

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Nitric oxide-independent vasodilation to endothelium-dependent agents like carbachol (CCh) is attributed to release of endothelium-derived hyperpolarizing factor (EDHF). Randall *et al.* (1996) suggested that anandamide, an endogenous cannabinoid, may be EDHF and this study compares vasorelaxation to EDHF and anandamide in the rat small mesenteric artery.

Segments (2 mm) of third generation mesenteric arteries from male Wistar rats (250-300g) were mounted in a myograph under a normalised tension in oxygenated Krebs buffer (Mulvany & Halpern, 1977). Vessels were precontracted with methoxamine (10 μM, a submaximal concentration) and >90% relaxation to 10 μM CCh was taken as showing a functional endothelium.

EDHF-induced relaxations were elicited by CCh (EC_{50} 0.65±0.01 μM; R_{max} 93.2±0.4%; n=10) in methoxamine- (5 μM) precontracted vessels in the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) and 10 μM indomethacin, and were sensitive to the cannabinoid receptor antagonist SR141716A [*N*-(piperidin-1-yl)- 5 -(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl]. Anandamide caused concentration-dependent relaxations of methoxamine- (10 μM) induced tone (EC_{50} 0.36±0.04 μM; R_{max} 90.6±4.3%; n=10) that were not affected by L-NAME. Precontraction of vessels with 60 mM KCl, or incubation with pertussis toxin (PTX; 400 ng ml^{-1} for 2 h) inhibited both CCh and anandamide relaxations (Table 1).

Glibenclamide (10 μM), an inhibitor of ATP-sensitive K^+ channels (K_{ATP}) and iberiotoxin (50 nM), a blocker of large conductance, Ca^{2+}-sensitive K^+ channels had no effect on either EDHF-mediated or anandamide-induced relaxation. 4-amino-pyridine (4-AP; at 1 mM, but not 50 μM), which blocks voltage-dependent K^+ channels (K_V) inhibited relaxation by CCh (EC_{50} 2.18±0.07 μM; n=5; P<0.01), but not anandamide.

Table 1. Effects of precontraction with 60 mM KCl or preincubation with PTX on relaxation to CCh.

	Relaxation of precontracted tone (%)		
	Control	KCl	PTX
CCh (1 μM)	67.8 ± 5.6	17.8 ± 7.1**	9.0 ± 3.2*
Anandamide (1 μM)	71.1 ± 7.0	5.2 ± 3.6**	17.6 ± 8.7**

Data are mean±s.e.mean; n=4; *P<0.05, **P<0.01 vs. control.

Apamin (1 μM), a blocker of small conductance, Ca^{2+}-sensitive K^+ channels, also inhibited CCh (EC_{50} 1.58±0.16 μM; n=7; P<0.05 and R_{max} 82.1±2.2%; n=7; P<0.01), but not anandamide relaxation. Charybdotoxin (CTX; 100 or 300 nM) had no significant effect on EDHF-mediated or anandamide-induced relaxation. Ciclazindol (10 μM), which blocks both K_{ATP} and K_V, inhibited EDHF-mediated relaxation (1.25±0.04 μM; n=4; P<0.01), but had no effect on that to anandamide.

Combinations of apamin (1 μM) with glibenclamide (10 μM), 4-AP (1 mM) or iberiotoxin (50 nM) did not have a significantly greater effect on EDHF-mediated relaxation than apamin alone. However, combinations of apamin with CTX (100 nM) or ciclazindol (10 μM) almost completely abolished EDHF-mediated relaxation. None of these combinations of inhibitors had any effect on anandamide-induced relaxation.

These results show that both EDHF, released by CCh in the presence of L-NAME, and anandamide cause relaxation by activation of K^+ channels via a PTX-sensitive mechanism. However, EDHF-mediated and anandamide-induced relaxations show differential sensitivity to K^+ channel blocking agents, and it is therefore concluded that anandamide is not identical with EDHF in the rat small mesenteric artery.

RW is an A.J. Clark student of the British Pharmacological Society.

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24P CHARACTERISATION OF THE RECEPTOR MEDIATING THE EXCITATORY ACTION OF ADENOSINE ON MESENTERIC AFFERENTS

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We have recently observed that adenosine stimulates jejunal afferent discharge in the anaesthetised rat (Kirkup *et al.*, 1997). Since this action of adenosine was insensitive to 1,3-dipropyl-8-cyclopentyl-xanthine it suggested that A_1-receptors were not involved. In the present study, we have used agonists selective for A_2- and A_3-receptors to characterise further the receptor(s) responsible for adenosine-mediated afferent stimulation.

Experiments were conducted using sodium pentobarbitone-anaesthetised (60mg/kg, i.p.) male Wistar rats (350-400g). A jugular vein and carotid artery were cannulated for systemic delivery of drugs and for continuous monitoring of haemodynamic parameters, respectively. Extracellular recordings were made from nerve bundles supplying a segment of mid-jejunum which was cannulated for measurement of intraluminal pressure. A cumulative intravenous (i.v.) dosing protocol was employed for each of the agonists. Data are presented as the mean±s.e.mean from 5-6 animals and the Students' paired *t*-test was used to test for significance.

The non-selective agonist, 5'-N-ethylcarboxamidoadenosine (NECA, 0.3-100μg/kg) induced dose-dependent increases in afferent discharge, an increase in intrajejunal pressure, hypotension and bradycardia (see Table 1 for effects of dose of 30μg/kg). The A_{2B}-selective agonist, N-[(2-methylphenyl)methyl] adenosine (metrifudil, 0.3-1000μg/kg; Gurden *et al.*, 1993) produced similar effects to NECA. However, its actions on afferent discharge and intraluminal pressure exhibited desensitisation at the maximum dose administered (see Table 1 for effects of dose of 300μg/kg). The A_{2A}-selective agonist, 2-[*p*-(carboxyethyl)phenylethylamine]-5-N-ethylcarboxamidoadenosine (CGS 21680, 0.3-300μg/kg), but not the A_3-selective agonist, N^6-(3-iodobenzyl)adenosine-5'-N-methyl-carboxamide (IB-MECA, 0.3-300μg/kg), evoked a significant increase in afferent discharge at the maximum dose administered (Table 1) but this was significantly less than the increase of 3473±977* impulses/30s elicited by subsequent challenge with adenosine (3mg/kg). Adenosine also evoked a significant increase in afferent discharge of 2979±805* impulses/30s after the maximum dose of IB-MECA had been administered. CGS21680 and IB-MECA each induced a dose-dependent hypotension but did not affect heart rate or intrajejunal pressure (Table 1). Nevertheless, administration of adenosine evoked significant decreases in heart rate of 296±21*** and 277±13*** beats/min and increases in intrajejunal pressure of 4.8±1.4* and 3.8±1.1* cmH_2O, post-CGS21680 and post-IB-MECA, respectively.

Table 1. Changes in mean arterial pressure (M.A.P.), heart rate (H.R.), intraluminal pressure (I.L.P.) and afferent discharge (A.D.) at dose of agonist (μg/kg) producing maximal effect on M.A.P.[1] or A.D.[2].

	ΔM.A.P. (mmHg)	ΔH.R. (beats/min)	ΔI.L.P. (cmH_2O)	ΔA.D. (imp/30s)
[1]NECA (30)	-80±5***	-233±16**	6.9±1.7*	4413±851*
[2]Metrifudil (300)	-57±10***	-59±8*	3.9±1.1*	1780±379*
[1]CGS21680 (300)	-63±6***	-3±7	0.5±0.3	424±129*
[1]IB-MECA (300)	-68±11*	-6±12	0.2±0.7	262±109

*P<0.05, **P<0.001, ***P<0.0001 *cf* basal values.

These data support our previous observation (Kirkup *et al.*, 1997), and suggest that adenosine-induced stimulation of afferent discharge is *via* an A_{2B} receptor in the anaesthetised rat.

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25P INTERACTIONS BETWEEN P2X RECEPTORS AND THE HYPERPOLARISATION-ACTIVATED CATIONIC CURRENT (I_H) IN SENSORY NEURONES OF THE RAT

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Sensory proprioceptive trigeminal mesencephalic nucleus (MNV) neurones of the rat express functional P2X receptors (Khakh *et al.*, 1997) as well as the hyperpolarisation-activated cation current (I_H) (Khakh & Henderson, 1997). Here we have investigated the interactions between P2X receptors and I_H.

The whole-cell patch clamp methods used have been described (Khakh *et al.*, 1997). Cells were allowed to dialyse with pipette solutions for at least 5 min before recordings were made. Data are shown as mean±s.e.mean from between 3-7 neurones;

Neurones of the MNV had resting membrane potentials of -55±1 mV and capacitance of 73±2 pF (n=131). Membrane hyperpolarising steps from -62 mV down to -132 mV activated slow inward currents with a half activation voltage (V_{50}) of -94±3 mV, slope value (k) of 8±2 mV and tail current (Imax) of -1261±293 pA.

Bath application of purine nucleotides evoked P2X receptor-mediated inward currents which were associated with a concomitant inhibition of I_H. ATPγS (10 μM), αβmeATP (300 μM) and ATP (300 μM) evoked inward currents of -496±51, -402±72, -369±105 pA which were associated with 41±9, 57±23 and 38±12 % inhibition of I_H tail current amplitude, respectively. Adenosine (100 μM), UTP (100 μM) and ADPβS (100 μM) were ineffective at evoking inward currents and inhibiting I_H. The P2X current evoked by ATPγS (10 μM) and the concomitant inhibition of I_H were completely blocked by suramin (100 μM), but were unaffected by the adenosine receptor antagonist DPCPX (10 μM).

ATPγS (10 μM) altered the voltage range over which I_H was activated such that the V_{50} changed from -97±3 mV to -109±2 mV but k and Imax were unchanged being 10±2 mV and -922±84 pA before and 8±3 mV and -833±68 pA in the presence of ATPγS, in these cells the P2X-evoked inward current was -567±35 pA. Including the protein phosphatase inhibitor okadaic acid (2 μM) or solutions with no added ATP/GTP in the pipette solution or applying the protein kinase inhibitor H89 (10 μM) in the bathing medium did not reduce the I_H inhibition by ATPγS (10 μM); the shifts in V_{50} produced by ATPγS were -8±2, -18±5 and -22±9 mV in the presence of okadaic acid, no ATP/GTP or H89, respectively.

When cells were dialysed with BAPTA (11 mM), ATPγS (10 μM) did not inhibit I_H (shift in V_{50} was -7±4 mV from -99±5 mV to -105±1 mV; $p>0.05$) although ATPγS still evoked a P2X-mediated inward current of -528±136 pA. Intracellular dialysis with BAPTA (11 mM) also decreased the maximal inhibition of I_H produced by ATPγS (30 μM) to 34±6 % without affecting the P2X-evoked inward current (-708±78 pA). Whereas in control cells with no intracellular BAPTA, ATPγS (30 μM) caused a larger inhibition of I_H of 72±13 % and the inward current was not changed being -694±119 pA. Furthermore, there was no direct correlation between I_H inhibition and the conductance change evoked by ATPγS (30 μM) (linear correlation coefficient r=0.1), suggesting that I_H inhibition was not secondary to a change in membrane conductance *per se*.

Thus Ca^{2+} entry through P2X receptors inhibits I_H in MNV neurones. This interaction may be important at sensory nerve terminals where P2X receptors and I_H are implicated in sensory transduction.

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26P THE EFFECT OF IBUPROFEN, MORPHINE AND AMITRIPTYLINE ON CARRAGEENAN-INDUCED CUTANEOUS HYPERSENSITIVITY IN THE ANAESTHETISED RAT

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Utilising extracellular recording techniques we have examined the effect of 3 analgesics: morphine, ibuprofen and amitriptyline, on baseline nociception and carrageenan-induced sensitisation of dorsal horn neurones (DHN) to peripheral electrical stimulation.

Male Wistar rats (250-350g) were anaesthetised with sodium pentobarbitone (60mgkg^{-1} ip; 4mgkg^{-1}h^{-1} iv) and prepared for single unit recording in the spinal cord (L4-5). DHN exhibiting wind-up to trials of 16 current pulses (0.5Hz, 3x c fibre threshold) were stimulated electrically (via A & c fibres, 0.1Hz) before and after intraplantar carrageenan (2%; 0.1 ml). All compounds tested were administered intravenously 15 minutes before carrageenan. Results (sum of action potentials per trial) were expressed (%control, mean±s.e.mean). Repeated Measures ANOVA and Students paired and unpaired t-tests were used to determine statistical significance, $p<0.05$.

The DHN responses (Table 1), compared to time matched controls, to electrical stimulation of A and c fibres were significantly enhanced at 2 and 4h following carrageenan (Repeated Measures ANOVA $F1,14 = 5.38$ & 7.23, Students unpaired t-test, $p<0.05$). Ibuprofen (30mgkg^{-1} iv, n=5) and morphine (1mgkg^{-1} iv, n=4), doses not significantly reducing baseline responses, significantly reduced DHN responses at 2 and 4h to both A and c fibre stimulation while amitriptyline (1mgkg^{-1} iv, n=5), a dose not significantly reducing baseline reponses, significantly reduced DHN responses to A fibre stimulation only.

In summary, these 3 analgesic drugs, at these doses, reversed the carrageenan-induced hypersensitivity though amitriptyline had only limited effect, reducing the sensitised A fibre evoked DHN responses only.

Table 1. Effects of drugs on baseline nociception and the carrageenan-induced cutaneous hypersensitivity

Drug	Baseline (%control)		+ Carrageenan (%control)			
	A fibres	c fibres	A fibres (2h)	A fibres (4h)	c fibres (2h)	c fibres (4h)
			132.2±25.5	173.3±40.7	141.9±16.2	162.6±26.9
Ibuprofen	102.8±22.1	91.0±10.1	90.0±15.3#	75.9±7.0#	62.5±15#	49.7±9.0#
Morphine	94.8±25.4	98.7±25.4	85.4±12.4#	101.6±16.6#	95.6±20.5#	94.5±26.0#
Amitriptyline	87.2±13.9	93.5±25.1	93.1±22.0	90.2±13.4#	115.1±33.5	118.7±31.9

Students t-test, paired*/unpaired#, $p<0.05$.

27P EFFECT OF THE NEUROKININ 1 (NK_1) RECEPTOR ANTAGONIST GR205171 ON ADJUVANT-INDUCED INFLAMMATORY PAIN IN THE RAT

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Afferents present within joint nerves in both animals and man, contain pro-inflammatory peptides including substance P which can increase during inflammation and arthritis (Scott et al., 1994.). NK_1 antagonists may therefore be useful in the treatment of chronic inflammatory disease such as arthritis. We have investigated the effect of the selective NK_1 antagonist GR205171 (rat pKi 9.5, human pKi 10.5, Gardner et al 1996), and its low affinity enantiomer GR226206 (human pKi 6.8 [2-2-methoxy-5-(5-trifluoromethyl-tetrazol-1-yl)-benzyl]-(2S-phenyl-piperidin-3R-yl)-amine dihydrochloride) in adjuvant induced inflammatory pain in the rat. Freunds complete adjuvant was injected intraplantar (100µl into the left paw of male Random Hooded rats (180-220g) to induce the arthritis. GR205171, or GR226206, 10mg/kg s.c. or vehicle (mannitol), were administered 1 hr before adjuvant and then three times a day for 17 days. GR205171 was also dosed alone in non adjuvant treated rats, 3 times a day for 17 days. The effect of the compounds or vehicle on adjuvant induced oedema was assessed using a plethysmometer, and the pain was assessed by measuring both paw withdrawal threshold (Randall & Selitto, 1957) and weight bearing on the inflamed hind paw (dual channel weight averager. Clayton et al., 1997). GR205171 significantly inhibited the decrease in mechanical paw withdrawal threshold and weight bearing ($p<0.05$; unpaired student t test.) and had a small but significant effect at some of the time points, on the adjuvant induced oedema. GR226206 had no significant effect on either pain readout, and appeared to potentiate the adjuvant induced increase in paw volume (data not shown). GR205171 alone had no significant effect on the paw withdrawal threshold, weight bearing or paw volume (data not shown). In conclusion, GR205171 exhibits analgesic and some anti-inflammatory activity in a model of chronic inflammatory pain. The less active enantiomer GR226206 showed no such activity. GR205171 may therefore be useful in the treatment of chronic inflammatory pain.

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Table 1. Effect GR205171 on adjuvant induced decrease in paw withdrawal threshold, weight bearing and increase in paw volume. (* $p<0.05$; unpaired t test).

	Data presented as % inhibition of adjuvant induced decrease in paw withdrawal threshold, weight bearing and the increase in paw volume (Mean±sem), n=10.		
Day	GR205171 10mg/kg s.c		
	Paw withdrawal	weight bearing	paw volume
1	0±13.3	30.4±25.3	26.3±7.5*
9	89.8±19.6*	110.0±6.2*	41.9±6.2*
17	71.4±3.4*	94.7±19.9*	15.5±9.1

28P THE EFFECT OF NOCICEPTIN, NOCICEPTIN ANALOGUES AND A NOVEL HEXAPEPTIDE AGONIST AT THE ORL_1 RECEPTOR IN CENTRAL AND PERIPHERAL SITES

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Nociceptin is the endogenous ligand for the ORL_1 receptor. Transcripts for the ORL_1 receptor have been shown to exist in the central nervous system and in some peripheral areas including the vas deferens.

We have shown that nociceptin is a potent and efficacious agonist in the vas deferens of the rat (RVD) and rabbit (Nicholson *et al.*, 1996) and have characterised the binding of [^{3}H]-nociceptin at the ORL_1 receptor site in guinea-pig, rabbit and rat brain membranes (Paterson *et al.*, 1997). We have now tested some analogues of nociceptin in the vas deferens and in radioligand binding studies and have begun behavioural studies with these compounds following intracerebroventricular (icv) administration in the rat.

Hooded Lister rats (~300g) were implanted with cannulae into the right lateral ventricle under isofluorane anaesthesia. The coordinates for cannula implantation (mm), measured from Bregma, were: 1.5 lateral, 0.8 caudal and 3.2 ventral from the surface of the scull. Animals were housed individually and allowed 1 week to recover before use. Icv injections were in a volume of 2 µl, administered over 30 seconds. Animals were placed in a novel environment, and scored for horizontal locomotion and rearing behaviour.

Icv administration of nociceptin (5 nmoles, $n\geq6$) produced a decrease in horizontal locomotor activity and rearing when compared to vehicle controls ($p < 0.05$). Administration of this dose of nociceptin was associated with an impairment of motor function that was confined to the hind limbs. Nociceptin(2-17) (5 nmoles, n=9) did not alter horizontal locomotion and rearing behaviours and the nociceptin-induced motor impairement was not evident. This lack of effect is consistent with data obtained from the rat vas deferens where nociceptin(2-17) was found to be inactive as an agonist at concentrations up to 3 µM (n=3) and in radioligand binding studies with [^{3}H-]nociceptin where it was inactive up to 10 µM (n=3).

Screening of hexapeptides from combinatorial libraries led to the identification of the potent agonist action of Ac.RYYRWK.NH_2 at ORL_1 (Dooley *et al.*, 1996). In the RVD this compound was more potent than nociceptin itself (EC_{50} values 0.80nM, range 0.75-0.89, and 7.88nM, 6.99-9.24, n=6), although not as efficacious (E_{max} 0.71±0.02 and 0.94±0.09). In radioligand binding studies the novel hexapeptide had as high an affinity for the ORL_1 receptor as nociceptin (pK_i 9.94±0.21, n=5 and 9.96±0.16, n=8 respectively). However, when this novel hexapeptide was administered icv to rats, (5 nmoles, n=6) it did not alter the locomotor or rearing behaviour of the animals when compared to vehicle-treated controls.

Thus, we have shown nociceptin is a high affinity ligand of the ORL_1 receptor in the brain and vas deferens, and icv administration of nociceptin decreases locomotor activity through a mechanism that remains to be elucidated. Although radioligand binding studies have shown the novel hexapeptide displaying high affinity for ORL_1 and we have found it to be a potent agonist in the RVD, we did not observe a nociceptin-like reduction in locomotor activity following icv administration in rats until in recent experiments, the peptide was coadministered with a cocktail of peptidase inhibitors.

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29P ANTAGONIST POTENCY OF THE NK_1 RECEPTOR ANTAGONIST CGP49823 AT SPINAL MOTONEURONES

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The novel tachykinin NK_1 receptor antagonist CGP49823 (2R,4S)-2-benzyl-1-(3,5-dimethylbenzoyl)-N-[(4-Quinolinyl)methyl]-4-piperidineamine (CGP49823) (Vassout et al 1994; Hauser et al 1994) has been compared in the present experiments with three other selective tachykinin non-peptide NK_1 antagonists. The drugs were tested as antagonists of the depolarization of spinal motoneurones induced by bath application of the selective NK_1 agonist septide (0.3 μM) for 120 seconds at 15 minute intervals. The latter depolarizations were near maximal. The composition of the bathing medium was (mM): NaCl 120; KCl 2.5; KH_2PO_4 1.25; $CaCl_2$ 2.5; $MgSO_4$ 2; $NaHCO_3$ 30, and glucose 10. It was gassed with 95% O_2, 5% CO_2 and maintained at 28 °C. The antagonists were bath applied and the depolarizations were recorded from lumbar ventral roots of 7 to 12 day old rat and gerbil hemisected spinal cords in vitro. The gerbil preparation is considered to model the human species variant of the NK_1 receptor. Spinal cords were removed from animals of either sex following decapitation under deep urethane anaesthesia.

Two hours was required to reach steady state depression of septide-induced depolarizations with each antagonist. IC_{50} values for this depression were estimated following cumulative increase in antagonist concentration. With the exception of SR140333 the antagonists were approximately thirtyfold more potent on gerbil preparations. The respective mean IC_{50} values from gerbil preparations produced by CP96345, CGP49823, SR140333 and CP99994 were μM ± s.e. (n) 0.10 ± 0.02 (6), 0.22 ±0.03 (6), 0.30 ±0.10 (5) and 0.38 ±0.02 (5) and the corresponding values from the rat preparations were 3.7 ±0.4 (5), 7.8 ±1.3 (5), 1.06 ±0.16 (6) and 10.5 ±2.2 (7). Dominance of NK1 receptor activity in the measured responses was confirmed by low potency of the NK_2-selective antagonist SR48968 which yielded an IC_{50} value of 12.0 ±2.8 (5) on gerbil preparations and produced less than 50% depression of septide-induced depolarization of rat motoneurones at the highest concentration (100 μM) tested.

The present study confirms that NK_1 receptors of gerbil motoneurones are more sensitive to CP96345 than those of the rat and that CGP49823 has a profile of NK_1 receptor antagonism similar to that of CP96345 and CP99994.

It may be significant that the latter three antagonists, unlike the NK_2 antagonist, possess a secondary amino group. CP9994 has the simplest chemical structure amongst the present antagonists (C_{19} compared to at least C_{28}). It is surprising that introduction of an additional bulky quinolyl moiety as in CGP49823 produced no significant change in potency. The binding site must have a high degree of steric tolerance.

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30P THE EFFECT OF DEOXYCHOLIC ACID ON DORSAL HORN RESPONSES TO COLORECTAL DISTENSION AND CUTANEOUS MECHANICAL STIMULATION, AND THE EFFECT OF MK-801, IN THE ANAESTHETISED RAT

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Most dorsal horn neurones responsive to colorectal distension (CRD) have convergent somatic receptive fields (Ness and Gebhart, 1987). The bile salt, deoxycholic acid (DCA), potentiates the hypotensive response to CRD (Procter et al.,1995). We have investigated the ability of rectal DCA to potentiate dorsal horn neurone responses to both visceral and cutaneous mechanical stimuli, and the possible role of NMDA receptors. Male Wistar rats (250-320g) were anaesthetised with sodium pentobarbitone (60mgkg^{-1} ip; 3-5mgkg^{-1}h^{-1} iv) and prepared for single unit extracellular recording from the lumbosacral spinal cord (L5-S1). A colorectal balloon was inflated to 80mmHg for 30s, at 5min intervals, to locate neurones responsive to CRD. Von Frey Hairs (VFH) were used to map convergent cutaneous receptive fields. Responses to 60mmHg CRD and submaximal VFH were recorded before and for 90 min following DCA/saline treatment (60mM, 1.7ml, ir). Data are given as mean ± s.e.mean. An unpaired Student's t-test was used to assess statistical significance ($P<0.05$ significant).

Eighteen neurones (0.1-1.2mm from cord dorsum) responded to CRD; 17/18 neurones showing a graded response to increasing pressure (20-100mmHg, 30s). One neurone responded maximally at 10mmHg. Cutaneous receptive fields (CRFs) identified in 16/18 neurones were located on the lower back, scrotum and tail, with VFH thresholds between 0.45-12.5g. In 15 neurones, a VFH stimulus-response curve was constructed to identify a submaximal stimulus pressure. DCA treatment caused a biphasic increase in responses to both visceral and cutaneous stimuli compared with time-matched controls (saline ir). At 40 and 90 min post DCA, responses were significantly increased to CRD (174±33% vs 67±22% and 202±50% vs 67±24% respectively [$P<0.05$; n=5-7]) and VFH (179±25% vs 118±18 and 211±25% vs 75±15% respectively [$P<0.05$; n=4-7]). Responses at 55 min were not changed. Pretreatment with MK-801 (0.3mgkg^{-1}iv) blocked the sensitisation to CRD (81±16 and 105±12%; n=5) and VFH (112±26% and 121±43%; n=4) at 40 and 90 min respectively. MK-801 had no effect on baseline responses. Following DCA treatment, CRF size was increased in 6/8 neurones, and VFH threshold reduced in 6/7 neurones. In MK-801 pretreated animals, CRF size was still increased, and VFH threshold reduced in 2/3 neurones tested. In conclusion, rectal DCA administration induces a central sensitisation, illustrated by the potentiation of both visceral and cutaneous responses. NMDA receptors are involved in the sensitisation process, but do not appear to mediate changes in receptive field properties in this study.

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31P ELECTROPHYSIOLOGICAL STUDIES ON THE EFFECTS OF LAMOTRIGINE ON RAT DORSAL ROOT GANGLION NEURONES *IN VITRO*

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The anticonvulsant drug lamotrigine (Lamictal®, LTG), has recently been shown to exhibit analgesic effects both in animal models and in man (Nakamura-Craig & Follenfant, 1995; Eisenberg *et al.*, 1996). However, the precise cellular and molecular mechanisms that underlie these actions are unclear. In the present study we have used single cell electrophysiological techniques to evaluate the effect of LTG on primary afferent neurones of the rat.

Dorsal root ganglia from 3-4 week old rats (AHA) of either sex were enzymatically dissociated (0.125% collagenase, 60min followed by 0.025% trypsin, 15min). Neurones were plated on poly-DL-ornithine and laminin treated glass coverslips for whole cell patch clamp recording using an Axopatch 200B amplifier. Under current clamp in standard physiological salt solution (PSS), small diameter neurones (10-25μm) had input resistances of between 80 and 300MΩ and resting membrane potentials of between -47 and -62mV (n=26). Trains of action potentials were evoked by supramaximal current injection (range 0.15-0.60nA) for 800ms. The latency and amplitude of the first spike were 8±1ms and 104±2mV, respectively, and the mean number of spikes per train was 11.6±1.6 (n=7). In the presence of LTG (100μM) there was no significant change in the latency to the first spike (10±2ms; NS paired Students *t*-test) and only a modest reduction in the spike amplitude (89±4mV, $P<0.05$). In contrast, LTG substantially inhibited subsequent spikes in a train and dramatically enhanced action potential accommodation. The number of spikes per train was significantly reduced to 9.0±2.4 (n=5) and 4.3±1.3 (n=6) at concentrations of 30μM and 100μM LTG, respectively.

Under voltage clamp and conditions designed to isolate slow tetrodotoxin-resistant Na^+ currents (TTX_R; Elliott & Elliott, 1993), 40ms depolarising steps from a holding potential (V_h) of -90mV evoked graded inward currents at potentials positive to -40mV and peak current occurred at around 0mV. LTG produced only a weak inhibition of the peak TTX_R under these conditions (IC_{50} value 536μM). However, when cells were held at a V_h of -60mV, LTG was significantly more potent (IC_{50} value 229μM), indicating a voltage-dependent action (data from 5-11 cells). Under these conditions 10μM and 100μM LTG reduced the whole cell current by 9±3% (n=4) and 34±6% (n=5), respectively. Using trains of test pulses to 0mV from a V_h of -90mV at a frequency of 5Hz, the ratio of the 10th to 1st pulse under control conditions was 0.76±0.05 for pulses of 20ms duration and 0.28±0.02 for 100ms pulses. In the presence of LTG (100μM) these ratios were significantly reduced to 0.72±0.05 and 0.19±0.05, respectively ($P<0.05$, paired *t*-test), indicating a use-dependent effect of the drug. The steady state inactivation curve for TTX_R, as determined using 4s conditioning pulses, was shifted in a hyperpolarising direction by LTG (100μM) from a $V_{1/2}$ value of -32.6±1.2mV to -37.5±0.8mV (n=6; $P<0.05$).

In summary, LTG reduces the excitability of rat sensory neurones by preferentially inhibiting the later spikes in a train of action potentials. The voltage- and use-dependent block of TTX-resistant Na^+ channels observed under voltage-clamp may underlie this phenomenon. Further studies are in progress to determine the effects of LTG on TTX-sensitive Na^+ currents in these neurones (see Elliott & Elliott, 1993 and Xie *et al.*, 1995). Attenuation of high frequency firing of nociceptors by LTG may contribute to the analgesic effects of this compound observed *in vivo*.

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32P KINETICS OF MEMBRANE BOUND, SOLUBILIZED MEMBRANE BOUND AND SOLUBLE FORMS OF RAT LIVER CATECHOL-*O*-METHYLTRANSFERASE

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The soluble (S) and membrane bound (MB) forms of catechol-O-methyltransferase (COMT) have many properties in common, but differ in their affinity for the substrate by a factor of 100. MB-COMT contains the whole primary structure of S-COMT with an extension of 43 (rat) and 50 (human) amino acid residues at its terminus, carrying the hydrophobic anchor domain (Ulmanen & Lundström, 1991). The present work was aimed at study the effect of solubilization of rat liver MB-COMT on its affinity for the substrate. Saline perfused tissues (liver), obtained from pentobarbitone (60 mg kg^{-1}) anaesthetized 60 days old male Wistar rats, were used in the experiments. Tissues were homogenised in 5 mM phosphate buffer, pH 7.8, and the soluble and membrane bound fractions of COMT obtained by the method described by Nissinen *et al.* (1988). COMT activity was evaluated by the ability to methylate adrenaline (0.1 to 2000 μM) to metanephrine, as previously described (Vieira-Coelho & Soares-da-Silva, 1996). K_m and V_{max} values (n=4) for COMT activity were calculated from non-linear regression analysis. Arithmetic means are given with s.e.mean; statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. Rat liver S- and MB-COMT were treated with Triton X-100 (1.0 %) and their kinetic parameters determined for the *O*-methylation of adrenaline. Triton X-100 was found to affect neither the affinity of S-COMT for the substrate (K_m = 257±55 vs 295±47 μM) nor the activity of the enzyme (V_{max} = 139±11 vs 119±7 nmol mg $protein^{-1}$ h^{-1}). The solubilized MB-COMT as compared to the native protein was found to be endowed with a lower affinity for the substrate , as evidenced by a 3.7 times higher K_m (9.3±1.3 vs 2.5±0.7 μM; $P<0.05$); an increase in V_{max} was also observed (64±3 vs 40±3 nmol mg $protein^{-1}$ h^{-1}). Using tight-binding inhibitors, it is possible to estimate the enzyme concentration allowing the evaluation of the efficiency of the enzyme in the *O*-methylation reaction, by determining the catalytic number (k_{cat}). This was obtained by plotting the enzyme concentration against initial velocities, using tolcapone (Ro 40-7592) as the titrating inhibitor (Lotta et al., 1995). Again, no differences were observed for the S-COMT in relation to the Triton X-100-treated enzyme (k_{cat} = 5.3±0.6 vs 5.8±0.2 min^{-1}) . In opposition, the solubilized MB-COMT was more efficient in methylating the substrate (k_{cat} = 5.3±0.4 min^{-1}) than the native MB-COMT (k_{cat} = 3.1±0.2 min^{-1}); this is agreement with the increase in V_{max} values observed for the solubilized MB-COMT. This data strongly suggests that the solubilized MB-COMT does not possess the kinetic characteristics of S-COMT.

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33P SENSITIVITY TO INHIBITION BY TOLCAPONE OF SOLUBLE AND MEMBRANE- BOUND FORMS OF CATECHOL-*O*-METHYLTRANSFERASE FROM RAT BRAIN, LIVER AND KIDNEY

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In the periphery, the soluble form of catechol-O-methyltransferase (S-COMT) is generally assumed as the predominant form of the enzyme, but in the CNS there is evidence to suggest that the membrane-bound COMT (MB-COMT) may be the most abundant form of the enzyme. Although there is evidence that nitrocatechol COMT inhibitors are endowed with similar inhibitory potencies against both human recombinant S- and MB-COMT expressed in baculovirus-infected insect cells (Lotta et al., 1995), there is no information, to our knowledge, on the potency of these compounds in inhibiting brain and peripheral COMT. The present study was aimed to evaluate the potency of tolcapone (Ro 40-7592) in inhibiting both S- and MB-COMT in brain and peripheral tissues of the rat. Saline perfused tissues (brain, liver and kidney), obtained from pentobarbitone (60 mg/kg) anaesthetised 60 days old male Wistar rats, were used in the experiments. Tissues were homogenised in 5 mM phosphate buffer, pH 7.8, and the soluble and membrane bound fractions of COMT obtained by the method described by Nissinen *et al.* (1988). COMT activity was evaluated by the ability to methylate adrenaline (0.1 to 2000 μM) to metanephrine, as previously described (Vieira-Coelho & Soares-da-Silva, 1996). K_m and V_{max} values for COMT activity were calculated from non-linear regression analysis. The constant $k_{enzyme} = V_{max} / K_m$, indicates the higher the V_{max} and the lower K_m, the higher the activity of the enzyme. For the calculation of the IC_{50}'s the parameters of the equation for one site inhibition were fitted to the experimental data. Geometric means are given with 95% confidence limits and arithmetic means are given with s.e.mean; statistical analysis was performed by one-way analysis of variance (ANOVA) using Newman-Keuls multiple comparison test. The kinetics of COMT are indicated in the table.

Kinetic parameters (K_m in μM; V_{max} in nmol mg protein^{-1} h^{-1}) of liver, kidney and brain S- and MB-COMT activity and IC_{50} values (in nM) for inhibition by tolcapone (n=4-5).

	K_m	V_{max}	k_{enzyme}	IC_{50}
Liver				
S-COMT	345±78	58±4	0.17	601 (214, 1684)
MB-COMT	3.0±0.6	24±1	8.0	21 (1, 39)
Kidney				
S-COMT	300±44	44±1	0.15	230 (77, 689)
MB-COMT	2.6±0.3	10±1	4	18 (7, 46)
Brain				
S-COMT	168±37	1.4±0.1	0.008	6 (3, 12)
MB-COMT	0.9±0.1	4.8±0.1	0.39	2 (1, 4)

The data obtained suggests that brain MB-COMT has a high affinity for the substrate and is also more sensitive to tolcapone, than MB-COMT from liver and kidney. The sensitivity of S-COMT from both liver and kidney to tolcapone was lower than that for the corresponding MB-COMT, whereas the sensitivity of brain S-COMT to tolcapone did not differ significantly from that for MB-COMT. In conclusion, both brain S- and MB-COMT differed considerably from its corresponding congeners in the periphery.

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Lotta, T., et al. (1995) Biochem., 34, 4202-4210.
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34P UPTAKE AND INTRACELLULAR FATE OF L-3,4-DIHYDROXYPHENYLALANINE, THE DOPAMINE PRECURSOR, IN PORCINE RENAL LLC-PK$_1$ CELLS

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LLC-PK$_1$ cells are endowed with considerable aromatic L-amino acid decarboxylase (AADC) activity (Soares-da-Silva et al., 1996) and this constitutes an important factor for their use as an *in vitro* model of the renal dopaminergic system. The present work was aimed at studying the uptake of L-3,4-dihydroxyphenylalanine (L-DOPA) and its intracellular decarboxylation to dopamine. LLC-PK$_1$ cells (ATCC CRL 1392; passages 198-206) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Medium 199 supplemented with 3% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, LLC-PK$_1$ cells were preincubated (30 min) with Hanks medium with added pargyline (100 μM) and tolcapone (1 μM). L-DOPA and dopamine were assayed by h.p.l.c. with electrochemical detection. Results are arithmetic means with s.e.mean, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Student's t test. The accumulation of L-DOPA from the apical side in cells cultured in collagen-treated plastic was found to be a saturable process with a K_m of 123±17 μM and a V_{max} of 6.0±0.2 nmol/mg protein/6 min. The uptake of L-DOPA applied from either the apical or basal cell borders in cells cultured in polycarbonate filters was also found to be saturable; non-linear analysis of saturation curves for apical and basal application revealed K_m (in μM) values of 63.8±17.0 and 42.5±9.6 and V_{max} values (in nmol/mg protein/6 min) of 32.0±5.8 and 26.2±3.4, respectively. Cell monolayers incubated with L-DOPA, applied from either the apical or the basal side, in the absence of benserazide, led to the accumulation of newly-formed dopamine. The intracellular accumulation of newly-formed dopamine was a saturable process with an apparent K_m value of 20.5±8.2 and 247.3±76.8 μM, when the substrate was applied from the apical and basal side, respectively. Some of the newly-formed dopamine escaped to the extracellular milieu. The basal outward transfer of dopamine did not depend on the intracellular levels of the amine, whereas the apical outward transfer of the amine depended on the intracellular concentration of dopamine and was a non-saturable process. The apical and basal outward transfer of dopamine were insensitive to cocaine (10 and 30 μM) and GBR 12909 (1 and 3 μM). The accumulation of exogenous dopamine in LLC-PK$_1$ cells was found to be saturable; non-linear analysis of the saturation curves revealed for the apical and basal application of dopamine a K_m of 17.7±4.3 μM and 169.1±55.2 μM a V_{max} of 2.0±0.1 and 2.7±0.5 nmol/mg protein/15 min, respectively. However, both cocaine (10, 30 or 100 μM) and GBR 12909 (1 or 3 μM) were found not to affect the uptake of 100 μM dopamine applied from either the apical or the basal cell border. In conclusion, the data presented here show that LLC-PK$_1$ are endowed with considerable AADC activity and transport quite efficiently L-DOPA through both the apical and basal cell borders. On the other hand, our observations support the possibility of a basal-to-apical gradient of AADC activity and that LLC-PK$_1$ cells might constitute an interesting *in vitro* model for the study of the renal dopaminergic physiology.

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35P TRANSFECTION OF THE NORADRENALINE TRANSPORTER INTO THE CATH.a CELL LINE

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The cell bodies of ascending noradrenergic neurones in the brain are located predominantly in the locus coeruleus. The CATH.a cell line has been termed a locus coeruleus-like cell line on account of its derivation from a brainstem tumour of a transgenic mouse in which the transgene was targeted towards tyrosine hydroxylase containing cells (Suri *et al.*, 1993). An *in vitro* model of locus coeruleus neurones could prove to be a useful tool in the investigation of noradrenergic networks and their associated pathophysiologies. Work to date has indicated that the CATH.a cell line demonstrates some of the properties expected of a locus coeruleus neurone (Bundey *et al.*, 1997a). However, a recent report has shown that it fails to take up [^{3}H]noradrenaline ([^{3}H]NA) (Bundey *et al.*, 1997b). This communication presents data demonstrating that transfection of the human noradrenaline transporter gene into the CATH.a cells confers the ability to take up [^{3}H]NA.

CATH.a cells (passage 20-30) were incubated in Hank's HEPES buffer containing pargyline (200μM), ascorbic acid (200μM) and [^{3}H]NA 40nM for 1 hour at 37°C. Unaccumulated [^{3}H]NA was then removed by two washes with buffer. The [^{3}H]NA taken up by the cells was extracted with perchloric acid (200mM). The reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a Titan RT-PCR system kit, employing 3 sets of 17-25bp primers. Transfection of the CATH.a with the noradrenaline transporter, ligated into a pWE.2 vector, was by electroporation (150V, 950μF). The transfects were seeded at a concentration of 1cell/well in 96 well plates and grown in RPMI 1640 containing 10% FCS and 250μg/ml geneticin. The successful monoclonal transfects are termed RUNTs.

[^{3}H]NA taken up by the untransfected CATH.a cells was 19.2±0.3 and 17.2±1.2 fmols/mg protein for cells incubated without and with the NA transport inhibitor, cocaine (10μM), respectively (n=3). RT-PCR supported these functional studies by demonstrating a lack of noradrenaline transporter messenger RNA in the CATH.a. [^{3}H]NA taken up by the RUNT.2 transfect (40.8±4.8 fmols/mg protein) was significantly different from the native CATH.a cell line (Students t test, $P<0.05$, n=3). CATH.a cells incubated with dibutyryl cyclic AMP (1mM) for 24 hours displayed no significant difference in [^{3}H]NA uptake from control. However, RUNT.2 transfects differentiated with dibutyryl cyclic AMP demonstrated a significant increase in [^{3}H]NA uptake (630±114 fmols/mg protein, n=3). This increase was inhibited by cocaine (10μM).

In conflict with the contention that the cell line may be an *in vitro* model of noradrenergic neurones the present data confirm that the CATH.a cell line does not take up [^{3}H]NA. Transfection of the cell line with the noradrenaline transporter was able to confer noradrenaline uptake and this was enhanced when the cells were differentiated with dbcAMP supporting the evidence that a cAMP response element is involved in noradrenaline transporter expression. These results suggest that the use of the CATH.a cell line as a model noradrenergic neurone should be done with caution and that the transfected cell line maybe a more useful tool for the study of noradrenergic transmission *in vitro*.

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36P EFFECT OF DEXMEDETOMIDINE ON NMDA RECEPTOR-MEDIATED SYNAPTIC TRANSMISSION IN THE NEONATAL RAT HEMISECTED SPINAL CORD PREPARATION *IN VITRO*

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Clonidine and other alpha-2 adrenoceptor agonists depress excitatory spinal transmission consistent with their central analgesic actions (Kendig *et al.*, 1991). Several of these agonists, including clonidine, are known to interact with nonadrenergic binding sites (NABS) and it has been suggested that NABS may be involved in their spinal depressant action. Dexmedetomidine, the (+) isomer of medetomidine, is a selective alpha-2 adrenoceptor agonist which also has an action at nonadrenergic binding sites (Sjoholm *et al.*, 1995). The purpose of the present experiments was to compare the relative potencies of dexmedetomidine and clonidine in the present preparation with those from *in vivo* analgesic tests and with IC_{50} values of binding at NABS.

Synaptic responses were recorded from the L4 or L5 lumbar ventral root of hemisected spinal cords from 3 to 6 day old Wistar rats (unsexed, approximately 10g) in response to electrical stimulation of the corresponding dorsal root as previously described (Otsuka & Konishi, 1974). The bathing solution consisted of (mM) NaCl 118, $NaHCO_3$ 24, KCl 3, $CaCl_2$ 1.5, $MgCl_2$ 1.25 and glucose 12 maintained at 25°C and gassed with 95%/5% O_2/CO_2. The dorsal root was stimulated at both low intensity, sufficient for activation of only A fibres to evoke the low intensity EPSP and at supramaximal intensity such that all dorsal root fibres were activated to evoke the high intensity EPSP. The NMDA receptor-mediated components of these reflexes were measured as previously described by Faber *et al.* (1997).

Cumulative increases in concentration of dexmedetomidine depressed both types of reflex with mean EC_{50} values of 2.7 ±0.6nM (n=4) for the low intensity EPSP and 0.84 ±0.1nM (n=4) for the high intensity EPSP.

Comparison of these values with our previous data (Faber *et al.*, 1997) yields a potency ratio showing dexmedetomidine to be 11 times greater than clonidine in depressing the low intensity EPSP and 47 times greater in depressing the high intensity EPSP. This correlates well with the relative potency of the analgesic actions of these two drugs *in vivo* (Sullivan *et al.*, 1992). The relative affinities of clonidine: dexmedetomidine binding at NABS is 100,000 (Sjoholm *et al.*, 1995). Thus the poor correspondence between the relative affinities for NABS and the synaptic depressant activity by dexmedetomidine and clonidine suggests that NABS are very unlikely to be involved in the spinal depressant activity of this class of drugs.

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37P CHARACTERISATION OF NOVEL PHENYLGLYCINES FOR SUBGROUP I METABOTROPIC GLUTAMATE RECEPTORS ON THE NEONATAL RAT SPINAL CORD PREPARATION

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Metabotropic glutamate receptors (mGluRs) have been the focus of intense investigation in recent years. Molecular cloning studies have revealed the presence of eight distinct subtypes, mGluRs 1-8 (Pin and Duvoisin, 1995). These can be classified into three groups according to their second messenger mechanism and agonist selectivities. Subgroup 1 (mGluRs 1 and 5) includes those receptors which are positively linked to phosphoinositide hydrolysis. Subgroups 2 and 3 comprising mGluRs 2,3 and mGluRs 4,6,7 and 8 respectively are all negatively coupled to adenylyl cyclase. (Pin and Duvoisin, 1995). Our understanding of the physiological role of mGluRs has been hindered due to a lack of selective agonists and antagonists. A series of novel phenylglycine derivatives have been developed as new agonists and antagonists and have recently been investigated in Chinese hamster ovary (CHO) cell lines expressing mGluR1, mGluR2 and mGluR4 subtypes (Sekiyama *et al*, 1996).

The present study investigates the activity of some of these novel compounds on the isolated spinal cord of 1-5 day old Sprague Dawley rats in a medium containing 0.1μM tetrodotoxin. Recordings were made from a spinal ventral root of motoneurones.

The following phenylglycine derivatives depolarised motoneurones with equieffective molar concentrations (relative to (1S,3R)-ACPD=1) of (S)-3,5-DHPG 0.4 ± 0.02 (n=9); (RS)-2F5HPG 14.4 ± 1.6 (n=10); (S)-3HPG 17.5 ± 2.5 (n=6); (RS)-2Cl5HPG 29.2 ± 0.7 (n=4); (RS)-4Cl3HPG 31.6 ±2.3 (n=9) and (RS)-3HPG 34.0 ± 4.6 (n=7). In contrast, the following phenylglycine derivatives had no agonist activity up to a concentration of 1mM:-(R)-3HPG (n=4); (R)-2F5HPG (n=3); (RS)-2Cl3HPG (n=4); (RS)-2,6-DCl3HPG (n=4) and (RS)-2,4-DCl5HPG (n=3).

The present study has identifed that for optimal activity at subgroup I mGluRs in the spinal cord either substitution of 3HPG with an additional hydroxy group at the 5-position ((RS)-3,5-DHPG) or mono-halogen substitution ((RS)-2F5HPG, (RS)-2Cl5HPG or (RS)-4Cl-3HPG) is favoured. Consistent with the above observations it has been reported that in CHO cells expressing mGluR1 and in rat cerebral cortical slices the 3HPG analogues have a similar rank order of potency ((S)-3,5-DHPG>(S)-6F3HPG>(R,S)-4Cl3HPG) (Sekiyama *et al*, 1996, Bedingfield *et al*, 1994).

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38P ZINC INHIBITION OF β3 SUBUNIT CONTAINING $GABA_A$ RECEPTORS RELIES ON A HISTIDINE RESIDUE LOCATED WITHIN THE SECOND TRANSMEMBRANE DOMAIN

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Zinc is an essential ion that is concentrated within the central nervous system and can act as an inhibitor at $GABA_A$ receptors. This inhibition may be mediated by binding to histidine residues near the GABA-gated ion channel and is also dependent upon the receptor subunit composition as γ subunit-containing receptors are markedly less sensitive (Smart *et al.*, 1994). Native $GABA_A$ receptors are believed to be hetero-oligomers composed of subunits from 5 distinct families, α, β, γ, δ and ε (Sieghart, 1995; Davies *et al.*, 1997). To date, the exact binding site for Zn^{2+} on a $GABA_A$ receptor is unknown. This study utilises the expression of α1 and β3 $GABA_A$ receptor subunits in *Xenopus* oocytes and site-directed mutagenesis to locate an important determinant of the Zn^{2+} binding site. The β3 subunit was selected for its ability to form functional GABA-insensitive, spontaneously-gated homomeric ion channels that are modulated by Zn^{2+}, picrotoxinin (PTX) and pentobarbitone (PB; Wooltorton *et al.*, 1996). A histidine residue (H292) within the putative ion channel of β3 subunits was mutated to alanine (A). Oocytes were injected with murine $GABA_A$ receptor cDNAs encoding for wild-type β3, mutant β3(H292A) or a 1:1 combination of α1β3 or α1β3(H292A) subunits and studied using a two-electrode voltage clamp technique.

Wild-type (w.t.) or mutant β3 homomers were insensitive to GABA up to 1 mM (n=4-9 oocytes). In contrast, PB concentration-response curves for w.t. and mutant β3 subunits revealed that mutating H292 shifted the curve leftwards and reduced the EC_{50} from 168.8±19.5 to 53.5±3.3 μM (n=3-21; mean±s.e.mean; $P<0.05$, Mann-Whitney). Similarly, the sensitivity of the spontaneously-opening β3 ion channels to PTX was slightly increased with the IC_{50} from the concentration-inhibition curve reduced from 83.5±6.2 to 23.3±1.5 nM (n=3-5; $P<0.05$). In comparison, the most profound change caused by mutating H292 was the reduced sensitivity to inhibition by Zn^{2+}. The Zn^{2+} IC_{50} was increased over 900-fold from 0.33±0.02 to 307.2±32.6 μM (n=4-10). Comparative experiments with GABA-sensitive α1β3 constructs indicated that for α1β3(H292A), the GABA concentration response curve was shifted with an EC_{50} of 5.70±0.36 μM, *c.f.* 3.24±0.20 μM for w.t. (n=3-8; $P<0.05$). However, the sensitivity of α1β3(H292A) receptors to Zn^{2+} was clearly reduced by 200-fold compared to w.t. with the IC_{50} changing from 0.11±0.01 to 22.7±4.2 μM (n=3-5). Interestingly, raising the Zn^{2+} concentration to 1 mM for the β3(H292A) and α1β3(H292A) constructs still resulted in substantial inhibition (81 and 92% of the Zn^{2+}-sensitive current and response to 5 μM GABA, respectively).

The involvement of a histidine residue is in accordance with the predictions made previously for a Zn^{2+} binding site on a $GABA_A$ receptor (Smart *et al.*, 1994). Interestingly, histidine residues are involved in the Zn^{2+} binding site on ρ1 subunits (Wang *et al.*, 1995). The effect of mutating H292 on the β3 homomer was more profound compared to the α1β3 combination which may reflect a greater number of binding sites for Zn^{2+} on the homomer. Finally, the shift in the sensitivities of β3 and α1β3 receptors to Zn^{2+} following the mutation of H292 indicates this residue either forms part of the Zn^{2+} binding site within the $GABA_A$ receptor-ion channel or indirectly affects the inhibitory action of Zn^{2+}

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39P INCREASED EXPRESSION OF mRNA ENCODING $GABA_A$ RECEPTOR SUBUNITS α1 AND α4 ASSOCIATED WITH HIPPOCAMPAL SCLEROSIS IN HUMAN PATIENTS WITH TEMPORAL LOBE EPILEPSY

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We have previously demonstrated a change in affinity of the $GABA_A$ receptor for the benzodiazepine antagonist flumazenil, using receptor autoradiography, in several subregions of hippocampal tissue resected from patients with refractory temporal lobe epilepsy (TLE) and hippocampal sclerosis (HS) relative to post-mortem controls (Hand *et al.*, 1996). In order to test the hypothesis that these changes in affinity may reflect an altered subunit composition and possibly efficacy of the native $GABA_A$ receptor, we have recently performed and reported quantitative *in-situ* hybridisation studies using [^{35}S]-labelled antisense oligonucleotides for mRNA encoding the α2, α5 and γ2 subunits of the $GABA_A$ receptor (Hand *et al.*, 1997). We have now extended this study to include estimation of the $GABA_A$ α1 and α4 subunit mRNA in hippocampi resected from 12 patients (average age 33y, range 22-42y, 10 female/2 male) and 10 neurologically normal controls (average age 72y, range 43-77y, 4 female/6 male).

Hippocampi samples were frozen in an embedding matrix on dry ice within minutes of resection and stored at -80°C. Control hippocampi were frozen 5.5-28 hours post-mortem. Cryostat sections (10μm) were cut at -15°C to -20°C and mounted onto charged microscope slides. Sections were fixed with 4% paraformaldehyde in ice-cold phosphate buffered saline (PBS) pH 7.2 for 5min, washed in fresh PBS, dehydrated using a series of alcohols and stored in 95% ethanol at 4°C until assayed. Oligonucleotides were labelled with [^{35}S] on the day of the hybridisation (1.3-2.5x10^{9}dpm/μg) and diluted to a concentration of 5x10^6 dpm/ml in hybridisation buffer (Sirinathsinghji *et al., 1995*). Sections were removed from alcohol, allowed to dry, and hybridised overnight at 42°C. Sections were then washed in standard saline citrate (SSC) 2 x 30min at 55°C, rinsed in 1 x SSC and 0.1 x SSC (1min at room temp), dehydrated using a series of alcohols, air-dried and apposed to Hyperfilm (Amersham) for 21 days at room temperature. Film optical densities (converted to atmol/mm^2 [^{35}S]) were measured in six hippocampal subregions identified by cresyl violet-staining following hybridisation. Non-specific hybridisation was assessed in the presence of 100-fold excess of unlabelled oligonucleotide. Neuronal densities of hippocampal subregions were obtained, using a 3-D counting method (Williams & Rakic, 1988) on paraffin-embedded samples of epileptic and control hippocampus.

Overall expression of mRNA encoding the $GABA_A$ α1 subunit was found to be significantly increased in the dentate gyrus stratum granulosum (215±31% of control, $p<0.01$ Student's *t*-test) and in the subiculum (267±29% of control) with no overall change evident in the hilus or Ammon's Horn. No significant change in overall expression of $GABA_A$ α4 mRNA was observed in any of the hippocampal subfields. Neuronal density estimates obtained from HS (n=7) and control (n=5) hippocampal specimens (except subiculum) were used to assess relative changes in mRNA expression in surviving neurons in HS. Significant upregulation of α1 mRNA levels per neuron was evident in the dentate gyrus, hilus and CA2 in HS (538±129%, 2715±901% and 624±209% of control respectively, $p<0.05$). Neurons of the dentate gyrus also demonstrated an upregulation of α4 mRNA in HS (356±93% of control).

Pooling the data from all five subunits analysed thus far, revealed a significant increase in mRNA per neuron in all hippocampal subfields in HS with the exception of CA3 and CA2 (ANOVA $p<0.01$). This may suggest an overall non-selective increase in $GABA_A$ receptor mRNA expression in surviving neurons in HS. Howevcr, the effects of age or difference in sampling method between groups cannot be excluded.

Control post-mortem brain tissue was provided by the Parkinson's Disease Society Brain Bank, Institute of Neurology, London and support was provided by the Royal Pharmaceutical Society.

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40P NMDA RECEPTOR AUTORADIOGRAPHY AND DIRECT 3D-CELL COUNTING IN RESECTED HIPPOCAMPUS OF PATIENTS WITH TEMPORAL LOBE EPILEPSY

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Autoradiographic studies of the *N*-methyl-D-aspartate (NMDA) receptor in hippocampal tissue resected from patients with intractable temporal lobe epilepsy (TLE) have reported both increased and decreased binding (Geddes *et al.*, 1990; McDonald *et al.*, 1991). These changes have not been directly related to the neuron densities in individual patients. We have performed saturation autoradiographic studies, using [^{3}H]MK-801, and 3D-cell counting, to compare receptor binding density and neuron density in 11 patients (mean age 34 years) whose TLE was due to unilateral hippocampal sclerosis (HS), with 6 neurologically normal post-mortem controls (mean age 74 years).

Hippocampal tissue collected post-resection was frozen in embedding matrix over dry ice within 15 min and stored at -80°C. Control hippocampi were frozen 5.5-27.5 h post-mortem. Cryostat sections (10 μm) were mounted onto charged slides and stored at -80°C until assayed. All sections were pre-incubated for 20 min in ice-cold buffer (50 mM Tris-HCl, pH 7.4, 50 μM glutamate, 50 μM glycine, 50 μM spermidine). Incubation was for 1 h at 25°C in fresh buffer solution containing 3-30 nM [^{3}H]MK-801. Unlabelled MK-801 (10^{-4} M) was used to define non-specific binding. Slides were rinsed (3 x 10 s) in fresh buffer (4°C), dipped in distilled water (4°C), dried with cold air and apposed to [^{3}H]-sensitive film for 28 days at room temperature. For each film, mean optical densities (converted to fmol/mg protein) were measured in six hippocampal subregions (identified in adjacent sectons stained with luxol fast blue/cresyl violet) by outlining the entire subregion. Bmax values were determined by non-linear regression analysis (GraphPad Prism). Quantitative neuropathology, using stereological techniques (3D-cell counting) (Williams & Rakic, 1988), was performed on 16-30 μm paraffin embedded sections of sclerotic and control hippocampus to determine neuron densities in each of the hippocampal regions investigated by autoradiography, excluding the subiculum.

Bmax values for [^{3}H]MK-801 binding in HS (CA1 51 ± 9, CA2 172 ± 28, CA3 94 ± 25, CA4 83 ± 17 fmol/mg protein mean ± s.e.mean) were significantly reduced compared with controls (CA1 439 ± 61, CA2 315 ± 30, CA3 232 ± 20, CA4 197 ± 30 $p<0.05$, Student's *t*-test). Compared to controls, patients with HS also showed significantly reduced neuronal densities in all areas measured (CA1 -91 ± 1.1%, CA2 -36 ± 6.0%, CA3 -61 ± 8.6%, CA4 -71 ± 8.0%, $p<0.05$, Student's *t*-test). In the dentate gyrus, the neuron density was reduced (-46.5 ± 7.3%) to a significantly greater extent than the Bmax (-8.9 ± 7.8%) ($p<0.01$), while in all other regions the percentage reduction in Bmax was not significantly different to the reduction in neuron density. No significant differences in binding K_D values were detected between HS and control hippocampi.

Thus, autoradiography combined with 3D-cell counting showed that decreased NMDA receptor binding in HS appeared to be mainly due to neuronal loss in regions other than the dentate gyrus. The results from the dentate gyrus support the theory (Sloviter 1987) that previously dormant excitatory receptors are incorporated into the excitatory pathway following epileptic activity, representing an apparent upregulation in receptor function.

Control tissue was provided by Dept of Pathology, University of Birmingham & Parkinson's Disease Society Brain Bank, Institute of Neurology, London.

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41P EFFECT OF THE CALCIUM CHANNEL ANTAGONIST, NIMODIPINE, IN DECREASING RESPONDING FOR THE ORAL SELF ADMINISTRATION OF LOW CONCENTRATIONS OF ETHANOL

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The dihydropyridine calcium channel antagonist, nimodipine, decreases intravenous self-administration of morphine and cocaine (Kuzmin *et al.*, 1992) and lowers voluntary alcohol drinking in rats (Engel et al., 1988). The present study examined effects of nimodipine on self-administration of alcohol by an operant technique in which rats press a lever to obtain alcohol.

Male Lister hooded rats were used (starting weights 400 - 450g), singly housed with free access to food and water and reverse phase lighting. They were trained in standard operant chambers, which were equipped with levers and a dipper receptacle for fluid presentation. Initial training used the "sucrose-fading" procedure to introduce the rats to responding for alcohol (adapted from Schwarz-Stevens *et al.*, 1991). Once trained, the rats pressed the levers to obtain solutions of dilute alcohol. A test time of 90 min per day was used, during the dark phase. A progressive ratio schedule was applied, in which the number of lever presses needed to obtain a dipper of alcohol was increased from 2, in increments of 4 (with two reinforcments at each ratio), until the rats gave up pressing. Different concentrations of alcohol (5 to 40%) were supplied for each period of 5 days testing. At the end of these tests, the alcohol solution was changed to water. The ratio at the end of each test session was used as a measure of how hard the rats were prepared to "work" for the presentation of the solutions. Alcohol intake was measured by weighing the dipper reservoirs and correcting for evaporation. For each ethanol concentration, the following treatment schedule was used: on the first day, no prior treatment was given, on the second and third days, saline injections were given to all rats, to test if the injection procedure affected the responding. On the fifth day, either nimodipine, 10 or 50 mg/kg or its vehicle were given 1h prior to the start of the session (n=9-10 per treatment group). Statistical comparisons were by one-way ANOVA with post-hoc analysis by Dunnett's test.

Nimodipine, i.p. at 10 mg/kg, significantly decreased the ratios when rats ceased to respond for the 5%, 10% and 15% ethanol concentrations. Nimodipine i.p. 50 mg/kg significantly decreased responding for 5% ethanol. No effects were seen on responding for ethanol concentrations higher than 15%. Nimodipine at 10 mg/kg significantly decreased ethanol intake at 5 to 20% ethanol. When water was substituted for ethanol, rats stopped responding at significantly lower ratios, and returned to previous values when the ethanol was reinstated, suggesting that they were responding for the pharmacological properties of alcohol.

Nimodipine did not affect the self-administration of water. The effects of nimodipine may be due to action on the reinforcing properties of alcohol, but the mechanism of action is not known

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Table 1. Ratios at which rats ceased responding, and intake of ethanol, mg/kg, at the different concentrations mean ± s.e.m. *P< 0.05 compared with values after vehicle administration. nim = nimodipine

Treatment	ratio 5% ethanol	ratio 10% ethanol	ratio 15% ethanol	ratio 20% ethanol	mg/kg ethanol at 5% ethanol	mg/kg ethanol at 10% ethanol	mg/kg ethanol at 15% ethanol	mg/kg ethanol at 20% ethanol
Tween vehicle	14.9±1.8	16.0±2.3	13.6±2.9	14.5 ± 2.6	52 ± 8	125±19	156±42	227± 64
10 mg/kg nim.	7.6±1.8*	6.4±1.3*	9.6±2.1*	14.0± 2.5	35 ± 7*	93±18 *	94 ± 35*	116± 44*
50 mg/kg nim.	7.8± 2.2*	11.3±2.6	10.9±4.3	16.2± 4.3	38 ± 12*	123±19	158±40	286± 60

42P INCREASED STIMULANT EFFECTS OF AMPHETAMINE AND COCAINE ARE SEEN SEVEN DAYS AFTER CESSATION OF CHRONIC BARBITAL TREATMENT IN MICE

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We have previously demonstrated that prolonged ethanol consumption by mice increases the locomotor stimulant actions of, and sensitisation to, amphetamine and cocaine. This effect lasted long after the cessation of the acute withdrawal signs and was demonstrated up to 2 months after cessation of the chronic ethanol treatment (Manley and Little, 1997). In the present study, we investigate the effects of amphetamine and cocaine following a chronic barbital ingestion.

Groups of 8, male mice of the TO strain (25-30g) were used. They were made physically dependent on barbital by a 10 day powdered diet schedule (Rabbani et al., 1994). The daily intake of barbital was 0.9 to 1.3 g/kg/24h. Controls were pair-fed powdered food without barbital. Following the chronic barbital treatment, the mice were given ad libitum access to standard laboratory chow, and left undisturbed for 7 days (the acute withdrawal signs last only 48h). They were then injected with amphetamine (3 mg/kg i.p.), cocaine (20 mg/kg) or saline and their locomotor activity for the next 30 min measured by infra-red beam breaks. They were then injected with these drugs once daily for 7 days, in their home cages, before a final test in the locomotor meters on day 7 after an acute injection of the drug they had been receiving. Statistical analysis was by ANOVA followed by Student -Newman-Keulls test.

The results showed that, after 6 daily injections, the locomotor stimulant effects of amphetamine (Table 1) and of cocaine (Table 1) were significantly greater in mice that had previously been given the chronic barbital diet (*P<0.05, compared with saline injections; ¥ P<0.05, compared with results from animals that received control diet). This pattern is similar to that seen previously after chronic ethanol treatment in mice (Manley and Little, 1997) and rats (Smith et al., unpublished results). The possibility of a pharmacokinetic interaction must be taken into consideration, but we previously showed that changes in amphetamine or cocaine metabolism did not account for the prolonged effects of chronic ethanol (Manley and Little, 1997). The results may therefore indicate that prolonged barbiturate intake may alter the adaptive responses to psychostimulant drugs. Although alcohol and barbiturates share a common action in potentiating the effects of GABA, they differ in their other acute actions and their routes of metabolism. The similar pattern of prolonged effects on the actions of psychostimulant drugs may be related to common mechanisms involved in dependence.

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Table 1: Locomotor activity in response to 3 mg/kg amphetamine, 7 days after barbital treatment

Chronic diet/ stimulant injections	Locomotor activity (1st Injection)	Locomotor activity (7th injection)
Control/ saline	4526 ± 429	3211 ± 471
control/amphetamine	9703 ± 1433 *	9416 ± 755 *
Barbital/saline	4028 ± 343	3352 ± 469
barbital/ amphetamine	10265 ± 782 *	12528 ± 1420*¥

Table 2: Locomotor activity in response to 20 mg/kg cocaine, 7 days after chronic barbital treatment

Chronic Diet/ stimulant injections	Locomotor activity (1st Injection)	Locomotor activity (7th injection)
Control/ saline	5047 ± 486	3972 ± 420
control/cocaine	11741± 678*	10840 ± 718*
Barbital/saline	4249 ± 432	4323 ± 725
barbital/cocaine	13256 ±908*	13657 ± 663*¥

43P MICRODIALYSIS STUDY OF THE MODIFICATION OF STRIATAL DOPAMINE RELEASE BY CHRONIC TREATMENT WITH SELECTIVE INHIBITORS OF MAO-A AND MAO-B IN THE GUINEA-PIG

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Dopamine (DA) has similar affinity for monoamine oxidase (MAO) types A and B. Since the proportion and affinity of MAO subtypes in guinea-pig brain is closer to that of the human than is the rat, the guinea-pig may well be a superior experimental model for study of the effect of MAO inhibitors on amine metabolism and release. Our previous study in the rat (Lamensdorf *et al.*, 1996) showed that chronic, but not acute, treatment with the selective MAO-B inhibitor deprenyl elevated striatal microdialysate DA. The effect of chronic and acute treatment with deprenyl on striatal DA release has accordingly been studied in the guinea-pig, and compared with the effects of the selective MAO-A inhibitor, clorgyline.

Microdialysis probes (4mm active length) were implanted in striatum of male English Short Hair guinea-pigs under pentobarbital/chloral hydrate anaesthesia, and perfused (2 µl/min) with artificial CSF as described by Lamensdorf *et al.* (1996). Control microdialysate collections (4 X 20 min each) were commenced 24 h after operation, followed by perfusion with a 20 min bolus of KCl (90 mM) or GBR-12909 (100 µM). Drugs were given i.p., with last dose 24 h before microdialysis.

In contrast to our study in the rat (Lamensdorf *et al.*, 1996), neither acute nor chronic treatment with deprenyl (2 mg/kg; 0.25 mg/kg daily for 21 days respectively), increased basal or KCl-stimulated striatal microdialysate DA in guinea-pig, but increased basal HVA (see Table 1 for chronic, basal level data). Acute and chronic treatment with clorgyline (4mg/kg; 0.2 mg/kg daily for 21 days, respectively), reduced microdialysate levels of dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), but also without change in basal or KCl-stimulated DA levels. Perfusion with GBR-12909 increased microdialysate DA by 167±7% in guinea-pigs and 388±34% in rats (s.e. mean, n=3 for each), but IC_{50} of GBR-12909 for inhibition of synaptosomal uptake of ^{3}H-DA was the same in both species (1nM). The results show a reduced effect of uptake inhibition on overflow of released neurotransmitter in the guinea-pig with relation to the rat, possibly because of greater synaptic cleft width in guinea-pig. These results are consistent with our proposal that the increased DA levels seen in rats following chronic deprenyl treatment are the result of uptake inhibition by endogenous β-phenylethylamine, which accumulates following MAO-B inhibition.

Table 1. Basal striatal microdialysate levels of DA and metabolites (pmol/20 min ± s.e. mean) in guinea-pigs treated chronically with MAO inhibitors. *=P<0.01 with respect to control, by ANOVA + Dunnett test.

treatment (n)	DA	DOPAC	HVA
control (12)	0.1±0.010	5.25±0.6	12.1±1.1
deprenyl (10)	0.1±0.007	6.6±0.06	19.2±0.3*
clorgyline (10)	0.09±0.01	2.7±0.35*	4.3±0.5*

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44P NEUROPROTECTIVE EFFECT OF MODAFINIL AGAINST MPTP TOXICITY IN MPTP-TREATED COMMON MARMOSETS

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Modafinil, [(diphenylmethyl)-sulphinyl-2-acetamide] is a psychostimulant which protects nigral dopamine neurones from MPTP toxicity in the mouse (Fuxe et al., 1992). We now report on the effects of modafinil on motor disability in MPTP-treated common marmosets and its ability to prevent MPTP induced nigral cell death in this species.

In 4 common marmosets (*Callithrix jacchus*), 11-15 months following MPTP treatment, modafinil (10, 30 or 100 mg/kg, po) was assessed for its ability to reverse motor deficits and to increase locomotor activity. Subsequently, adult common marmosets (n=16) were divided into 4 groups and treated with MPTP 2 mg/kg SC once daily for 5 days. One group received only MPTP treatment, while the others received in addition either 10 mg/kg, 30 mg/kg or 100 mg/kg of modafinil once daily by gavage during dosing with MPTP and for 2 weeks after the last dose of MPTP. The animals were graded daily on a disability rating scale and locomotor activity assessed in photocell cages once weekly. An additional 4 normal animals served as controls. At the end of the experiment, the brains were removed and quantitative evaluation of nigral tyrosine hydroxylase-immunoreactive (TH-IR) cells, glial fibrillary acidic protein (GFAP)-positive astrocytes and striatal [^{3}H]-mazindol binding were performed. Behavioural results were analysed by Mann-Whitney's U-test and biochemical data by one way ANOVA followed by post hoc Dunnett's test.

Modafinil (10, 30 and 100 mg/kg, po) produced a dose-dependent reversal of motor deficits and increased locomotion in MPTP treated common marmoset with established motor deficits. Similarly, modafinil dose-dependently prevented the establishment of disability when administered during and after MPTP treatment. MPTP treatment caused a 76% loss of nigral dopamine cells (Table 1). This was prevented dose-dependently by modafinil so that following treatment with 10, 30 and 100 mg/kg, cell loss was 67%, 54% and 23% (P<0.05, vs MPTP marmosets) respectively. Similarly, MPTP treatment reduced specific [^{3}H]-mazindol binding by 95% (P<0.01 vs normal controls) Modafinil treatment reduced the loss of [^{3}H]-mazindol binding dose-dependently, so that following 10, 30 and 100 mg/kg, the reduction in [^{3}H]-mazindol binding was 92%, 74% and 22% (P<0.01, vs MPTP treatment) of control value respectively. GFAP staining showed no significant difference between groups.

The results showed that modafinil reversed motor deficits in MPTP-treated common marmosets and prevented nigral cell death induced by MPTP. The mechanism by which this is achieved is not clear. It does not appear to involve inhibition of MAO-B or dopamine reuptake.

Fuxe K, Janson AM, Rosen L, Finnman UB, Tanganelli S, Morari M, Goldstein M, Agnati LF (1992) *Exp Brain Res*, 88, 117-130.

Table 1. The mean No. of TH-IR cell counts and mean density of [^{3}H]mazindol bindings of normal marmoset

TH-IR cell counts	320±21
[^{3}H]mazindol bindings	56.05±6

45P EFFECTS OF CORTICOSTERONE ON THE ACTIVITY OF DOPAMINE-SENSITIVE NEURONES IN THE VENTRAL TEGMENTAL AREA

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Glucocorticoid hormones have important effects in the CNS, and recent studies have suggested these influences are of importance in drug dependence. However, there has been little investigation of the effects of these hormones on neuronal function. We investigate the effects of corticosterone (the major glucocorticoid hormone in rats) on neurones in the ventral tegmental area (VTA). This area is thought to be involved in reward mechanisms and motivation, and has been implicated in dependence on alcohol, psychostimulants and other drugs.

Male hooded Lister rats (200-225g) were used throughout. Midbrain slices containing the VTA were prepared, two slices from each rat, after cervical dislocation. Recordings were made, after an settling period of 60 min, from slices perfused with aCSF at 2.3 ml/min, using the interface method of slice perfusion (one slice per chamber). Single unit recordings were made from dopamine-sensitive cells with firing frequencies less than 5 Hz and action potential duration over 2 ms. Either dopamine, 5 μM or NMDA (N-methyl-DL-aspartate) 5 or 15 μM were added to the perfusion medium for 5 min, at 15 min intervals; recordings were obtained during the last 1 min of dopamine or NMDA application. After baseline measurements had been made, corticosterone was added at increasing concentrations, from 50 nM to 2 μM to one slice of each pair; 15min pretreatment. The two higher corticosterone concentrations were studied on a separate set of slices from the lower concentrations. The n values were 6 per treatment group (one pair of slices per rat, one neurone per slice). Student's t-test was used to compare the firing rates in the slices to which corticosterone was added with the rates in the parallel recordings from the slices which did not receive corticosterone.

The mean firing frequency (Table 1) was significantly higher after addition of NMDA in slices perfused with corticosterone 100, 500 nM or 1 μM, than in control slices. In the presence of 2 μM concentration of corticosterone, however, the firing rate in response to NMDA was reduced. There were no changes, at any of the concentrations of corticosterone, in the depressant effects of dopamine on the firing rate. Corticosterone did not alter the basal firing frequency of the cells. No changes were seen in action potential duration after any of the corticosterone concentrations.

The results indicate that selective alterations in firing rate in response to NMDA occur in dopamine-responsive cells in the VTA at concentrations of corticosterone similar to those circulating in the body during times of stress. These may be related to the psychological effects of glucocorticoid hormones

Table 1. Firing rates (Hz), mean ± s.e.m., cort = corticosterone. *P<0.05; **P<0.001 compared with parallel control slice recordings

Basal, (no NMDA) control slices	Basal, before addition cort		Controls	cort 50 nM	Controls	cort 100 nM	Controls	cort 500 nM
1.67 ± 0.05	1.76 ± 0.03	NMDA 5 μM	1.84 ± 0.05	1.92 ± 0.06	1.79 ± 0.08	2.29 ± 0.09*	1.71 ± 0.1	2.42 ± 0.19*
		NMDA 15 μM	2.25 ± 0.12	2.38 ± 0.07	2.06 ± 0.11	3.23 ± 0.11**	2.01 ± 0.17	3.24 ± 0.2**
Basal (no NMDA) control slices	Basal, before addition cort		Controls	cort 1 μM	Controls	cort 2 μM		
1.45 ± 0.17	1.52 ± 0.27	NMDA 5 μM	1.58 ± 0.21	2.88 ± 0.28*	1.8 ± 0.17	1.25 ± 0.18		
		NMDA 15 μM	2.0 ± 0.24	3.2 ± 0.24*	2.17 ± 0.21	0.22 ± 0.16**		

46P MODULATION OF CYCLIC GMP-MEDIATED DILATATION IN THE RAT ISOLATED MESENTERIC ARTERY BY BASALLY RELEASED NITRIC OXIDE

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We have previously shown that the mechanisms which mediate relaxation to the nitric oxide (NO) donor 3-morpholino-sydnonimine (SIN-1) are modulated by the basal release of endothelium-derived NO (Plane *et al.*, 1996). In the present study, we have investigated whether this modulation can be mimicked by pre-incubating endothelium-denuded tissues with agents which increase levels of cyclic GMP and may reflect an increase activity of phosphodiesterases (PDE).

Male Wistar rats (250-300 g) were stunned and then killed by cervical dislocation. Segments of third order branches of the superior mesenteric artery (D_{100} 290 ± 10 μm; n=40) were mounted in a myograph under a normalised tension as previously described (Plane *et al.*, 1996). In some experiments, the endothelial cell layer was removed mechanically by gently rubbing the intima with a hair. Tissues were maintained at 37°C in oxygenated Krebs buffer, containing indomethacin (2.5 μM). All data are expressed as mean ± s.e. mean and differences between mean values were calculated using the Students' t-test.

SIN-1 (0.01-10 μM) caused concentration-dependent relaxation of arterial segments pre-constricted with phenylephrine (3 μM). In intact arterial segments, relaxation to SIN-1 was abolished by exposure to the potassium channel inhibitor charybdotoxin (ChTX; 50 nM; n=5; P<0.01). In contrast, in endothelium-denuded tissues, relaxation to SIN-1 was unaffected by pre-incubation with either ChTX (n=6; P>0.05) or the inhibitor of soluble guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM; n=4; P>0.05) alone, but abolished by exposure to these two inhibitors together (n=6; P<0.01).

Pre-incubation of endothelium-denuded arterial segments with either glyceryl trinitrate (GTN; 4.4 nM; 10 mins) or 8-bromo cyclic GMP (8-Br-cyclicGMP; 1 μM; 10 mins) attenuated relaxation to SIN-1 but did not significantly reduce the maximum responses which were 81.6 ± 6.9 % (n=4; P>0.05) and 94.1 ± 0.4 %, (n=4; P>0.05), respectively. Exposure to ChTX, in the presence of either GTN or 8-Br cyclic GMP, significantly inhibited relaxation to SIN-1 reducing the maximum responses by around 40-50 % to 43.0 ± 7.2 % (n=7; P<0.01) and 49.5 ± 5.1 % (n=7; P<0.01), respectively.

Pre-incubation with the PDE inhibitor zaprinast (1 and 10 μM; 10 mins), significantly potentiated relaxations to SIN-1 in endothelium-intact arteries (n=3; P<0.01). In the presence of zaprinast, relaxation of intact arterial segments to SIN-1 was unaffected by ODQ (n=4; P>0.05) but significantly attenuated by. ChTX, although now the maximum response was unaltered (n=5; P>0.05). Exposure of zaprinast-treated tissues to ODQ and ChTX together abolished SIN-1-evoked relaxation (n=4; P<0.01).

These data indicate that, in intact arteries, basal release of NO leads to an inhibition of cyclic GMP-mediated pathway of relaxation to the NO donor SIN-1. This effect can be mimicked by pre-incubation of endothelium-denuded arterial segments with agents which raise cyclic GMP and can be partially reversed by the PDE inhibitor zaprinast. The basal release of endothelium-derived NO may, therefore, inhibit cyclic GMP-mediated relaxation via an up-regulation of PDE.

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47P COPPER INTERACTS WITH HOMOCYSTEINE TO INHIBIT NITRIC OXIDE FORMATION IN THE RAT ISOLATED AORTA

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Homocysteinaemia is an independent risk factor for premature atherosclerosis although the underlying mechanisms are unclear. The deleterious effects of homocysteine (HC) include the inhibition of nitric oxide (NO) formation and reactions with copper to generate superoxide (O^{2-}) (Berwanger *et al.*, 1995). As NO and O^{2-} react to form peroxynitrite ($ONOO^-$) (Koppenol, *et al.*, 1992), interactive effects of HC and Cu^{2+} on NO formation in the rat isolated aorta were examined using organ bath and biochemical methods (Plane *et al.*, 1997).

Male Sprague Dawley rats (250 g) were killed by decapitation. Segments of aorta (2mm in length) were mounted in organ baths for recording of isometric tension as previously described [Plane et al., 1997]. In parallel experiments cGMP was measured using radioimmunoassay (Plane et al., 1997). All data are expressed as mean ± s.e.mean and differences between mean values calculated using the student's t test.

Acetylcholine (ACh; 0.01-10μM) evoked concentration - dependent relaxation of isolated aortic rings precontracted with 3μM phenylephrine. Following pre-incubation for 30 min, 100 μM HC alone was without effect on ACh-stimulated relaxation or cGMP formation (table 1). However, both ACh - stimulated relaxation and cGMP formation were markedly inhibited by the presence of both 100 μM $CuCl_2$ and 100 μM HC (table 1). This inhibition was partially reversed by the presence of superoxide dismutase (SOD; 30 U/ml) and by catalase (CAT; 30 U/ml) (table 1). $CuCl_2$ plus HC had no significant effect on relaxation or cGMP formation in response to sodium nitroprusside (1-10 μM), demonstrating no effects on guanylyl cyclase (GC) activity.

Although HC alone had no effect, the presence of Cu^{2+} markedly augmented the inhibitory effect of HC on NO formation and effects. This could not be ascribed to a direct effect of Cu^{2+} alone since $CuCl_2$ promotes NO synthase and guanylyl cyclase activity in the rat aorta (Plane et al., 1997). As SOD and CAT reversed the interaction with Cu^{2+} and HC, it is concluded that this effect is due to the formation of both H_2O_2 and O^{2-}. NO reacts with O^{2-} to form $ONOO^-$, effectively reducing the NO available for the activation of GC. This mechanism may be important in the pathophysiology of homocysteinaemic angiopathy and points to the therapeutic use of Cu^{2+} chelators in this disorder.

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Table 1. Maximal relaxation (MR) and cGMP formation (fmoles/mg tissue/min) in response to Ach (mean ± SEM; n = 6-8) after incubation for 30 min with combinations of HC, Cu^{2+}, SOD and CAT (concentrations in text). * $p < 0.01$ relative to controls

	control	*HC alone*	*HC + $CuCl_2$*	*HC + $CuCl_2$ + SOD*	*HC + $CuCl_2$ + CAT*
MR	94 ± 2.5	86 ± 3	22 ± 9.7	67 ± 2.9	76 ± 7.13
cGMP	260 ± 30	230 ± 25	40 ± 5	220 ± 24	240 ± 38

48P ADENOSINE RECEPTOR mRNA LEVELS DURING POST-NATAL MATURATION OF RAT KIDNEY

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Adenosine is a paracrine mediator of many functions in the mature kidney. However, little is known about its role in the immature kidney. The aim of this study was to assess expression of adenosine receptors (AR) during post-natal renal development by measuring renal AR mRNA levels in rats aged 2 to 60 days.

Male Wistar rats were sacrificed and both kidneys removed. Total RNA was isolated from whole kidneys and β-actin and AR subtype mRNA levels were quantitated by the reverse transcriptase polymerase chain reaction (Robinson & Simon, 1991).

Transcript numbers (copies μg^{-1} total RNA x 10^7) at 60 days were: A_1, 1.25 ± 1.37 (mean ± s.e.mean); A_{2A}, 22.64 ± 29.72; A_{2B}, 1.42 ± 0.29; A_3, 0.23 ± 0.07 and β-actin, 270 ± 80 (n=5). β-actin mRNA did not show statistically significant variations during post-natal maturation (Table 1). A_1 receptor mRNA declined with increasing age. Levels at 42 days were 4.7-fold lower ($P<0.05$) than those at 2 days although levels then increased at 60 days. A_3 transcript numbers at 12 and 60 days showed statistically significant ($P<0.05$) elevations of 10 and 20-fold respectively, in comparison to levels at 2 days. A_{2A} transcripts increased by 60 % ($P<0.05$) from 2 to 12 days and then declined, such that at 60 days, levels were 4.5-fold ($P<0.05$) less than at 12 days. Changes in A_{2B} mRNA expression during maturation were less marked showing only a 38 % decrease at 42 days in comparison to levels at 2 days.

The present study indicates: (1) the most abundant AR transcript in the kidney of mature rats is that for the A_{2A} receptor and (2) there are significant changes in AR expression during renal maturation. The changes noted for A_{2A} and A_3 receptors transcripts may be of significance since these coincide with a period of rapid renal development which occurs at 16-20 days.

Robinson, M.O. & Simon, M.I. (1991) *Nucl. Acids. Res.* **19**: 1557-1562

Table 1. Levels of adenosine receptor and β-actin mRNA during renal maturation (% of levels in 60 day old rats).

Days	A_1	A_{2A}	A_{2B}	A_3	β-actin
2	177 ± 17	290 ± 1$^{\#}$	141 ± 10	5 ± 5	102 ± 26
12	165 ± 37	467 ± 11	131 ± 7	52 ± 7*	121 ± 28
18	112 ± 50	412 ± 135	136 ± 13	32 ± 5	89 ± 15
42	38 ± 13*	200 ± 52$^{\#}$	88 ± 7*	41 ± 8	95 ± 1
60	100 ± 48	100 ± 122	100 ± 18	100 ± 32*	100 ± 9

Values are mean ± s.e.mean (n=5). * $P<0.05$ relative to 2 day-old rats; $^{\#}$ $P<0.05$ relative to 12 day-old rats (ANOVA).

49P FURTHER CHARACTERISATION OF THE ANTAGONISTIC ACTION OF PYRIDYLISATOGEN ON THE ISOLATED TAENIA OF THE GUINEA-PIG CAECUM

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Relaxation of the taenia by ATP is antagonised by 2, 2'-Pyridylisatogen tosylate (PIT) in a time and concentration dependent manner consistent with irreversible antagonism of post-junctional ATP receptors (Spedding *et al.*, 1975). Other effects of PIT include potentiation of ATP responses at a recombinant purinoceptor from chick brain (King *et al.*, 1996) and an ability to trap hydroxyl and superoxide free radicals (Nepveu *et al.*, 1997). Other spin trap compounds, α-phenyl-tert-butylnitrone (tBPN) and dimethyl-pyrroline N oxide (DMPO), share structural properties to PIT in that all three compounds possess a nitrone moiety. A further characteristic of PIT was an unexplained inability to antagonise responses in certain preparations which showed an elevated EC_{50} value to ATP or ADP (Spedding and Weetman, 1976). The three fold aim of this study was to investigate the effect of low concentrations of PIT upon ATP-induced responses in guinea-pig isolated taenia caecum; to determine whether the spin traps, tBPN and DMPO, demonstrate any effect upon ATP responses and to investigate the inability of PIT to antagonise ATP responses with the possible reason being an age-related insensitivity of taenia isolated from older guinea-pigs.

Taenia caeci preparations were obtained from male guinea-pigs (185-300g) or in age studies (400-550g) in a first series of experiments and (800-1000g) in a second series. Preparations were suspended in a 10ml organ bath containing McEwen's solution at 36 ± 1°C and gassed with 95% O_2/ 5% CO_2. After an equilibration period of 30 min, responses were recorded isotonically on a Grass 3F recorder (load 1.5g).

Cumulative concentration-response curves were obtained to ATP (1-1000 μM) at 20 min intervals. All test compounds were incubated for 30 min prior to ATP responses.

PIT (0.1 μM) failed to potentiate the response to ATP producing a control EC_{50} value of 26.61 ± 5.70 μM and after incubation with PIT an EC_{50} of 34.94 ± 6.56 μM (n=8; P>0.05). DMPO (50 μM), but not tBPN, produced a small significant potentiation of the response to ATP reducing control EC_{50} of 19.63 ± 0.53 to 15.14 ± 0.99 μM (P<0.05; n=3). Only PIT (50 μM) significantly antagonised responses to ATP producing a dose ratio (DR) of 19.91 ± 5.65 (P<0.01; n=10). Incubation of taenia, derived from older guinea-pigs, with PIT (50 μM), failed to produce any significant antagonism of ATP responses emphasised by a DR of 1.67 ± 0.41 (P>0.05; n=7). Repeating the experiments, using taenia obtained from matched parts of the caecum in both young and old animals, produced improved antagonism in older guinea-pigs (DR 4.89 ± 1.39) but this was still significantly less than that obtained in younger animals (DR 13.24 ± 2.22; P<0.05; n=7).

In conclusion low concentrations of PIT failed to potentiate responses to ATP. The presence of the nitrone moiety, in the spin trap molecules, failed to produce an antagonistic effect upon ATP responses, however a small potentiation of ATP responses was produced by DMPO. The antagonism produced by PIT is dependent upon the age of animal from which the taenia is isolated.

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50P 5-HT RECEPTOR INVOLVEMENT IN THE FACILITATION OF PERISTALSIS ON MUCOSAL APPLICATION OF 5-HT IN THE GUINEA-PIG ISOLATED ILEUM

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The facilitation of peristalsis on the mucosal application of 5-HT to the guinea-pig ileum was first reported by Bulbring & Crema (1958). In the present study we investigate the 5-HT receptor(s) mediating this effect.

Four segments of ileum (taken 5-30 cm from the ileo-caecal junction) were obtained from guinea-pigs of either sex (500-1000g) and cannulated at the oral and aboral ends and secured horizontally in a bath containing Krebs-Henseleit solution kept at 37°C and oxygenated with 95% O_2 and 5% CO_2. Peristalsis was measured and recorded using the methodology previously described (Costall *et al.*, 1993) with some modifications: the lower outlet was at the level of the fluid in the bath and an arrangement was made to automatically stop the pump introducing the Krebs-Henseleit solution for 10s as soon as peristalsis was obtained. The solution was introduced at a constant rate of 1-1.5 ml min^{-1} into the lumen. Non-cumulative concentration response curves to 5-HT were constructed approximately 1 hour after the mounting of the tissues as follows: peristalsis was elicited (by closing the lower outlet) in cycles of 10-12 min duration with resting periods of the same duration between each cycle of peristalsis. An increasing concentration of 5-HT was applied in each cycle to the mucosal side. This was achieved by changing the solution being pumped into the lumen with one containing 5-HT 5-6 min after closure of the lower outlet and changing back to normal Krebs-Henseleit solution once 3 ml of the solution has been injected. Peristalsis was observed for five min once 5-HT had reached the ileum, after which the lower outlet was opened to start the resting period. The concentration response curves to 5-HT were expressed as a percentage decrease in peristaltic threshold below the minimum threshold in the 2 min period before the addition of 5-HT. Antagonists were added to the Krebs-Henseleit solution pumped into the lumen and equilibrated for one hour. The comparisons of the concentration response curves to 5-HT in the absence and presence of an antagonist were made in a tissues taken from the same animal.

5-HT (3-100 μM) caused concentration-dependent reductions in the threshold for peristalsis with a pD_2 (mean±s.e.mean) value of 5.61±0.21 (n=5). The concentration response curve to 5-HT was shifted dextrally in the presence of ondansetron 5 μM (pD_2 values in the absence and presence of ondansetron 5.42±0.07 and 4.12±0.10 respectively n=6, P<0.05 Students' paired t-test) and granisetron 1 μM (pD_2 values in the absence and presence of granisetron 5.45±0.12 and 4.50±0.10 respectively n=5, P<0.05 Students' paired t-test). No significant shift of the concentration response curve to 5-HT was observed in the presence of the 5-$HT_{1/2}$ receptor antagonist methiothepine (0.1 μM, n=4), the 5-HT_2 receptor antagonist ritanserin (0.1 μM, n=5) or the 5-HT_4 receptor antagonist SB 204070 (0.1 μM, n=5) (Wardle *et al.*, 1994).

The results of this study suggest the involvement of the 5-HT_3 receptor in the facilitation of peristalsis on the mucosal application of 5-HT. The data also suggest the lack of involvement of 5-HT_1, 5-HT_2 and 5-HT_4 receptors in the above response.

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51P CHARACTERISATION OF MUSCARINIC RECEPTORS IN THE RABBIT IRIS SPHINCTER MUSCLE

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According to a previous report (Bognar *et al.*, 1992), muscarinic agonist contracts the rabbit iris sphincter muscle (RISM) via a receptor different from the M_1, M_2, M_3 and M_4 subtypes. In the present study, we re-examined the pharmacological characteristics of muscarinic receptors in RISM and compared them to M_3 receptors in rabbit urinary bladder smooth muscle (RUBSM) (Tobin, 1995).

Female New Zealand white rabbits were euthanised by CO_2 asphyxiation. Two strips of iris sphincter muscle were cut from each eye (ciliary margin removed) and four strips of urinary bladder smooth muscle were cut from the supratrigonal portion of the bladder (longitudinal section, mucosa removed). The tissues were mounted in 10 ml organ baths containing Tyrode solution (with 10 μM indomethacin, at 37° C, constantly aerated with 95% O_2/5% CO_2). A resting tension of 150 mg and 2 g was applied to the iris and the bladder, respectively. Cumulative concentration-response curves to (+)-cis-dioxolane (1nM - 0.3mM) were established in the absence and presence of antagonists (90 min equilibration). Antagonist affinities (pA_2) were determined with three concentrations of the antagonist using Schild analysis.

(+)-Cis-dioxolane induced concentration-dependent contractions in RISM and in RUBSM (pEC_{50} = 6.50±0.05, max. tension=40-476mg and pEC_{50} = 6.73±0.06, max. tension=1.1-12.0g, respectively). No time-dependent loss of sensitivity was observed in either tissue. The effects of (+)-cis-dioxolane in RISM and RUBSM were competitively antagonized by a range of muscarinic receptor antagonists: atropine, 11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one (AQ-RA 741), darifenacin, methoctramine, oxybutynin, pirenzepine, secoverine, p-fluoro-hexahydrosiladifenidol (p-F-HHSiD) and zamifenacin. The pA_2 estimates are summarized in Table 1.

Table 1. pA_2 values for muscarinic antagonists in rabbit iris sphincter muscle and urinary bladder smooth muscle

Antagonist	pA_2(IRIS)		pA_2 (BLADDER)
Atropine	9.24±0.25		9.63±0.21
Pirenzepine	6.80±0.10	6.95 *	7.02±0.12
Methoctramine	5.80±0.17		5.85±0.15
p-F-HHSiD	7.42±0.21	6.43 *	7.71±0.20
Darifenacin	9.42±0.09		9.09±0.07
Zamifenacin	8.62±0.26		8.19±0.11
Secoverine	7.49±0.12	6.43 *	7.78±0.16
Oxybutynin	7.88±0.15		8.29±0.14
AQ-RA 741	6.25±0.05	6.22 *	6.96±0.11

Values shown are means ± s.e.m., n≥5.
Schild slopes were not significantly different from unity.
*: pA_2 values in iris from Bognar *et al.* (1992).

The data from this study show that the antagonist profile in RISM is similar, although not identical, to that in RUBSM. We conclude that, contrary to a previous proposal (Bognar *et al.*, 1992), M_3 muscarinic receptors mediate contraction of RISM.

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52P RAPID DESENSITIZATION OF MUSCARINIC M_3, BUT NOT HISTAMINE H_1, RECEPTORS IN PRIMARY HUMAN DETRUSOR SMOOTH MUSCLE CELLS

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In recent years, it has become evident that receptor desensitization plays a significant role in the regulation of cellular signal transduction. We have previously demonstrated rapid desensitization of muscarinic M_3 receptors in primary human detrusor smooth muscle (HDSM) cells (Marsh et al., 1995). Desensitization of the histamine H_1 receptors may be important in chronic inflammatory diseases of the bladder where histamine is present at persistently high concentrations (Kastrup et al., 1983). In this study we set out to compare the desensitization characteristics of the histamine H_1 and muscarinic M_3 receptors.

Primary cultures of HDSM were established from biopsies taken from six patients undergoing bladder endoscopy. All patients had no history of urinary tract dysfunction and had not been subjected to anticholinergic or antihistamine agents. Accumulation of total ^{3}H-inositol phosphates was measured as described previously (Harriss et al., 1995).

All cell lines demonstrated a concentration-dependent response to carbachol and histamine (EC_{50} = 16±2 μM and 3.7±0.3 μM respectively) and the maximum responses were 12.5±2.9 and 12.9±1.8 fold over basal levels respectively (n=6 detrusors). pK_B values for inhibition of carbachol responses by atropine, 4-DAMP and pirenzepine were 9.4±0.1, 9.0±0.1 and 6.9±0.2 respectively. pK_B values for inhibition of histamine responses by mepyramine and chlorpheniramine were 8.1±0.1 and 7.9±0.2 respectively while tiotidine 30μM and thioperamide 1μM had no effect (n=6 detrusors). A rapid and concentration-dependent desensitization of the carbachol response occurred following pretreatment with 100 μM carbachol. The response was reduced by 58±5% after pretreatment for 5 minutes and the maximum reduction was 89±2% after 30 minutes (n=6 detrusors). The IC_{50} for desensitization at 30 minutes was 6±1 μM carbachol. The onset of desensitization of the histamine response following pretreatment with 100 μM histamine was slow. No significant reduction was observed after pretreatment for 10, 20 and 30 minutes ($p>0.05$, one way ANOVA). After pretreatment for 40 minutes the response was reduced by 31±6% and a maximum reduction of 55±3% was only achieved after pretreatment for 2 hours (n=6 detrusors). The IC_{50} for desensitization at 2 hours was 18±7 μM histamine.

These results confirm our previous reports of the expression of muscarinic M_3 receptors which are susceptible to rapid desensitization. Histamine H_1 receptors are also present in these cells. In contrast to the muscarinic M_3 receptors, these histamine H_1 receptors seem to desensitise slowly and to a lesser extent. We aim to explore the possible factors underlying these dissimilarities in future studies.

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53P FACILITATION OF NEURONALLY-MEDIATED CONTRACTIONS OF THE RAT URINARY BLADDER *IN VITRO* BY TACHYKININ NK_1 AND NK_2 BUT NOT NK_3 RECEPTOR ACTIVATION

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Activation of bladder tachykinin receptors induces contraction (Hall *et al*, 1992), sensitisation of afferent neurones (Kibble & Morrison, 1996), modulation of neuromuscular transmission (Patra & Westfall, 1996) and activation of the micturition reflex (Bushfield *et al*, 1995). In this study we explored further the role of tachykinins in the bladder by examining the effects of tachykinin receptor activation on cholinergic and purinergic neuromuscular transmission in the rat detrusor *in vitro*.

Male CD rats were killed by stunning followed by cervical dislocation and bladders were removed, quartered and suspended between platinum electrodes under 1g initial tension in 15 ml tissue baths containing aerated Krebs solution. After a 1 hour equilibration period, tissues were subjected to electrical field stimulation (EFS) and contractile responses monitored. Trains (5s) of electrical pulses were delivered at intervals of 100s, pulse width 0.1msec with supramaximal voltage (40V) and submaximal frequency (4Hz). Contractile responses to EFS were abolished by pre-treatment with tetrodotoxin (1μM), indicating that they are neuronally-mediated. All studies were performed in bladders from 4 different animals and data shown are mean values ± s.e.mean or 95% CL.

Substance P-O-methylester (SPOME) (EC_{50} 21nM, 15-29nM) and β-ala^{8}NKA(4-10) (EC_{50} 49nM, 33-73nM) but not senktide (up to 3μM) induced concentration-dependent enhancement of the contractile response to EFS. The responses to SPOME were transient, being completely reversed within 8 minutes.The responses to β-ala^{8}NKA(4-10) were longer lasting and only partially reversed 50 minutes after treatment. The response to SPOME was blocked by the NK_1 antagonist SR-140,333, giving a pA_2 value of 7.7, slope = 1.46 (0.91, 1.83). The response to β-ala^{8}NKA(4-10) was blocked by the NK_2 antagonist, SR-48,968, giving a pA_2 value of 8.3 , slope = 1.0 (0.76, 2.1).

Atropine (1μM) produced a 54 ± 3.3% inhibition of the response to EFS but did not inhibit the potentiation by 300nM β-ala^{8}NKA(4-10) (76±3.1% enhancement) or SPOME (61±5.4%), indicating that the purinergic component of the response to EFS was potentiated. Similarly, αβ-methylene-ATP (1μM)-induced desensitisation of P_{2X} receptors produced a 39±4.5% inhibition of the response to EFS with no inhibition of the potentiation by 300nM β-ala^{8}NKA(4-10) (56±1.9% enhancement) or SPOME (55±6.0%), indicating that the cholinergic component of the response was also potentiated. Activation of NK_1 or NK_2 receptors did not potentiate the contractile effects of acetylcholine: EC_{50} values in the presence of vehicle, 300nM β-ala^{8}NKA(4-10) or SPOME were 246 (199-304), 209 (160-271) and 271 (209-351) nM, respectively. EC_{50} values for αβ-methylene-ATP in the presence of vehicle, 300nM β-ala^{8}NKA(4-10) or SPOME were 216 (93-502), 132 (58-304) and 197 (49-796) nM, respectively. These results suggest that NK_1 and NK_2 receptor activation results in pre-junctional potentiation of cholinergic and purinergic transmission with no effect on detrusor muscle sensitivity. Tachykinin NK_1 and NK_2 receptors may play a key role in the control of bladder function by prejunctional modulation of efferent neuronal activity, in addition to direct effects on smooth muscle tone and afferent nerve sensitivity.

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54P EFFECTS OF S-22068, AN IMIDAZOLINE DERIVATIVE, ON ACUTE GLUCOSE TOLERANCE AND PLASMA INSULIN LEVELS IN MICE

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Several α-adrenergic antagonists which are effective at stimulating insulin secretion also bind to non-adrenoceptor imidazoline binding sites. Binding studies in pancreas have suggested that the islet imidazoline binding site involved in insulin secretion is distinct from the I_1 and I_2 sites (Chan *et al.*, 1994). The imidazoline compound S-21663 (PMS 812), which has very low affinity for both α-adrenoceptors and I_1 and I_2 sites, has since been shown to improve glucose tolerance and stimulate insulin secretion in streptozotocin-diabetic rats (Wang *et al.*, 1996). Here we report the acute effects of the related imidazoline S-22068 (1,4-di-isopropyl-2-(4,5-dihydro-1-H imidazol-2-yl) piperazine) on glucose tolerance and insulin secretion in adult male CBA mice *in vivo*.

Glucose tolerance test: groups of 8 mice were challenged with glucose (1g kg^{-1} i.p.) following an overnight fast, and blood glucose (BGL) measured in samples of tail vein blood collected 60 min prior to glucose and at 0, 30, 60, 90 and 120 min thereafter. S 22068 (24 mg kg^{-1}), saline, or gliclazide (2 mg kg^{-1}) as a positive control, were administered p.o. immediately following the first blood sampling. Insulin sensitivity test: non-fasted mice received soluble insulin (2.5 IU kg^{-1} i.p.) 60 min following oral dosing with drugs or saline; BGLs were determined prior to dosing and at 60 min intervals thereafter. Endogenous insulin levels were measured by radioimmuno-assay in plasma samples collected immediately prior to drug dosing and at 30, 60, 120 and 180 min subsequently. No more than two samples were collected from a single animal. Differences between independent data groups, expressed as means ± s.e.mean, were analysed by Students t- test.

S 22068 significantly attenuated the rise in BGL following a glucose challenge: the mean rise was 3.64 ± 0.39 mM compared to 5.41 ± 0.58 mM in controls ($p < 0.05$), and was similar to that observed after gliclazide (3.01 ± 0.82 mM). In contrast to gliclazide however, S 22068 did not affect the basal BGL when administered to fed mice. The mean change in BGL from 0 to 60 min was +1.53 ± 0.44 mM compared to +0.44 ± 0.47 mM in controls and -2.67 ± 0.11 mM ($p < 0.001$) after gliclazide. The BGL following insulin injection was significantly lower at 120 min after both S 22068 (3.27 ± 0.44 mM) and gliclazide (4.45 ± 0.54 mM) compared to controls (5.73 ± 0.37); $p < 0.01$ and $p < 0.05$ respectively. Endogenous plasma insulin levels in fed mice were not significantly altered at any time point following S22068 treatment, but increased by 200-300% above zero time (control) at 30 min following gliclazide.

We conclude that S 22068, at an oral dose which is equipotent with the the sulphonylurea gliclazide in terms of improving glucose tolerance, is not acting by stimulating insulin release. Also, unlike gliclazide, S 22068 is not intrinsically hypoglycaemic and may therefore be acting at peripheral tissue sites to increase insulin sensitivity.

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55P DEVE LOPMENT AND CHARACTERIZATION OF POLYCLONAL ANTI-EFAROXAN ANTIBODIES FOR USE IN PURIFICATION OF ISLET IMIDAZOLINE RECEPTORS

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Efaroxan is the best characterized imidazoline insulin secretagogue but its receptor in the endocrine pancreas remains to be isolated and cloned (Morgan *et al.*, 1995). We have developed a photoactivable azide derivative of efaroxan (Az-Ef) to label the imidazoline receptor, and now describe the production and characterization of polyclonal anti-efaroxan antibodies for the detection of cross-linked efaroxan-receptor molecule(s).

5'-Amino-efaroxan (Am-Ef) coupled to BSA (with glutaraldehyde) was used as immunogen. Primary subcutaneous injections in female New Zealand white rabbits (3-5kg body weight) of the immunogen emulsified in Freund's complete adjuvant was followed at 3-4 week intervals by booster injections in Freund's incomplete adjuvant. Marginal ear vein bleeds were taken after each booster injection. The presence of anti-efaroxan antibodies in the sera was determined by ELISA. This ELISA was used to assess the specificity of the antibodies in competition with a series of α2-adrenoceptor ligands and imidazoline compounds.

Adrenaline, noradrenaline and yohimbine did not displace antibody binding to the coated Ef-protein conjugate, demonstrating that the antibodies do not recognise these ligands. In contrast, a series of imidazoline insulin secretagogues were able to displace with the following rank order; efaroxan > midaglizole > clonidine > antazoline > phentolamine > RX821002. Efaroxan, Az-Ef and Am-Ef were all able to displace antibody binding with a similar potency (EC_{50} approx. 5μM at 1:100 antiserum). The imidazoline antagonist KU14R (imidazole-efaroxan) was also recognised by the antiserum.

The putative endogenous ligand of imidazoline receptors Clonidine-Displacing Substance (CDS), which has recently been shown to be a potent insulin secretagogue (Chan *et al.*, 1997), was also recognised by the antiserum. A crude methanolic extract of rat brain containing CDS was able to displace the binding of anti-efaroxan antibodies to the coating efaroxan conjugate with an EC_{50} approximating to 2 units (One unit of CDS activity is defined as the amount required to displace 50% of specifically bound [^{3}H]clonidine (2nM) in the presence of 10μM noradrenaline, from rat brain membranes under standard conditions (Atlas & Burstein, 1984)). Although the structure of CDS still awaits confirmation, it has been proposed that its molecular weight approximates to 600Da. One unit of CDS activity has been calculated to correspond to approximately 5pmoles of active substance (Atlas, 1995) and thus, this implies that the EC_{50} value of 2 units of CDS in the ELISA correlates to 100nM. Meanwhile, agmatine, (decarboxylated arginine) an amine which binds to α2-adrenoceptors and imidazoline binding sites (Li *et al.*, 1994), was not recognised by the antiserum.

In conclusion, we have developed highly specific polyclonal anti-efaroxan antibodies which recognize Az-Ef with high affinity, and may be useful for isolation of receptor protein(s) mediating imidazoline regulation of insulin release. Furthermore, this polyclonal antiserum also recognise the putative endogenous ligand CDS and so, therefore, may be used in the purification of the putative endogenous ligand of imidazoline receptors.

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56P LEPTIN ACTIVATION OF K_{ATP} CHANNELS IS MIMICKED BY TYROSINE KINASE INHIBITORS IN CRI-G1 INSULIN SECRETING CELLS

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Leptin, the ob gene product, which regulates body weight, has effects on central and peripheral tissues, including pancreatic beta cells (Emilsson et al, 1997). Recently we have shown that leptin hyperpolarises CRI-G1 insulin secreting cells via activation of ATP-sensitive K^+ (K_{ATP}) channels (Harvey et al, 1997). In this study whole cell recording was used to examine the mechanisms underlying this action of leptin.

Recording pipettes contained (mM): 140 KCl, 0.6 $MgCl_2$, 2.73 $CaCl_2$, 10 EGTA, 5 ATP and 10 HEPES pH 7.2 (free Ca^{2+} of 0.1 μM) and the bath solution comprised (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES pH 7.4. Under current clamp conditions with 5 mM ATP in the pipette, the resting membrane potential (RMP) of CRI-G1 cells was -37 ± 1.4 mV (mean ± SEM; n=31). Application of the broad spectrum protein kinase inhibitor H-7 (10 μM) for 10-15 min had no effect on the RMP of CRI-G1 cells; the mean RMP in the presence of H-7 was -33 ± 2.0 mV (n=4). Furthermore, coapplication of leptin (10 nM) with H-7 (10 μM) hyperpolarised the cells to -68 ± 4.2 mV (n=4). Under voltage clamp conditions leptin increased the slope conductance from 0.56 ± 0.10 nS to 2.56 ± 0.34 nS and the reversal potential associated with this conductance increase was -79 ± 0.86 mV (n=4).

In contrast, application of the tyrosine kinase inhibitors, tyrphostin B42 (10 μM) and genistein (10 μM) hyperpolarised CRI-G1 cells to -73 ± 1.7 mV (n=10) and -58 ± 3.1 mV (n=8), respectively. Both these agents increased the slope conductance from 0.53 ± 0.06 nS to 5.27 ± 0.92 nS (n=10, tyrphostin) and from 0.56 ± 0.06 nS to 2.4 ± 0.68 nS (n=8; genistein) and the reversal potential associated with this increase in conductance was -79 ± 0.9 mV (n=10; tyrphostin B42) and -78 ± 0.8 mV (n=8; genistein), consistent with the activation of a K^+ conductance. The sulphonylurea, tolbutamide (100 μM) completely reversed the hyperpolarisation and increase in slope conductance induced by both tyrosine kinase inhibitors to pre-drug levels. The reversal potentials for this action of tolbutamide were -79 ± 1.1 (n=6; tyrphostin B42) and -78 ± 1.4 (n=4; genistein).

Application of leptin (10 nM), following the tyrphostin-induced hyperpolarisation had no significant effect on the RMP or conductance of CRI-G1 cells, such that the mean RMP was -76 ± 2.3 mV and -78 ± 2.9 mV (n=5; $p > 0.05$) and the slope conductance was 3.9 ± 0.6 nS and 4.2 ± 0.5 nS (n=5; $p>0.05$) in the absence and presence of leptin, respectively. However, in the presence of genistein which itself hyperpolarised cells to -52 ± 3.5 mV, leptin (10 nM) further hyperpolarised the cells to -80 ± 2.5 mV (n=4; $p<0.05$). This action of leptin was associated with an increase in conductance from 1.5 ± 0.4 nS to 6.5 ± 3.4 nS (n=4) and was completely reversed by tolbutamide (100 μM; n=4).

In conclusion, leptin activation of K_{ATP} channels in CRI-G1 cells is mimicked by two different inhibitors of tyrosine kinases. Further investigation is required to determine the mechanism by which leptin activates K_{ATP} channels.

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