1P PHORBOL ESTER TREATMENT INHIBITS PHOSPHOINOSITIDE 3-KINASE ACTIVATION BY, AND ASSOCIATION WITH, THE T CELL MOLECULE CD28

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Phosphoinositide 3-kinase (PI3K) appears to play a pivotal role in the regulation of CD28-dependent IL-2 production from T cells (Ward *et al.* 1996). Hence, CD28 ligation by its natural ligand B7.1 or antibody leads to increases in PI3K activity and accumulation of PtdInd(3,4,5)P_3 which is believed to act as an important intracellular mediator (Ward *et al.* 1996). The CD28 cytoplasmic tail contains a consensus site (^{177}TPR) for the serine/threonine kinase protein kinase C (PKC), which lies adjacent to the ^{173}YMNM motif responsible for coupling CD28 to PI3K. The precise role of this PKC phosphorylation motif is unknown, although PMA can supply the additional signal necessary for CD28-dependent IL-2 production, and thus, may modulate the enzymatic activity or physical association of accessory signalling proteins with the CD28 cytoplasmic tail. We investigated therefore, whether PKC can modulate PI3K binding to, and activation by, CD28.

Co-sedimentation of chinese hamster cell (CHO)-B7.1$^+$/B7.2$^+$ with [^{32}P]-labeled Jurkat cells at a ratio of 1:3, resulted in the accumulation of PtdIns(3,4,5)P_3 as determined by anion exchange HPLC (Ward *et al.* 1995). Pre-treatment with PMA (0.5-100 ng/ml) inhibited by 75.3±5.5% (mean±SEM, n=4) the B7.1-induced accumulation of PtdIns(3,4,5)P_3. The non-PKC activating phorbol ester 4α-phorbol (0.5-100 ng/ml) had no effect on CD28-induced PtdIns(3,4,5)P_3 accumulation. Complete inhibition of PtdIns(3,4,5)P_3 accumulation by PMA was not achieved, at least at the concentrations used. This may indicate that there may be a phorbol ester-resistant component of CD28-activated PI3K. Immunoblotting experiments revealed that B7.1- or B7.2-induced association of CD28 immunoprecipitates with the p85 sub-unit of PI3K was also partially inhibited by PMA (0.5-50 ng/ml). This correlated with a decrease in associated *in vitro* PI3K activity present in CD28 immunoprecipitates following activation. Pre-treatment with 0.5-50 μM of the PKC inhibitor Ro-31/8220 (Wilkinson *et al.* 1993) prior to the addition of PMA, prevented the inhibitory effects of PMA on B7.1-induced PtdIns(3,4,5)P_3 accumulation and CD28:PI3K interactions. Since we have previously reported that B7.1 stimulates serine/threonine phosphorylation of CD28 (Wilson *et al.* 1995), we investigated whether phosphorylation of CD28 is mediated by PKC. However, PMA (5-500 ng/ml) in the absence or presence of 10 μM ionomycin, does not detectably phosphorylate CD28 from resting or B7.1-activated [^{32}P]-labelled Jurkat cells. Ro-31/8220 had no effect on the phosphorylation of CD28 induced by B7.1 or B7.2. The disruption of CD28:PI3K interactions by PMA may not be due to direct PKC-mediated CD28 phosphorylation, but may instead be due to the action of PMA-activated PKC on PI3K itself, since p85 has been reported to be phosphorylated by PMA (Reif *et al.* 1993). The physiological relevence of these observations is unclear, although this mechanism may act as a negative-feedback signal to limit CD28-mediated PI3K activation.

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2P INTERLEUKIN-13 INHIBITS NITRIC OXIDE SYNTHASE INDUCTION BY THE ACTIVATION OF PHOSPHATIDYL-INOSITOL-3-KINASE IN HT-29 CELLS

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As part of our programme to understand the regulation of human inducible nitric oxide synthase (iNOS), we have characterised its induction in a colonic epithelial cell line, HT-29 (Kolios *et al*, 1995), cellular distribution in ulcerative colitis (Kolios *et al*, 1996a) and inhibition by the cytokine, IL-13 (Kolios *et al*, 1996b). We have shown that low concentrations of IL-13 potently inhibit nitrite generation and iNOS protein expression by HT-29 cells, while higher concentrations in addition block iNOS mRNA induced by the optimum combination IL-1α/IFN-γ/TNF-α. We now provide evidence to indicate that phosphatidylinositol-3-kinase (PI-3K) activity is a key intermediate for the inhibitory activity of IL-13.

Stimulation of HT-29 cells with a cytokine cocktail (IL-1α, TNF-α and IFN-γ) for 24 hours in the presence of either vehicle, or 30 ng/ml IL-13 plus wortmannin (Wo) vehicle, or 30 ng/ml IL-13 plus 300nM Wo, or 30 ng/ml IL-13 plus 100nM Wo, or 30 ng/ml IL-13 plus 30nM Wo produced 142.09 ± 2.82 nM, 73.04 ± 5.28 nM, 139.33 ± 4.19 nM, 109.42 ± 5.43 nM and 67.80 ± 1.76 nM nitrite/10^6 cells (mean±s.e.mean, n=3), respectively. In addition, Wo at 30, 100, 300nM reversed IL-13-induced inhibition of iNOS mRNA expression, determined by densitometric analysis as described (Kolios *et al*, 1995).

To determine directly whether IL-13 was an activator of PI-3K, HT-29 cell lysates pre-treated with or without IL-13 were immunoprecipitated with anti p85 PI-3K antibody followed by an *in vitro* lipid kinase activity (Ward *et al*, 1995). IL-13 produced a concentration (0.3, 3, 10, 30 ng/ml) and time dependent (30s, 3, 5, 10 mins) activation of PI-3K, which was inhibited by 33, 57, and 85% compared to maximally stimulated (30ng IL-13 at 30 secs) cells by the addition of 30, 100 and 300nM Wo, respectively.

To study PI-3K activity *in vivo*, the HT-29 cells were labelled with ^{32}P-orthophosphate, stimulated and the lipids extracted as described previously (Ward *et al*, 1995). An increase in PI-3K activity, as defined by the increase in the levels of the products phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate, showed a 292 ± 25.86 % (mean±s.e.mean, n=3) increase from control at 30 seconds in the case of PI (3,4,5) P_3.

This study has shown that the inhibition of iNOS transcription resulting in reduced NO production is induced by IL-13 and mediated by the activation of PI-3K in HT-29 cells.

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3P DIFFERENTIAL CONTROL OF L-ARGININE UPTAKE, ARGINASE AND NITRIC OXIDE SYNTHASE IN RABBIT ALVEOLAR MACROPHAGES (AMs)

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L-Arginine serves as substrate of nitric oxide synthase (NOS) and arginase, enzymes which appear to play a particular role in AMs. Whereas arginase appears to be expressed constitutively in AMs of different spezies, NOS has to be induced by bacterial toxins and/or cytokines. Protein kinase C (PKC) and adenylyl cyclase are important cellular signal transduction pathways, also in AMs. Therefore, the possible significance of these pathways for the regulation of arginine uptake and its metabolism by arginase and NOS was studied in rabbit AMs.

Freshly prepared rabbit AMs were resuspended in DMEM-F12 medium containing 5 % FCS and disseminated ($3*10^6$ cells per well). L-Arginine uptake and its metabolism were studied either after 2 hr adherence (acute) or after 20 hr culture. Arginine uptake was determined by measuring the cellular radioactivity after 2 min of incubation with ^{3}H-L-arginine (37 kBq, 100 nM), the metabolism by NOS and arginase by measuring the accumulation of ^{3}H-L-citrulline and ^{3}H-L-ornithine in incubation media during 1 hr incubation with ^{3}H-L-arginine.Values are means± s.e.m. of at least 6 experiments, absolute values are expressed per $3*10^6$ cells.

A significant formation of citrulline was not observed under any of the conditions studied. Acute arginine uptake amounted to 8,700±1,200 DPM, it was reduced by 81±4 % after culture. Presence of LPS (1 μg/ml) during the culture period slightly enhanced arginine uptake compared to the reduced level after culture (by 77±39 %). Acute ornithine formation amounted to 135,300±9,150 DPM, but it was enhanced by 119±2 % after culture and further increased by 36±5 % when LPS had been present. Forskolin (10 μM) present during the culture period caused a further reduction of arginine uptake by 49±3 %, but an increase in ornithine formation by 47±4 %. In the presence of LPS, forskolin stimulated ornithine formation by 166±57 %. The phorbol ester PMA (100 nM, present during culture) caused a 20±7 % reduction in ornithine formation, but enhanced arginine uptake by 1,860±254 % (compared to culture control value). α-PMA, a phorbol ester which does not activate PKC, had no effects. The effect of PMA on arginine uptake was inhibited by staurosporine (100 nM), cycloheximide (10 μM) and actinomycin (5 μg/ml) by 58±5, 68±14 and 99±0.2 %, respectively. When PMA was added after the 20 hr culture period for 0.25-6 hr, arginine uptake was enhanced by 149±44 % after 0.5 hr and maximally by 662±127 % after 2 hr. This acute effect of PMA was blocked by staurosporine, but not affected by cycloheximide or actinomycin.

In conclusion, in rabbit AMs arginine uptake and arginase activity are regulated by PKC- and cAMP-dependent pathways in an opposite direction. NOS activity was not detected and appears not to be induced by any of these pathways. In a time-dependent manner, activation of PKC appears to mediate rapid activation of a preexisting, silent arginine transporter as well as to en-hance its expression.

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4P AGONIST-INDEPENDENT INCREASES IN INOSITOL 1,4,5-TRISPHOSPHATE FOLLOWING PERTUSSIS TOXIN TREATMENT OF BABY HAMSTER KIDNEY CELLS EXPRESSING RECOMBINANT TYPE 1α mGluRs

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Of the eight G-protein coupled metabotropic glutamate receptor (mGluR) subtypes cloned to date, mGluR1 and mGluR5 preferentially couple to phospholipase C (PLC). We have recently shown that the basal (agonist-independent) activity of the type 1α mGluR expressed in baby hamster kidney (BHK) cells is dramatically increased following pre-treatment with pertussis toxin (PTx) and the resulting elevated phosphoinositide turnover can by reduced by mGluR antagonists (Carruthers *et al.*, 1996). Here, we have investigated whether PTx pre-treatment affects the accumulation of the second messenger Ins(1,4,5)P_3 in BHK-mGluR1α cells under basal and agonist-stimulated conditions, and whether the mGluR antagonist α-methyl-4-carboxyphenylglycine (MCPG) can reverse these effects.

BHK-mGluR1α cells (passage 3-40) were maintained in DMEM, 5% dialysed foetal calf serum, 2 mM glutamine, 50 μg ml^{-1} gentamicin, 0.5 mg ml^{-1} G418 and 1 μM methotrexate. Cells were washed in oxygenated Krebs-Henseleit buffer (KHB) and preincubated with KHB for 15 min ± mGluR antagonist. Agonists or vehicle were added and cells incubated at 37°C for 0-300 s and experiments terminated with trichloroacetic acid. Ins(1,4,5)P_3 was measured in neutral cell extracts (Challiss *et al.*, 1994). Where indicated, cell cultures were pre-incubated with PTx (100 ng ml^{-1}) for 22-24 h.

PTx pretreatment caused an approximate 2-fold increase in basal Ins(1,4,5)P_3 mass levels in BHK-mGluR1α cells (-PTx, 66 ± 6; +PTx, 130 ± 24 pmol mg^{-1} protein; n=6, P<0.05, Student's unpaired t-test). Challenge of control and PTx-pretreated cells with L-glutamate (300 μM) evoked a similar time-course of changes in Ins(1,4,5)P_3 accumulation (peak glutamate-stimulated level at 30 s, -PTx, 178 ± 20; +PTx, 276 ± 18 pmol mg^{-1} protein, n=3). For other mGluR agonists, quisqualate (30 μM) stimulated an Ins(1,4,5)P_3 mass accumulation essentially identical to that evoked by glutamate, whilst 1**S**,3**R**-ACPD (300 μM) failed to cause a significant increase in Ins(1,4,5)P_3 accumulation, in control or PTx-pretreated cells.

The (+)-enantiomer of MCPG (1 mM) decreased basal Ins(1,4,5)P_3 levels in both control (by 17.8 ± 6.4%) and PTx-pretreated (by 40.3 ± 5.1%) BHK-mGluR1α cells, whilst the (-)-enantiomer was without effect. In the case of PTx-pretreated cells, the (+)-MCPG inhibitory effect attained statistical significance (PTx basal, 183 ± 14; +MCPG, 107 ± 6 pmol mg^{-1} protein, n=3, P<0.01, unpaired Student's t-test), reducing basal Ins(1,4,5)P_3 to levels approaching those observed in control cells. (+)-MCPG (0.03-1 mM) concentration-dependently inhibited glutamate (50 μM)-stimulated Ins(1,4,5)P_3 mass accumulation in both control and PTx-pretreated cells.

These data extend our previous observations on PTx effects on basal rates of phosphoinositide hydrolysis in these cells (Carruthers *et al.*, 1996), and are consistent with a 'constitutive' activity of recombinant mGluR1α following PTx treatment of BHK cells which is manifest at the level of Ins(1,4,5)P_3. The possible effects of chronic elevation of Ins(1,4,5)P_3 levels in PTx-pretreated BHK-mGluR1α cells are under investigation.

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5P Ca^{2+}-INDEPENDENT CALMODULIN BINDING TO PURIFIED INOSITOL 1,4,5-TRISPHOSPHATE ($InsP_3$) RECEPTORS INHIBITS $InsP_3$ BINDING

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$InsP_3$ receptors mediate the release of Ca^{2+} from intracellular stores and are regulated by a variety of diffusible messengers, protein kinases and accessory proteins. We previously demonstrated that the $InsP_3$ binding properties of purified cerebellar $InsP_3$ receptors are similar whether assayed by conventional centrifugation binding assays or Scintillation Proximity Assay (SPA†) (Patel *et al.*, 1996a). Using SPA, we have also characterised both Ca^{2+}-independent and Ca^{2+}-dependent binding of calmodulin to $InsP_3$ receptors (Patel *et al.*, 1996b). Here, we examine the effects of calmodulin on binding of $InsP_3$ to its receptor.

Purified $InsP_3$ receptors were attached to wheat germ agglutinin-coated SPA beads as described previously (Patel *et al.*, 1996a). Binding of [^{3}H]$InsP_3$ (2 - 3 nM; 54 Ci/mmol) to the $InsP_3$ receptor-beads (10 mg beads/ml) was performed at 2°C in binding buffer containing 20 mM PIPES (pH 7), 1 mM EGTA, 5 mM KH_2PO_4 and 0.1% surfact-amps X-100 with the free $[Ca^{2+}]$ ($[Ca^{2+}]_m$) buffered between 2 nM and 30 μM. Non-specific binding (typically 10% of total binding) was determined in the presence of 1 μM $InsP_3$. Samples (200 μl) were counted for 60 s at 2°C.

In Ca^{2+}-free binding buffer, [^{3}H]$InsP_3$ bound specifically to $InsP_3$-receptor beads, but not to the beads alone. Calmodulin (50 μM) inhibited specific binding of [^{3}H]$InsP_3$ by 85 ± 4 % (n = 3). Half-maximal inhibition occurred at a calmodulin concentration of 3.3 ± 0.6 μM (n_H = 0.78 ± 0.06; n = 3). From equilibrium competition-binding experiments, the K_d for $InsP_3$ was 6.2 ± 0.4 nM (n_H = 0.95 ± 0.04; n = 3) in the absence of calmodulin and 15.2 ± 1 nM (n_H = 0.97 ± 0.05; n = 3) in the presence of 3 μM calmodulin.

We previously demonstrated that Ca^{2+} reversibly stimulates [^{125}I]calmodulin binding (Patel *et al.*, 1996b). Increasing $[Ca^{2+}]_m$ from 2 nM to 30 μM caused a small reduction in binding of [^{3}H]$InsP_3$ (15 ± 4 %; n = 3) to the receptor-beads in the absence of calmodulin. Ca^{2+} did not, however, affect the response to a submaximal calmodulin concentration; calmodulin (3 μM), inhibited $InsP_3$ binding by 57 ± 0.8 % (n = 3) when $[Ca^{2+}]_m$ was 2 nM and by 54 ± 3 % (n = 3) when $[Ca^{2+}]_m$ was 30 μM. Similar results were obtained at a range of intermediate $[Ca^{2+}]_m$.

We conclude that calmodulin inhibits binding of $InsP_3$ to purified $InsP_3$ receptors and that the effects of calmodulin are unaffected by increases in $[Ca^{2+}]_m$.

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6P INVERSE AGONIST ACTIVITY OF ATROPINE AT HUMAN M_2 AND M_4 MUSCARINIC ACETYLCHOLINE RECEPTORS REVEALED BY [^{35}S]-GTPγS BINDING

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The expansion of the classical ternary complex model to include a receptor isomerization step from the inactive to the active G-protein coupled state allows antagonists to be classified as either neutral antagonists or inverse agonists (Milligan et al., 1995). Here we present evidence that human M_2 and human M_4 muscarinic acetylcholine receptors expressed in Chinese hamster ovary cells display constitutive activity and that atropine acts as an inverse agonist.

The binding of [^{35}S]-GTPγS to the G-protein alpha subunit has been used as a measure of the activation of a number of G-protein coupled receptors including the muscarinic family (Lazareno et al., 1993). This method was adapted to investigate inverse and traditional agonists. Optimum assay conditions were found to be 30 min incubations at 30°C with 70 pM [^{35}S]-GTPγS, 10 μM GDP, 10 mM $MgCl_2$, 100 mM NaCl and 100μg/ml protein.

Expression levels were assessed using [^{3}H]-NMS saturation binding (n=5): Mean B_{max} ± SEM (fmol/mg) were found to be 1389±66 for CHO-M_4, 868±18 for the higher expressing M_2 clone CHO-SLM_2 and 604±80 for CHO-M_2. Upon stimulation with a maximal concentration of methacholine (1 mM), large increases in [^{35}S]-GTPγS binding above basal were observed in CHO-M_2 196±11.3%, CHO-SLM_2 224.2±5.2% and CHO-M_4 267±9.2% (all increases over basal, n=4). EC_{50} values for methacholine were: CHO-SLM_2 0.21±0.02 μM, CHO-M_2 0.43±0.03μM, CHO-M_4 0.90±0.09μM (n=4). A maximal concentration of atropine (1 μM) produced a significant ($p<0.01$ using ANOVA one-way) decrease in basal [^{35}S]-GTPγS binding in each of the cell lines (Table 1). Atropine decreased [^{35}S]-GTPγS binding in a concentration dependent manner with apparent IC_{50} values shown in Table 1. These values reflect the reported affinity constants for atropine and apparent affinity values for methacholine at these receptors (Caulfield , 1993).

Table 1. Measurement of atropine inhibited [^{35}S]GTPγS binding (n=4)

Cell line	Mean IC_{50} ± SEM (nM)	Maximum decrease of basal ± SEM
CHO-M_4	2.89±1.00	9.4±1.8
CHO-SLM_2	1.14±0.34	22.7±0.5
CHO-M_2	1.58±0.25	15.6±1.6

We conclude that [^{35}S-]GTPγS binding can be used in this recombinant system as a quantitative measure of both agonists and inverse agonist activity at the human M_2 and human M_4 muscarinic acetylcholine receptors. Agonists display a receptor reserve for this response upon receptor binding whereas inverse agonists do not.

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7P ROLE OF G-PROTEIN βγ SUBUNITS IN THE AUGMENTATION BY NEUROPEPTIDE Y-Y1 RECEPTORS OF ATP-MEDIATED INCREASES IN ARACHIDONIC ACID RELEASE FROM CHO-K1 CELLS

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We have previously shown that $G_{i/o}$ protein-coupled receptors, such as the transfected neuropeptide Y-Y1 receptor, can augment α_{1B}-adrenoceptor-stimulated arachidonic acid (AA) release from CHO-K1 cells (Selbie, et al., 1995). This augmentation was shown to be inhibited by pertussis toxin, suggesting a role for $G_{i/o}$ heterotrimeric proteins in such augmentation of $G_{q/11}$ protein-coupled receptor effects. Recent observations of βγ sensitive phospholipase C (PLC) and phospholipase A2 (PLA2) isoforms (Stephens, et al., 1994) indicate that the G protein βγ subunits may mediate some of the effects of $G_{i/o}$ on intracellular signalling. In this communication, we have investigated the role of these subunits in the augmentation by NPY Y-Y1 receptor stimulation on ATP-mediated release of AA by transient transfection of proteins which can scavenge βγ subunits.

[^{3}H]-AA was measured from CHO-K1 cells stably expressing the human neuropeptide(NPY) Y-Y1 receptor (CHO-Y1R), as described previously (Selbie, et al., 1995). Cells were transiently transfected, using a modified calcium phosphate precipitation method (Chen and Okayama, 1987), with either a minigene construst in the plasmid pRK5 containing residues G495-L689 of the C-terminus of β-adrenoceptor kinase 1 (βARK1, the putative βγ subunit binding domain of βARK1;Koch, et al., 1995) or with the control plasmids pRK5 and pcDNA3 (Invitrogen). Expression of the βARK1 minigene peptide was confirmed by Western blot analysis. Whole cell binding of [^{125}I]-peptide YY was measured as described previously (Selbie, et al., 1995].

In cells transfected with the control plasmid, stimulation of the NPY-Y1 receptor with the agonist peptide YY (PYY; 100nM) had no significant affect on PLA2 activity as measured by [^{3}H]-AA release from CHO-Y1R cells (1.08±0.11 fold increase over basal; n=4 experiments performed in triplicate). ATP (10μM) produced a 2.55±0.12 fold increase in [^{3}H]-AA release over basal (n=4) which was increased to 3.60±0.15 fold in the presense of PYY(100nM; n=4).

Transient expression in CHO-Y1R cells of the βARK1 minigene had no significant effect on either ATP (10μM)-stimulated AA release (2.1±0.18 fold increase in βARK1 minigene transfected cells and 2.55±0.12 fold in control vector transfected cells; n=4) or the response to PYY (100nM) alone (1.10±0.01 fold and 1.08±0.11 fold respectively; n=4). βARK1 minigene expression significantly inhibited the PYY-mediated augmentation of ATP-stimulated [^{3}H]-AA release. Co-incubation with PYY(100nM) and ATP(10μM) resulted in a 2.44±0.29 fold increase in βARK1 transfected cells compared to 3.60+0.15 in control cells (n=4)($p<0.01$, student's t-test). These data represented a 42.4%±9.3% increase over the ATP response(10μM) in control cells compared to a 15.3%±9.7% increase over the ATP(10μM) response in βARK1 transfected cells. These inhibitory effects did not appear to be a result of receptor down-regulation, since expression of βARK1 minigene did not greatly effect either ATP-stimulated AA release (as above) or NPY-Y1 receptor number. (B_{max}=2.8x10^4±0.55 and 1.9x10^4±0.21 receptors/cell; K_D= 3.06±0.54 and 2.29±0.87 nM; for n=3 in βARK1 transfected and control cells, respectively). The results of this study suggest that G protein βγ subunits contribute to the synergistic interactions between $G_{i/o}$ and $G_{q/11}$ protein-coupled receptor systems.

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8P SECOND MESSENGER RESPONSES OF THE RIGID GLUTAMATE ANALOGUES (RS)-DHPG AND (RS)-DHBAP IN GUINEA-PIG CEREBRAL CORTEX SLICES

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Glutamate receptors may be divided into the two superfamilies of G-protein-coupled and ligand-gated ion channel receptors. The former are more commonly referred to as metabotropic glutamate receptors (mGluR) which may couple to either stimulation of phosphoinositide turnover (Group I) or inhibition of cyclic AMP generation (Groups II and III, Knöpfel *et al.*, 1995). The rigid glutamate analogue (RS)-3,5-dihydroxyphenylglycine, (RS)-DHPG has been suggested to be a selective Group I mGluR agonist in rat brain (Schoepp *et al.*, 1994), and so we have analysed second messenger responses to this agent, together with its' phosphinic acid analogue (RS)-amino(3,5-dihydroxyphenyl)methylphosphinic acid, (RS)-DHBAP, synthesised as previously described (Boyd *et al.*, 1995).

Generation of [^{3}H]-inositol phosphates and [^{3}H]-cyclic AMP was assessed in guinea-pig (Dunkin-Hartley, 400-700g, either sex) cerebral cortex slices prelabelled with [^{3}H]-inositol or [^{3}H]-adenine, respectively (Alexander et 1989). All experiments were conducted in quadruplicate on at least three separate occasions.

Both (RS)-DHPG and (RS)-DHBAP evoked concentration-dependent accumulations of [^{3}H]-inositol phosphates with pEC_{50} values of 4.93 ± 0.12 and 4.60 ± 0.06, with maximal responses of 2.32 ± 0.31 and 4.13 ± 0.88 -fold basal, respectively. When analysed in the same experiment, maximal responses to (RS)-DHPG and (RS)-DHBAP were not different from one another.

(RS)-DHPG and (RS)-DHBAP evoked concentration-dependent enhancements of [^{3}H]-cyclic AMP accumulation in the presence of the stable adenosine analogue 5'-*N*-ethylcarboxamidoadenosine (NECA, 10 μM) with pEC_{50} values of 5.14 ± 0.18 and 4.91 ± 0.01, with maximal responses of 604 ± 118 and 178 ± 9 % NECA response, respectively. In contrast the response to 30 μM forskolin was not significantly inhibited by either agent at concentrations up to 1 mM (92 ± 7 and 143 ± 15 % forskolin response, respectively.

These results indicate that the Group I mGluR selectivity of RS-DHPG is maintained in the guinea-pig cerebral cortex. These results also allow the identification of a phosphinate glutamate analogue as a novel lead compound to investigate mGluR function.

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9P CYCLIC GMP-DEPENDENT PROTEIN KINASE ACTIVITY DOES NOT CORRELATE WITH NEGATIVE INOTROPY IN RAT CARDIOMYOCYTES

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It has been hypothesized that activation of cGMP-dependent protein kinase (PKG) by muscarinic receptor agonists is responsible for their ability to decrease contractile force in mammalian ventricles and that sodium nitroprusside (SNP) fails to decrease contractility, even though it increases cGMP levels, because it does not activate the kinase (Lincoln and Keely, 1981). This hypothesis is based on experimental results in intact rat hearts and it is not clear whether the PKG activity measured in those experiments was actually present in the cardiomyocytes themselves. The object of the present study was to test the above hypothesis using freshly isolated rat ventricular cardiomyocytes. Ventricular cardiomyocytes were prepared as previously described (MacDonell *et al.*, 1995). Cells isolated from a single heart were divided into two aliquots. One was treated with 1 nM isoprenaline alone and the other was treated with 1 nM isoprenaline plus either 10 μM carbachol for 2 min, 10 μM or 100 μM SNP for 6 min or 100 nM atrial natriuretic peptide (ANP) for 6 min. Soluble PKG activity was then measured in the cardiomyocytes using an improved PKG assay described by Jiang *et al.* (1992) which makes use of a novel substrate for PKG, BPDEtide.

As shown in Table 1, carbachol did not activate soluble PKG in isolated cardiomyocytes. This concentration of carbachol had previously been shown to induce a significant negative inotropic effect in isoprenaline-stimulated cardiomyocytes (MacDonell *et al.*, 1995). On the contrary, SNP and ANP significantly activated PKG. SNP has no effect on the contractility of isoprenaline-stimulated cardiomyocytes (MacDonell *et al.*, 1995) and several studies have failed to demonstrate an effect of ANP on contractility in rat cardiac preparations (e.g., Hütter, 1991). These results suggest that PKG activation is not responsible for the negative inotropic effects of muscarinic receptor agonists in isoprenaline-stimulated rat ventricular cardiomyocytes.

Table 1. Effects of carbachol, SNP and ANP on soluble PKG activity in rat isolated cardiomyocytes.

Treatment	Control PKG activity ratio[1]	Treated PKG activity ratio[1]	n
Carbachol 10 μM	0.19 ± 0.04	0.19 ± 0.02	6
SNP 10 μM	0.21 ± 0.05	0.31 ± 0.08	5
SNP 100 μM	0.10 ± 0.04	0.32 ± 0.08 *	6
ANP 100 nM	0.09 ± 0.02	0.31 ± 0.03 *	5

[1] endogenously activated PKG /total PKG activity.
* $P<0.05$, paired Student's *t*-test.

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10P PROTEINASE-ACTIVATED RECEPTOR-2-DEPENDENT ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES IN RAT AORTIC SMOOTH MUSCLE CELLS

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Proteinase-activated receptor-2 (PAR-2) is a novel G protein-coupled receptor which is activated by an unusual mechanism first described for the thrombin receptor. Proteolytic cleavage of the PAR-2 extracellular N-terminal exodomain reveals a new N-terminus containing the receptor-activating sequence SLIGRL which serves as a "tethered ligand" (Nystedt *et al.*, 1994). PAR-2 is activated by the serine-protease trypsin at nanomolar concentrations or by the synthetic peptide SLIGRL. We have shown previously that thrombin, the activator of the prototype tethered ligand receptor, stimulates the activation of mitogen-activated protein (MAP) kinase in cultured rat aortic smooth muscle cells (RASMCs)(Malarkey *et al.*, 1996). Members of this serine/threonine specific protein kinase family are believed to play roles in cell proliferation and regulation of smooth muscle contractility. MAP kinases are components of a well characterized signalling cascade and are activated on phosphorylation by the dual specificity kinases of the MEK (MAP kinase or ERK kinase) family (Malarkey *et al.*, 1995). As trypsin has been shown to evoke early signalling events in RASMCs (Kable *et al.*, 1995) and PAR-2 has been detected in rat aortic tissue (Al-Ani *et al.*, 1995), we have investigated whether trypsin stimulates activation of MAP kinase in RASMCs and if this response is dependent on PAR-2.

RASMCs were cultured and maintained as described previously by Malarkey *et al.* (1996). Cells were rendered quiescent in serum-free media for 48 h prior to stimulation. MAP kinase activity was quantified by *in vitro* [γ-^{32}P]ATP phosphorylation of a peptide substrate derived from a portion of the epidermal growth factor receptor ($EGFR^{661-680}$). MEK activity was measured by an *in vitro* kinase assay utilizing recombinant wild type MAP kinase and $EGFR^{661-680}$ as sequential substrates. PAR-2 mRNA expression was detected by reverse-transcriptase polymerase chain reaction (RT-PCR) amplification using primers directed against amino acid residues 222-232 and a C-terminal sequence of the published mouse PAR-2 sequence.

Exposure of RASMCs to trypsin (30 nM) evoked a time-dependent activation of MAP kinase. Stimulation of kinase activity was evident as early as 2 min, maximal at 5 min (4.1±0.4 fold increase) and returned to basal levels by 30 min. Trypsin also caused comparable activation of MEK. Activation of MAP kinase by 30 nM trypsin was abrogated by prior treatment of the protease for 5 min with 1 mg/ml soybean trypsin inhibitor. SLIGRL (300 μM) stimulated MAP kinase activity (4.6±0.3 fold increase at 5 min) and MEK activity with time courses which closely resembled activation by trypsin. Both trypsin and SLIGRL responses were also dependent on concentration with EC_{50} values of 12.1±3.4 nM and 62.5±4.5 μM respectively. Prior treatment of RASMCs for 20 min with 300 μM SLIGRL prevented 98.5±1.5 % of the increase in MAP kinase activity evoked by a subsequent 5 minute exposure to the peptide. Under these desensitization conditions, trypsin-stimulated MAP kinase activity was inhibited by 78.9±15.1 % while the same response to thrombin was attenuated by only 16.6±12.1 %. RT-PCR analysis of mRNA extracted from RASMCs yielded a product of approximately 507 base pairs which corresponded to the PCR product obtained from the mouse PAR-2 cDNA clone and which DNA sequencing analysis confirmed was PAR-2. Values above represent mean ±s.e.mean for n=3-5 experiments.

Our results show that in RASMCs, trypsin stimulates the activation of components of the MAP kinase cascade in a manner consistent with an interaction with PAR-2.

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11P THE HUMAN RECOMBINANT SOMATOSTATIN SST5 RECEPTOR COUPLES TO PERTUSSIS TOXIN-SENSITIVE AND -INSENSITIVE G PROTEINS

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The human recombinant somatostatin (SRIF) sst_5 receptor mediates both inhibition and stimulation of cAMP accumulation (Akbar *et al.*, 1994). In the present study the effects of SRIF and selective ligands (Raynor *et al.*, 1993) on forskolin-stimulated cAMP accumulation were used to characterise these responses in detail.

CHO-K1 cells (~200 000) expressing the human recombinant sst_5 receptor (Wilkinson *et al.*, 1996) were suspended in DMEM/Ham's F-12 (1:1) media supplemented with 15mM Hepes (pH7.4), 0.02% bacitracin and 0.5mM IBMX and co-incubated with ligand and forskolin for 10min at 37°C. Reactions were terminated and cAMP content was determined by a radioligand binding assay (Brown *et al.*, 1971). Data are the mean ± s.e.mean, n>3.

SRIF and SRIF-28 potently inhibited forskolin-stimulated cAMP production, with maximal inhibitions of 77% at 10nM and 76% at 3nM, respectively (see table). The putative sst_5 receptor selective ligand, L-362,855, potently inhibited cAMP accumulation whilst BIM23027, an sst_2 selective ligand, was a weak agonist in agreement with previous studies on this receptor (Williams *et al.*, 1996).

Agonist effects on forskolin-stimulated cAMP accumulation

	Control		*Pertussis toxin treated*	
	pIC_{50}	% Inhibition	pEC_{50}	% Stimulation
SRIF	9.2±0.1	77±2	7.2±0.2	216±128
SRIF-28	9.7±0.2	76±1	7.7±0.0	252±5
L-362,855	9.7±0.2	74±4	-	
BIM23056	8.3±0.3	57±5	-	
BIM23027	6.7±0.7	74±1	-	

BIM23056 had a smaller maximal effect than the other agonists ($P<0.05$, unpaired t-test), suggesting that it acts as a partial agonist. At concentrations above 10nM the concentration-effect curves to SRIF and SRIF-28 were bell-shaped. Thus 10μM SRIF produced 48±3% inhibition of the forskolin response and 10μM SRIF-28 only inhibited cAMP levels by 37±5%.

Pre-treatment of cells with 100 $ngml^{-1}$ pertussis toxin for 18hr abolished the inhibitory effect of all SRIF analogues on forskolin-stimulated cAMP accumulation. Furthermore, following pertussis toxin treatment, SRIF and SRIF-28 increased forskolin-stimulated cAMP accumulation although with about 100-fold lower potency than observed for inhibition of cAMP production (see table). L-362,855 was a weak agonist, 10μM producing 152.0±13.2% of the forskolin response whilst BIM23056 did not increase cAMP accumulation at all.

This study demonstrates the potent coupling of the sst_5 receptor to inhibition of cAMP accumulation compared to its weaker coupling to stimulation of cAMP accumulation and confirms the findings of Akbar *et al.*, (1994) which suggested the sst_5 receptor is promiscuous in the G proteins it activates. BIM23056 acted as an agonist with low intrinsic activity in causing inhibition of cAMP accumulation. The lower potency of SRIF and SRIF-28 at stimulating cAMP accumulation, the weak agonist profile of L-362,855 and the lack of stimulation with BIM23056 are consistent with a lower coupling efficiency for stimulation compared to inhibition of forskolin-stimulated cAMP accumulation.

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12P THE EFFECT OF MAPKK INHIBITOR PD98059, THE P38MAPK INHIBITOR SB203580 AND THE SMALL G PROTEIN Rac 1 ON INTERLEUKIN 1 SIGNAL TRANSDUCTION IN T LYMPHOCYTES

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Interleukin 1 (IL1) is a proinflammatory cytokine implicated in pathological conditions such as rheumatoid arthritis. An array of cell signalling pathways have been proposed for IL1 (reviewed in O'Neill, 1995) though the precise molecular mechanisms involved remain to be determined. Recently three MAP kinase (MAPK) cascades have been implicated, namely 'classical' p42/p44 MAPK, p38/HOG1 MAPK and p54/JNK MAPK. In this study we have examined the effect of the MAP kinase kinase inhibitor, PD98059 (Dudley *et al.*, 1995) and the p38MAPK inhibitor, SB203580 (Cuenda *et al.*, 1995) on IL1 signalling in T lymphocytes. In addition the involvement of the small G protein Rac1, which has been postulated to be upstream of p38 (Zhang *et al.*, 1995) has been investigated.

As a model system the murine thymoma cell line EL4.NOB-1 was used. This is an IL1 receptor rich strain producing IL2 in response to IL1 or the phorbol ester PMA. Cells ($1x10^6$) were pretreated for 1h at 37°C with either PD98059 (0.1-30μM), SB203580 (0.1-3μM) or vehicle control (DMSO) and then stimulated with IL1 (10ng/ml), PMA (100ng/ml) or appropriate vehicle control for 24h. Following this time period the cells were centrifuged and supernatants removed for determination of IL2 levels by ELISA. Cells were also transfected with a gene construct comprising the IL2-promoter from -293 to -5 linked to a reporter gene chloramphenicol acetyl transferase (CAT). Following a 24h recovery period the cells were pretreated with inhibitor and stimulated as detailed above. CAT activity was subsequently determined as % acetylation of ^{14}C-chloramphenicol by cell cytosolic fractions. For studies involving Rac1, cells were also transfected with plasmids containing genes for constitutively active Rac1, V12Rac1, or a dominant negative mutant, N17Rac1.

PD98059 dose-dependently inhibited both IL1 and PMA stimulated IL2 production (Table 1) though the effect on PMA was less potent. SB203580 also inhibited IL1 stimulated IL2 production (Table 2), but not that induced by PMA. In addition SB203580 partially inhibited IL1 stimulated CAT activity in transfected EL4.NOB-1 cells (Table 2). This inhibition was less than that of native IL2 in the transfected cells; native IL2 inhibition in transfected and non-transfected cells being similar. This suggested an effect of SB203580 on translation of IL2 mRNA which, unlike that for CAT, is subject to regulatory control. This was also suggested from a lack of effect on the activation of NFκB (as judged by gel shift analysis), a transcription factor important for IL2 gene expression.

Table 1. The effect of PD98059 (μM; mean ± s.e.mean, n=1-4) on IL1 and PMA stimulated IL2 (ng/ml).

	0	0.1	0.3	3	10	30
IL1	4.8±1.6	2.9±1.1	1.9	1.4±0.6	0.4±0.1	0
PMA	21.2±4.2	29.7	29.5	9.5	1.6±0.4	0

Table 2. The effect of SB203580 (μM) on IL1 stimulated IL2 (ng/ml) and CAT activity (% acetylation). Results from a single experiment carried out in duplicate (mean ± spread) representative of 3-4 experiments are shown.

	0	0.1	0.3	3
IL2	20.8±3.1	8.8±2.3	6.4±0	7.5±1.1
CAT	2.1±0.2	-	1.5±0.1	-

Finally, constitutively active Rac1 had an 8-12 fold potentiating effect on CAT and native IL2 expression, whilst dominant negative Rac1 was partially inhibitory (~15%). The latter construct however also inhibited control expression (~50%).

These results therefore suggest that the classical MAPK pathway and the p38MAPK pathway are important in IL1 signalling in T lymphocytes. The p38MAPK pathway may function at least in part at the level of translation. In addition, IL1 appears to engage Rac1, which may be a receptor proximal signal leading to p38 activation.

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13P REGULATION OF TNFα-MEDIATED EXPRESSION OF ICAM-1 AND VCAM-1 IN EA.hy 926 CELLS

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Increased adherence of monocytes to endothelium is an important early event in the pathogenesis of atherosclerosis. TNFα is a proatherogenic cytokine elaborated by cells of the vascular wall. Up-regulation of adhesion molecule expression TNFα involves a complex cascade of intracellular signalling and transcription factor activation steps. In the present study we have used a range of inhibitors directed against proteases and signalling enzymes to investigate the regulation of ICAM-1 and VCAM-1 in response to TNFα in the human endothelial cell line EA.hy 926. We measured monocyte binding to EA.hy 926 using a myeloperoxidase-based 96 well plate assay, and ICAM-1 and VCAM-1 expression with an ELISA.

In response to TNFα (10ng/ml), expression of ICAM-1 increased 10-14 fold after 4h (n=3) and remained constant for 24h. VCAM-1 expression increased 10-12 fold after 24h (n=3). The protease inhibitors tosyl phenylalanine chloromethyl ketone (TPCK), tosyl lysine chloromethyl ketone (TLCK), calpain1-inhibitor (C1-I), are all putative nuclear factor-κB (NFκB) inhibitors (Finco *et al.*, 1994), and inhibited TNFα-induced ICAM-1 and VCAM-1 expression after 6h (table 1). The phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor, tricyclodecan-9-yl xanthogenate.K (D609), or the RNA synthesis inhibitor, actinomycin D (AD), also inhibited both ICAM-1 and VCAM-1 expression 6h post-TNFα incubation (table 1). The phospholipase A_2 (PLA_2) inhibitor, 7,7-Dimethyleicosadienoic acid (DEDA) (50μM), only inhibited VCAM-1 expression (table 1). TLCK (50μM), TPCK (25μM), and C1-I (20μM) also inhibited monocyte adhesion after 6h 30.2±5%, 48.3±2.6%, 45.6±2.7% (mean±s.e.mean) respectively (n=3). The protein kinase C (PKC) inhibitors, staurosporin (10nM) or chelerythrine (2μM) as well as phospholipase D (PLD) and phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin (3.2μM), had no effect on either ICAM-1 or VCAM-1 expression. The inhibitors used had no effect on cell viability as measured by MTT assay.

TABLE 1

Inhibitor	Time (h)	% Inhibition of control (±s.e.mean, n=3)	
		VCAM-1	ICAM-1
TPCK (25μM)	6	79.4±11.6	55.8±1.7
TLCK (50μM)	6	68±4.2	13.7±3.5
C1-I (20μM)	6	89±3.4	36±6.4
D609 (50μg/ml)	6	110±8.2	52.8±3.5
AD (0.05μg/ml)	6	92.8±6.5	28.2±4.3
DEDA (50μM)	6	44.1±6.0	No effect

Our data suggest that PKC, PLD and PI3K are not involved in the up-regulation of ICAM-1 and VCAM-1 in EA.hy 926 cells in response to TNFα. Inhibition of ICAM-1 and VCAM-1 by protease inhibitors indicate that NFκB is required for their expression induced by TNFα. Involvement of PC-PLC in ICAM-1 and VCAM-1 expression indicates a role for the diacylglycerol-activated ceramide cascade (Schütze *et al.*,1992) in TNFα-induced NFκB activation. Inhibition by AD confirms that *de-novo* protein synthesis is required. Inhibition of PLA_2 (activated by neutral sphingomyelinase present in the membrane) by DEDA, suggests that VCAM-1 expression requires PLA_2-dependent signalling. This finding further supports the notion of a key role for ceramide-mediated NFκB activation in ICAM-1 and VCAM-1 expression. However additional regulatory mechanisms, possibly involving PLA_2, are required to account for the differences observed in ICAM-1 and VCAM-1 expression.

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14P CLASSIFICATION OF P_2-PURINOCEPTORS ON CYSTIC FIBROSIS SUB MUCOSAL EPITHELIAL (CFSME) CELLS

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UTP has been suggested as a possible therapy for the treatment of lung dysfunction in cystic fibrosis. $P2Y_2$- purinoceptors have been shown to be present in cystic fibrosis nasal epithelial cells (Mason *et al.*, 1991). CFSME cells are likely to be of greater pathophysiological relevance in cystic fibrosis and have been shown to contain P_1-purinoceptors (McCoy *et al.*, 1995). This study was designed to characterise the P_2-purinoceptor subtypes present in CFSME cells.

Changes in $[Ca^{2+}]_i$ in cell suspensions (2-4 x 10^5 cells ml^{-1}) were measured using standard fluorescent techniques. After loading with Fura-2 or, in suramin studies, Fluo-3, agonist concentration-effect (E/[A]) curves were constructed in log unit increments. Each cuvette containing 2ml of cells was used for a single response determination. Compounds with no agonist activity were tested as antagonists by addition 15s before the agonist. Responses were expressed as a % of that to 10μM UTP.

For agonists producing a response > 25% at 100μM, E/[A] curves gave the potency ($p[A]_{50}$) order: UTP ≥ATP>adenosine>ATPγS≥ UDP, ADP, AMP (Table 1). 2-MeSATP and benzoyl-benzoyl-ATP (bbATP) produced maximal responses (α) < 25% of UTP. UMP, uridine (both 1mM), bbATP (10μM) and 2-MeSATP (100μM) had no effect on UTP responses. The P2T-purinoceptor antagonist ARL 66096 (10μM; Humphries *et al.*, 1994) had no effect on the ADP response. Neither apyrase (2U ml^{-1} pre-incubated during Fura-2 loading to prevent tonic desensitisation of P_2-purinoceptors by ATP) nor the ectoATPase inhibitor ARL 67156 (300μM; Dainty *et al.*, 1994b) had any effect on responses to UTP or 2-MeSATP. In the presence of UTP (10μM), responses to ATP, ATPγS, UDP and ADP but not adenosine or histamine (all 100μM) were abolished. Suramin antagonised UTP (apparent pA_2=4.6 ± 0.1, n=4) but not histamine.

Agonist	α	$p[A]_{50}$	Agonist	α	$p[A]_{50}$
UTP	106.4 (2.6)	6.0 (0.1)	UDP	> 70*	< 5.0
ATP	95.3 (6.0)	5.8 (0.2)	ADP	> 50*	< 4.0
Ado	65.6 (5.4)	5.4 (0.3)	AMP	> 30*	< 4.0
ATPγS	> 80*	< 5.0			

Table 1 Mean (± s.e.) n= 4. * incomplete curves @ 100μM

This study demonstrates the presence of purinoceptors mediating increases in $[Ca^{2+}]_i$ in CFSME cells. UTP did not act via P2X-, $P2Y_1$- or P2Z-purinoceptors though the responses (albeit small) to 2-MeSATP and bbATP may indicate their presence. P2T-purinoceptor activity is precluded since ARL 66096 did not affect responses to ADP. Purinoceptor desensitisation by endogenously released ATP did not occur and agonist potencies were unaffected by ectoATPase activity. The response to adenosine and cross-desensitisation studies confirmed the presence of P_1-purinoceptors, at which UTP did not act. As in the rat aorta, UTP responses were suramin sensitive (Dainty *et al.*, 1994a). Thus, the general pharmacology and agonist potency order indicates the presence of a $P2Y_2$-purinoceptor (Lustig *et al.*, 1993) in CFSME cells.

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15P MODULATION OF VASOCONSTRICTION IN THE HEPATIC CIRCULATION BY NITRIC OXIDE

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Nitric oxide (NO) has a variety of interactions with vasoactive agents and it is unclear if some of the differences are species or method dependent. It was our objective to determine the vasodilator effect of adenosine on the hepatic (HA) and superior mesenteric (SMA) artery before and after blockade of NO production to evaluate the possibility of organ specificity for vascular regulation by NO. Vascular circuits supplied blood flow under constant flow or pressure to the liver or intestine in cats under sodium pentobarbital anesthesia (32.5 mg/kg, intraperitoneal). The NO synthase antagonist, N^G-nitro-L-arginine methyl ester (L-NAME) (2.5 mg · kg^{-1}, i.v.), increased arterial pressure from 106.4 ± 7.6 mmHg to 141.4 ± 8.1 mmHg (mean ± SE, n = 6) and raised basal vascular tone in the SMA but not the HA. The NO synthase substrate, L-arginine (75 mg · kg^{-1}), reversed these effects. Maximal response to adenosine was determined at control (pre-L-NAME) pressure and flow adjusted using the vascular circuit (Macedo & Lautt, 1996). The decrease in perfusion pressure was the index of vasodilation. Infusion of adenosine decreased SMA pressure from the same basal, pre L-NAME pressure (106.4 ± 7.6 mmHg) by 51.7 ± 2.9, 64.7 ± 5.3, and 42.2 ± 5.2 mmHg respectively for control, L-NAME, and L-arginine. From the same pre L-NAME basal flow, the decrease in pressure in response to adenosine was 51.7 ± 2.9, 135.2 ± 6.1, and 16.7 ± 2.4 mmHg respectively. Adenosine was not potentiated in the HA by L-NAME. In conclusion, L-NAME increased basal tone for the SMA and potentiated the dilation induced by adenosine in the SMA but had no effect on the HA. This study provides evidence that there is a highly organ-specific compensatory mechanism in which the absence of NO promotes potentiation of other vasodilators. This mechanism offers compensatory protection to the intestine in the event of endothelial dysfunction but does not interfere with the adenosine-dependent regulation of hepatic arterial blood flow that tends to maintain hepatic blood flow constant (Lautt et al., 1985).

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16P SHEAR-INDUCED MODULATION OF VASOCONSTRICTION IN THE HEPATIC CIRCULATION BY NITRIC OXIDE

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Nitric oxide (NO) has been implicated in shear rate dependent vasodilation in response to increases in blood flow. The aim of this study was to investigate the role of NO in the hepatic vasculature during sympathetic nerve stimulation under conditions of varying shear stress in the pentobarbital anesthetized cat (intraperitoneal, 32.5 mg/kg). When shear stress was increased by keeping the blood flow constant at the site of the constricted vessels (obtained by using a perfusion circuit) (Macedo & Lautt, 1996), there was a potentiation of the vasoconstriction induced by sympathetic nerve stimulation (2-4 Hz) when the NO synthase blocker, L-NAME, was given as a bolus (2.5 mg/kg^{-1}, i.v.). At constant flow, the increase in hepatic arterial (HA) perfusion pressure due to nerve stimulation was 28.8 ± 6.5 mmHg (mean ± SE, n = 6) under control state, 62.7 ± 14.6 ($p < 0.01$) after L-NAME infusion, and was reversed by L-arginine (6.3 ± 3.0). The same patttern of effects was observed for the portal vein (PV) (1.5 ± 0.5 mmHg for control, potentiated to 3.3 ± 0.5 mmHg after L-NAME and reversed to 1.2 ± 0.5 mmHg $p < 0.05$, repeated measures ANOVA, by L-arginine). When shear stress was held constant by decreasing blood flow during vasoconstriction, L-NAME did not affect the vasoconstriction of the resistance vessels. We conclude that when vasoconstriction results in elevated shear stress at the site of constriction, nitric oxide is released, resulting in suppression of the constriction thus serving to protect the endothelial cells within the constricted vessel.

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17P THE ROLE OF COPPER IN MEDIATING NITRIC OXIDE AND PROSTACYCLIN SYNTHESIS IN THE RAT AORTA

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Through its capacity to catalyze the generation of free radicals, copper has been widely implicated in the pathophysiology of cardiovascular disease (CVD). The synthesis of both nitric oxide (NO) and prostacyclin (PGI_2) involve haem-mediated redox reactions. Since impairment of PGI_2 and NO synthesis is associated with CVD (Gryglewski et al., 1995), it is possible that the deleterious influence of copper may involve effects on NO synthase (NOS) and cyclooxygenase (COX). In order to test this hypothesis, the effect of the high affinity copper chelator, diethyldithiocarbamic acid (DEDCA) on NO and PGI_2 synthesis by the rat aorta was investigated using both organ bath and biochemical methods.

Aortae were obtained from Sprague Dawley rats (300g) and prepared for in vitro organ bath studies (Karatapanis et al.,1994). Contraction was elicited with phenylephrine (PhE) and relaxation with calcium ionophore A23187 (A23187) and sodium nitroprusside (SNP). PGI_2 and cGMP synthesis were assessed biochemically with radioimmunoassay as previously described (Jeremy et al., 1996; Miller et al., 1994).

At up to $1x10^{-4}$ M, DEDCA had no effect on PhE-stimulated contraction but inhibited SNP-induced relaxation (Table 1). At $1x10^{-5}$ M DEDCA inhibited relaxation induced by A23187 but not SNP in aortic rings pre-contracted with PhE. $CuCl_2$, but not Fe_2Cl_3, reversed the inhibitory effect of DEDCA on both A23187- and SNP-stimulated relaxation (Table 1). DEDCA inhibited cGMP synthesis when stimulated with A23187 (IC_{50}; $5x10^{-7}$ M) and SNP (IC_{50}; $5x10^{-5}$ M). DEDCA inhibited PGI_2 synthesis when stimulated with 10μM arachidonate (IC_{50}; $4.9x10^{-5}$M), 10μM adrenaline (IC_{50}; $5x10^{-5}$M) and 10 μM A23187 (IC_{50}; $4.8x10^{-5}$M). At equimolar concentrations, $CuCl_2$ but not Fe_2Cl_3 reversed the inhibitory effect of 100 μM DEDCA on arachidonate-stimulated PGI_2 synthesis. The effect of DEDCA on PGI_2 synthesis could not be reversed by repeated washing of the tissue, indicating that DEDCA binds irreversibly to an active site in the COX holoenzyme.

The present study suggests that divalent copper may play a role in mediating the activities of NOS, COX and guanylyl cyclase. The reversal of the DEDCA-mediated effects with divalent copper, but not iron, indicates that these effects are not due to removal of the iron contained within haem which in turn is integral to NOS, guanylyl cyclase and COX activity. Finally, these data indicate that the pathophysiological impact of copper in CVD (as well as inflammatory disease) may involve effects on these key enzymes.

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Table 1. Effect of $CuCl_2$ and Fe_2Cl_3 (both 100 μM) on DEDCA (100 μM) inhibited relaxation (% maximal; mean ± S.E.M., n = 6)

	control	DEDCA	DEDCA + $CuCl_2$	control	DEDCA	DEDCA + Fe_2Cl_3
A23187-induced relaxation	85 ± 3.9	5 ± 4.3	90 ± 7	87 ± 4.2	5 ± 2.9	3.7 ± 1.5
SNP-induced relaxation	95 ± 4.2	10 ± 2.5	97 ± 3.3	92 ± 8.4	7 ± 4.9	10 ± 5.2

18P LIPOTEICHOIC ACID FROM *S. AUREUS*, BUT NOT FROM *B. SUBTILIS*, SYNERGISES WITH *B. SUBTILIS* PEPTIDOGLYCAN TO CAUSE HYPOREACTIVITY TO NORADRENALINE AND ORGAN INJURY IN RATS

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The cell wall components of the pathogenic Gram-positive bacterium *Staphylococcus aureus* (*S.aureus*), lipoteichoic acid (LTA) and peptidoglycan (PepG), synergise to induce nitric oxide synthase (iNOS) and to cause circulatory failure and multiple organ injury in the rat (De Kimpe *et al*, 1995). Structural differences in the LTAs from *S.aureus* and the non-pathogenic Gram-positive bacterium *Bacillus subtilis* (*B.subtilis*) may be responsible for the lack of synergy between LTA and PepG of *B.subtilis* in inducing iNOS activity in macrophages (this meeting, Kengatharan *et al.*, 1996). Here, we investigate the effect of co-administration of *S.aureus* LTA or *B.subtilis* LTA with *B.subtilis* PepG on the haemodynamics, iNOS activity (lung) and organ injury in the rat.

Male Wistar rats (250-325g) were anaesthetised with thiopentone sodium (120 $mg.kg^{-1}$, i.p). The trachea was cannulated to facilitate spontaneous respiration. The carotid artery and the femoral vein were cannulated to monitor mean arterial blood pressure (MAP) and for administration of compounds, respectively. The pressor response to noradrenaline (NA, $1\mu g.kg^{-1}$, i.v.) was assessed prior to and every 60 min after the injection of the bacterial components (time 0). At 360 min, rats were killed and (i) serum samples were taken for the determination of biochemical markers of organ injury, and (ii) lungs were removed to determine iNOS activity by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in lung homogenates (De Kimpe *et al*, 1995).

Treatment of rats with LTA (*S.aureus*, $3mg.kg^{-1}$) and PepG (*B.subtilis*, $1mg.kg^{-1}$) caused significant (i) vascular hyporeactivity to NA, (ii) increase in the serum levels of alanine aminotransferase (ALT) (indicating liver injury), urea and creatinine (indicating renal failure), and (iii) increase in lung iNOS activity; compared to LTA (*S.aureus*, $3mg.kg^{-1}$) treated or sham-operated animals (Table 1). However, the observed fall in MAP was not significantly different from the one elicited by LTA (*S.aureus*, $3mg.kg^{-1}$) alone (Table 1). In contrast, co-administration of LTA (*B.subtilis*, $3mg.kg^{-1}$) and PepG (*B.subtilis*, $1mg.kg^{-1}$) did not produce significant changes in NA response, iNOS activity (lung) or serum levels of ALT, urea or creatinine compared to sham-operated animals (Table 1).

Thus, *S.aureus* LTA, but not *B.subtilis* LTA, synergises with PepG (from *B.subtilis*) to induce hyporeactivity to NA, iNOS activity and organ injury in anaesthetised rats. The structural differences between *S.aureus* LTA and *B.subtilis* LTA may account for the inability of *B.subtilis* LTA to synergise with *B.subtilis* PepG to induce iNOS and to elicit various pathological features of septic shock.

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Table 1. Effect of LTA (from *S.aureus* or *B.subtilis*) and PepG (from *B.subtilis*) on haemodynamics, iNOS activity and organ injury (in the rat).

Experimental group	MAP (mmHg)	NA response (mmHg.min)	Lung iNOS activity (pmol L-citrulline min^{-1} g tissue)	ALT (iuL^{-1})	Serum urea (mM)	Serum creatinine (μM)
sham	117±3	38±5	24±15	63±6	6±1	35±4
LTA (*S.aureus*, $3mg.kg^{-1}$)	97±6	35±6	195±36*	74±5	8±1	38±4
LTA (*S.aureus*, $10mg.kg^{-1}$)	77±3*#	23±3	253±30*	60+4	12±1	39±5
LTA (*S.aureus*, $3mg.kg^{-1}$)+PepG (*B.subtilis*, $1mg.kg^{-1}$)	84±6*	10±3*#	419±58*#	257±54*#	15±1*	69±6*#
LTA (*B.subtilis*, $3mg.kg^{-1}$)+PepG (*B.subtilis*, $1mg.kg^{-1}$)	89±7*	42±10	142±49	115±29	10±2	48±9

Values for 360 min after injection of bacterial components are given as mean ± s.e.mean (n=4-8); *$P<0.05$ vs sham & #$P<0.05$ vs LTA (*S.aureus*, $3mg.kg^{-1}$) alone by oneway-ANOVA (Bonferroni's test).

19P EFFECTS OF POLYCLONAL ANTIBODIES AGAINST TNFα OR IL-1β ON THE MULTIPLE ORGAN FAILURE SYNDROME ELICITED BY ENDOTOXIN IN THE ANAESTHETISED RAT

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A distinct isoform of nitric oxide synthase (inducible NOS, iNOS) can be induced by proinflammatory cytokines such as tumour necrosis factor-α (TNFα) or interleukin-1β (IL-1β) *in vitro* and *in vivo* (Busse and Mülsch, 1990; Kosaka et al., 1992.). Here, we investigate the role of endogenous TNFα and IL-1β on (i) the induction of iNOS, (ii) the circulatory failure and (iii) the multiple organ failure syndrome (MODS) caused by endotoxaemia in the anaesthetised rat.

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The carotid artery was cannulated for the measurement of mean arterial pressure (MAP) and the femoral vein for the administration of compounds. Sheep polyclonal antibodies (PAb) against human TNFα (100 mg·kg^{-1}, i.v., n=6), IL-1β (100 mg·kg^{-1}, n=6) or a mixture of PAb against human TNFα and IL-1β (both 30 mg·kg^{-1}, i.v., n=7) was administrated 30 min prior to *E.coli* 0127:B8 lipopolysaccharide (LPS; 10 mg·kg^{-1} i.v., n=12). At 90 min, an arterial blood sample was collected to measure the concentration of TNFα by ELISA. At 6 h, serum samples were taken and analysed for alanine aminotransferase (ALT) and bilirubin for liver function, and creatinine for renal function by a contract laboratory for veterinary chemistry. In addition, lungs were removed to determine iNOS activity by measurement of the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline.

In the anaesthetised rat, LPS caused hypotension, renal and liver dysfunction, and an increase in iNOS activity in the lung ($p<0.05$, Table 1). These effects of LPS were significantly reduced (except for the increase in creatinine) by pretreatment of LPS-rats with PAb to TNFα and PAb to IL-1β, the PAb to TNFα alone and to a lesser extent with the PAb to IL-1β alone. The increase in plasma TNFα levels elicited by LPS was significantly inhibited by treatment of LPS-rats with the PAb against TNFα, either given alone or in combination with PAb to IL-1β, but not with the PAb to IL-1β alone (Table 1).

Thus, inhibition of both TNFα and IL-1β by PAb reduces (i) the induction of iNOS, (ii) the circulatory failure and (iii) the liver dysfunction elicited by LPS. We suggest that both TNFα and IL-1β are important mediators in the pathogenesis of liver injury in endotoxaemia.

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Table 1.

Treatment	MAP at 6h (mmHg)	TNFα at 90 min (ng ml^{-1})	ALT (iu l^{-1})	Bilirubin (μM)	Creatinine (μM)	iNOS activity (nmol/min/mg protein)
Sham	109 ± 4	0.2 ± 0.05	64 ± 4	2.1 ± 0.2	31 ± 5	0.04 ± 0.03
LPS	84 ± 4	5.3 ± 0.5	510 ± 94	6.8 ± 0.9	68 ± 8	8.1 ± 0.6
+ PAb TNFα	95 ± 3*	1.9 ± 0.4*	122 ± 29*	3.3 ± 0.3*	62 ± 8	4.4 ± 0.7*
+ PAb IL-1β	101 ± 3*	4.9 ± 0.6	336 ± 69*	5.3 ± 0.7	59 ± 6	4.0 ± 0.8*
+ PAb IL-1β + Pab TNFα	95 ± 4*	2.7± 0.4*	129 ± 28*	4.1 ± 0.3*	71 ± 12	4.3 ± 0.5*

mean ± s.e.mean. * $p<0.05$ vs. LPS + saline, unpaired Student's *t* test.

20P ENHANCED HAEMODYNAMIC EFFECTS OF SB 209670 AND LOSARTAN IN CONSCIOUS, ENDOTOXAEMIC RATS

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Although the non-selective endothelin antagonist, SB 209670, enhances the hypotensive and mesenteric and hindquarters vasodilator effects of lipopolysaccharide (LPS) in conscious rats, it does not influence renal haemodynamics, indicating the involvement of other factors in this vascular bed (Gardiner *et al.*, 1995). To determine any possible influence of angiotensin II under these conditions, we have now assessed the effects of a continuous co-infusion of SB 209670 (600 μg kg^{-1} bolus, 600 μg kg^{-1} h^{-1}) and losartan (10 mg kg^{-1} bolus, 10 mg kg^{-1} h^{-1}) beginning 1 h before continuous infusion of saline (Group a, n = 8) or LPS (150 μg kg^{-1} h^{-1}) (Group b, n = 8) for 23 h, or a continuous infusion of saline beginning 1 h before LPS for 23 h (Group c, n = 8), in conscious male, Long Evans rats (350-450 g) chronically instrumented with pulsed Doppler probes (renal, mesenteric and hindquarters) and intravascular catheters (Gardiner *et al.*, 1995). All surgery was carried out under sodium methohexitone anaesthesia (40-60 mg kg^{-1} i.p., supplemented as required). Experiments began 24 h after catheterization. Resting cardiovascular variables in Group a, Group b and Group c, respectively, were:- heart rate (HR, beats min^{-1}), 342 ± 11, 348 ± 5 and 334 ± 7 (mean ± s.e. mean); mean arterial blood pressure (MAP, mm Hg), 102 ± 1, 102 ± 1 and 103 ± 1; renal vascular conductance (RVC, [kHz mm Hg^{-1}]10^3), 68 ± 8, 54 ± 3 and 59 ± 3; mesenteric vascular conductance (MVC), 68 ± 5, 55 ± 3 and 70 ± 4; hindquarters vascular conductance (HVC), 46 ± 2, 42 ± 2 and 38 ± 2. Changes at 1, 3, and 24 h during the experiments are shown in Table 1.

SB 209670 and losartan caused significant and persistent hypotension and vasodilatation. In the presence of SB 209670 and losartan, the hypotension and increases in RVC, MVC and HVC caused by LPS were significantly increased. The results indicate that endothelin and angiotensin II influence haemodynamic status in conscious, Long Evans rats, and this influence is amplified in endotoxaemia. It appears the relative lack of effect of SB 209670 on the renal haemodynamic responses to LPS (Gardiner *et al.*, 1995) is due to a marked vasoconstrictor action of angiotensin II in the kidney.

Table 1. Cardiovascular changes in conscious rats. * $P < 0.05$ for change (Friedman's test); superscripts $P < 0.05$ versus change in corresponding group (Kruskal-Wallis test).

		1h	3h	24h
ΔHR (beats min^{-1})	a	32 ± 9*	26 ± 15	32 ± 14
	b	54 ± 8*[a]	48 ± 10*	86 ± 22*[a]
	c	3 ± 4[ab]	11 ± 8[b]	76 ± 4*[a]
ΔMAP (mm Hg)	a	-15 ± 2*	-17 ± 2*	-25 ± 2*
	b	-14 ± 2*	-43 ± 2*[a]	-41 ± 4*[a]
	c	0 ± 1[ab]	-7 ± 2*[ab]	-3 ± 2[ab]
ΔRVC (%)	a	36 ± 5*	30 ± 6*	31 ± 13*
	b	33 ± 5*	158 ± 12*[a]	148 ± 13*[a]
	c	6 ± 3[ab]	71 ± 7*[ab]	80 ± 9*[ab]
ΔMVC (%)	a	24 ± 10*	17 ± 8*	44 ± 9*
	b	26 ± 6*	170 ± 17*[a]	151 ± 18*[a]
	c	2 ± 3[ab]	-14 ± 5*[ab]	-6 ± 6[ab]
ΔHVC (%)	a	11 ± 8	18 ± 8	45 ± 12*
	b	31 ± 5*[a]	48 ± 8*	81 ± 8*[a]
	c	-6 ± 4[ab]	21 ± 13	40 ± 9*[b]

Gardiner, S.M. *et al.* (1995) *Br.J.Pharmacol.* **116**, 1718-1719.

21P EFFECT OF THE ET_A RECEPTOR ANTAGONIST BQ123 ON INFARCT SIZE AND ENDOTHELIN RELEASE IN ISOLATED PERFUSED RABBIT HEARTS

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Endothelin-1(ET-1) belongs to a family of potent endogenous vasoconstrictor peptide (Yanagisawa et al.,1988), mediating its actions via two receptor subtypes, namely ET_A and ET_B. A pathophysiological role for ET-1 has been postulated in myocardial ischaemic/reperfusion injury and endothelin receptor antagonists have been shown to reduce infarct size and enhance post-ischaemic myocardial recovery in several animal models of acute ischaemic/reperfusion injury (Opgenorth 1995). However, studies using ET_A receptor antagonists have shown contradicting results. For instance, BQ123 (0.03 and 10μg $kg^{-1}min^{-1}$ intracoronary) reduced infarct size in dogs (Grover et al 1993) whereas FR 139317 (0.2mg $kg^{-1}min^{-1}$, preceded by a loading dose of 1.0mg kg^{-1}, i.v.) does not in rabbits (McMurdo et al.,1994). Whether or not this discrepancy is related to a dose-dependent effect of the antagonists is not known and neither is the extent to which these antagonists influence the release of ET-1 from the myocardium. Therefore, the aims of the present study were to evaluate the effect of different concentrations of the ET_A receptor antagonist BQ123 on ischaemic/reperfusion injury and extent of endothelin release in isolated perfused rabbit hearts.

Male New Zealand White rabbits (2.0-2.5kgs) were divided into four treatment groups: I) Control(vehicle; n=10), ii) BQ123(0.1μM; n=7), iii) BQ123(0.5μM; n=9), iv) BQ123(1μM; n=7). The rabbits were anaesthetised with sodium pentobarbitone (30mgkg^{-1} i.v.) containing heparin (500iukg^{-1}) and the hearts were rapidly excised and perfused in Langendorff mode with modified Krebs Henseleit buffer (37°C, gassed with 95% O_2/CO_2) at a constant flow rate (10ml min^{-1} kg^{-1} body weight). Hearts were stabilised for 10min, followed by 15min perfusion with BQ123 (dissolved in DMSO) in the buffer, prior to 30min regional ischaemia (by occluding the Left coronary artery) and 180min reperfusion. Heart rate, perfusion pressure and the surface electrocardiogram were continuously monitored throughout the experiment. Samples of coronary effluents were collected at regular intervals and analysed for endothelin-1 content using a Biotrak® ET-1 ELISA assay kit. At the end of the experiment the coronary ligature was re-tied and infarct size was measured using planimetry as previously described (Chokkukannan et al., 1995). Haemodynamics (% change from baseline) and infarct size (% of AAR) were expressed as mean ± sem. ET-1 release ($pg^{-1}min^{-1}g^{-1}$ wet heart weight) expressed as median (Q_1-Q_3). Statistical analysis was performed using ANOVA/Dunnett tests (haemodynamics) and Mann-Whitney test (infarct size and ET-1 data).

In a preliminary set of experiments in isolated perfused rabbit hearts (n=4), BQ123 at concentrations 0.1, 0.5 and 1μM produced 20±2, 55±2 and 20±2% inhibition respectively of the maximum increase in coronary perfusion pressure induced by ET-1 (0.1nmol). Heart rate remained unchanged in the control and BQ123 (0.1μM) treated hearts throughout the experiment. BQ123 (0.5μM) caused a significant decrease (to -18±4%; $p<0.05$ from 191±10 beats min^{-1}) and 1μM BQ123 caused an increase (to 83±14%; $p<0.05$ from 174±3.4 beats min^{-1}) in heart rate before the onset of ischaemia. Coronary perfusion pressure (27.2 ± 2.5 mmHg) increased significantly to (120±12%; $p<0.05$) at the end of 30 min of occlusion in control hearts. A similar increase was measured in all the BQ123 treated group of hearts. Reperfusion restored the perfusion pressure to baseline in control hearts. However, it remained elevated in all the BQ123 treated hearts following reperfusion.

Infarct size expressed as a percentage of area at risk was reduced significantly in BQ123 (0.5μM) treated group of hearts (41±3%; $P<0.01$) when compared to control group (69±2%). There was no reduction in infarct size with 0.1 or 1μM BQ123 (65±3% and 61±2% respectively). Endothelin-1 release into coronary effluents in control hearts was significantly increased by 1.6 fold ($P<0.05$) at the end of 180min reperfusion from the pre-ischaemic value [0.8 (0.6-1.3) pg min^{-1} g^{-1})]. This increase was not seen in perfusates from BQ123 (0.5μM) treated hearts. In contrast to this, in hearts treated with BQ123 (0.1 and 1μM), there was a significant decrease in ET-1 release both at the end of 30min ischaemia (to 3.3 and 5.4 fold respectively; $p<0.05$) and at the end of 180min reperfusion (3.7 and 3.8 fold respectively; $p<0.05$) from pre-ischaemic values [1.1 (0.9-1.4) and 1.0 (0.9-1.2) pg $min^{-1}g^{-1}$), respectively]. In conclusion, the results of this study shows that the selective ET_A receptor antagonist is only protective against ischaemic/reperfusion injury within a very narrow concentration range. Furthermore, the effects of BQ123 on both haemodynamics and ET-1 release suggest that either an action of ET-1 at receptors other than ET_A is unmasked or BQ123 has partial agonist activity.

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22P SEASONAL DIFFERENCES IN THE ACTIONS OF VASOACTIVE AGENTS ON SEGMENTS OF DIGITAL ARTERIES OF THE FALLOW DEER

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Segments of digital arteries from fallow deer respond to a wide range of agents, including noradrenaline (NA), phenylephrine (PHE), 5-hydroxytryptamine (5-HT) and histamine (Scarlett, 1995; Fusi *et al.*, 1995). Digital arteries, collected in the winter, on mounting in organ baths, were found to be more constricted than those collected in the summer. A study was thus begun to determine if seasonal differences might be found in the responses of these vessel segments to vasoactive drugs.

Fallow deer (*Dama dama*), kept in open surroundings, were slaughtered on the farm. Digital arteries from the left foreleg were dissected, placed in cold aerated physiological salt solution and transported to the laboratory within 90 min. Eight segments at a time were mounted separately in organ baths containing modified Krebs-Henseleit solution at 37° C, aerated with 95% O_2 + 5% CO_2. The segments were left under a resting tension of 40 mN for 60 min before application of drugs and recording of isometric tension. Vessels were collected in January and February (winter) and May - September (summer).

Table 1. EC_{50} values for the vasoconstrictor actions of L-phenylephrine, 5-hydroxytryptamine and noradrenaline on deer digital arteries collected in winter and in summer

Agonist	EC_{50} summer (μM ± s.e.m)	EC_{50} winter (μM ± s.e.m)	
L-phenylephrine	2.75 ± 0.49 (8)	0.48 ± 0.13***	(11)
5-hydroxytryptamine	1.01 ± 0.16 (8)	0.23 ± 0.11*	(5)
L-noradrenaline	1.35 ± 0.27 (8)	0.80 ± 0.11	(13)

* $P < 0.05$, *** $P < 0.001$ (unpaired Student's t-test). Figures in brackets indicate the number of animals.

Arteries collected in the winter were not only more sensitive to the vasoconstrictor actions of PHE and 5-HT but they were also less sensitive to agents known to cause vasodilation. Of 31 winter vessels pre-contracted with PHE, 26 showed no relaxation to the endothelium-dependent agent, histamine. In these same vessels, the endothelium-independent agent, sodium nitroprusside produced a maximum relaxation in tone of only 3.8 ± 1.2%. In the 5 vessels that showed some relaxation to histamine, sodium nitroprusside produced a significantly greater relaxation of 47.9 ± 11.2% ($P < 0.05$).

Comparisons were also made with tension responses of segments of guinea-pig aorta. When the responses to NA and PHE were compared with those from winter deer artery segments, no significant differences in EC_{50}, Hill coefficient or normalised maximum responses were seen. The maximum relaxation produced by sodium nitroprusside of PHE-contracted vessels was 3.8 ± 1.2% for winter deer artery (n = 5) and 84.8 ± 3.5% (n = 4) for guinea-pig aorta ($P < 0.0001$). No detectable relaxation was produced by another nitric oxide donor, 3-morpholinosydnonimine, in deer artery (n = 4) compared with 40.9 ± 2.5% relaxation in guinea-pig aorta (n = 4); $P < 0.001$). A significant difference was also seen when the effect of forskolin on PHE-contracted vessels was examined. Maximum relaxation of deer vessels (n = 4) was 28.4 ± 11.5% compared with 73.9 ± 6.4% in guinea-pig aorta ($P < 0.05$).

It is possible that fallow deer, in adapting to lower ambient temperatures in winter, reduce the effectiveness of endothelium-dependent and -independent vasodilation so as to decrease blood flow to the limb extremity thereby limiting heat loss.

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23P 5-HT$_1$-LIKE RECEPTORS MEDIATING VASOCONSTRICTION IN EQUINE DIGITAL BLOOD VESSELS: EVIDENCE FOR DIFFERENT RECEPTOR SUBTYPES

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Equine laminitis is a common condition characterised by decreased perfusion of the sensitive hoof laminae and reperfusion injury. 5-HT is a very potent vasoconstrictor of equine digital arteries(EDA) and veins (EDV) which both possess 5-HT$_1$-like and 5-HT$_2$ receptors mediating vasoconstriction (Weller et al 1994, Bailey and Elliott 1996). We have suggested previously that EDA and EDV 5-HT$_1$-like receptors may differ on the basis of differing potency and efficacy of the 5-HT$_{1D}$ selective agonist, sumatriptan. Here we examine the effects of antagonists on EDA and EDV 5-HT$_1$-like receptors .

Rings of EDA and EDV, from a mixed population of healthy adult horses killed at abattoir, were denuded of their endothelium, prepared for isometric tension recording and preincubated with pargyline (0.5 mM) and benextramine (0.1 mM) for 30 min before washing. The contractile response to depolarising Krebs solution (DKS, 118 mM KCl) was obtained. Cumulative concentration response curves (CRCs) to 5-HT were then constructed over the concentration range 0.1 nM to 0.1 mM, 30 min after the addition of ketanserin (KET; 0.1 μM), together with yohimbine (YOH; 0.1 - 1 μM) and propranolol (PROP; 0.3 -3 μM). Additional CRCs were constructed for 5-carboxamidotryptamine (5-CT; 0.1 nM to 0.1 mM) in the presence of PROP(1 μM) and YOH(1 μM). Increases in tension were expressed as a percentage of the DKS response, (n=6 for each antagonist).

The CRCs to both 5-HT (in the presence of 0.1 μM KET) and 5-CT were biphasic. For both agonists, the first part of the CRC in the presence and absence of the antagonists were analysed and the results are shown in Table 1. For 5-HT the antagonism shown was dose dependent. In the presence of YOH and PROP, the exact position of the plateau in the 5-HT CRC became difficult to estimate. The apparent pK_b values were therefore calculated from the dose of 5-HT required to give 15% of the DKS response, rather than from estimates of the EC_{50} values for 5-HT.

These data support our suggestion that 5-HT$_1$-like receptors found in EDA and EDV are different subtypes. Both receptors show similar affinity for yohimbine, the 5-HT$_{1D}$ selective antagonist. Yohimbine also inhibits responses of EDV to sumatriptan with a similar pK_b value (Weller et al 1994). Propranolol (5-HT$_{1A/B}$ antagonist) has little or no effect on the EDV, but inhibits the 5-HT$_1$-like responses in EDA. Our previous studies showing the low potency and efficacy of 8-hydroxy-2-(N,N-dipropylamine) tetralin (5-HT$_{1A}$ selective agonist) suggest the EDA 5-HT$_1$-like receptor is not a 5-HT$_{1A}$ receptor. Equine digital vascular tissue appears to possess at least two subtypes of 5-HT$_1$-like receptor which can be distinguished by their affinity for propranolol.

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Table 1

agonist	antagonist (all 1 μM)	Arteries: $EC_{15\%}$ (5-HT) or EC_{50} (5-CT) * control	treated	apparent pK_b value	Veins : $EC_{15\%}$ (5-HT) or EC_{50} (5-CT) * control	treated	apparent pK_b value
5-HT	PROP	$5.46(4.2\text{-}6.8)\times10^{-9}$	$4.21(3.6\text{-}4.8)\times10^{-8}$ †	6.87 ±0.1	$5.43(4.1\text{-}6.7)\times10^{-9}$	$8.21(6.1\text{-}10.3)\times10^{-9}$	not determined
+KET	YOH	$5.46(4.2\text{-}6.8)\times10^{-9}$	$3.37(2.9\text{-}3.8)\times10^{-8}$ †	6.69 ±0.1	$2.83(2.1\text{-}3.6)\times10^{-9}$	$2.32(1.5\text{-}3.2)\times10^{-8}$ †	6.93 ±0.1
5-CT	PROP	$1.67(1.5\text{-}1.9)\times10^{-9}$	$7.35(3.9\text{-}10.9)\times10^{-9}$ †	6.49 ±0.1	$1.35(0.7\text{-}2.0)\times10^{-9}$	$1.29(0.2\text{-}2.4)\times10^{-9}$	not determined
	YOH	$1.67(1.5\text{-}1.9)\times10^{-9}$	$9.54(5.5\text{-}13.6)\times10^{-9}$ †	6.71 ±0.1	$1.35(0.7\text{-}2.0)\times10^{-9}$	$5.88(1.2\text{-}10.5)\times10^{-9}$ †	6.34 ±0.2

† significant, ANOVA with Dunnett's comparison
* Molar concentration, Geometric mean (95% confidence interval)

24P TYROSINE KINASE INHIBITORS REDUCE THE CONTRACTILE RESPONSE OF THE RABBIT ISOLATED RENAL ARTERY TO 5-HT

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5-Hydroxytryptamine (5-HT) is a potent vasoconstrictor and in the majority of blood vessels this action is mediated via the activation of 5HT$_2$ receptors (Van Nueten *et al.*, 1984). However, in the rabbit renal artery, 5-HT-evoked contraction appears to be mediated by the 5-HT$_{1D}$-like receptor (Choppin & O'Connor, 1994). Contraction via this receptor requires partial precontraction of the vessel with an agonist or raised extracellular potassium chloride. Recent studies in our laboratory have shown that stimulation of the 5-HT$_{1D}$-like receptor in rabbit mesenteric arteries leads to activation of phospholipase-D, an effect which in other systems has been shown to be sensitive to tyrosine kinase inhibitors (Uings *et al.*, 1993). In this study, we have examined the sensitivity of the contractile response of rabbit renal artery to tyrosine kinase inhibitors.

Female New Zealand white rabbits (1.5-2.5kg) were anaesthetised with an intravenous injection of sodium pentobarbitone (60mg.kg^{-1}) and killed by rapid exsanguination. Isolated segments (1.5-2mm) of renal artery were denuded of endothelium, mounted in a Mulvany-Halpern myograph for isometric force recordings. The tissues were set at a resting tension of 1g, and maintained at 37 °C in oxygenated Krebs buffer. Arterial segments were stimulated with phenylepherine (100μM) to assess the maximum response of the tissue. The tissues were incubated with ketanserin (1μM), prazosin (1μM) and different tyrosine kinase inhibitors (40μM) for 30 min, then precontracted with equimolar-substituted potassium Krebs buffer (20 mM), prior to cumulative application of 5-HT (1nM-10μM). The effect of the tyrosine kinase inhibitors on contraction to raised extracellular potassium (60 mM) was also examined. All 5-HT responses are expressed as a percentage of maximal phenylepherine contraction ± s. e. mean and differences calculated using the Students t-test.

The tyrosine kinase inhibitors genistein, and tyrphostins A23 and A47 caused a significant, non-competitive inhibition of the response to 5-HT (n=4; $P > 0.05$). In contrast, the inactive analogues of genistein and the tyrphostins, daidzein and tyrphostin A1, respectively, failed to cause any significant reduction of the response to 5-HT (n=4; $P < 0.05$). Both the active tyrosine kinase inhibitors and their inactive analogues did cause a significant inhibition (approximately 19%; n=4; $P > 0.05$) of contraction to potassium Krebs buffer (60mM). However, the difference between the inhibition with the active and inactive analogues was not significant (n=4; $P > 0.05$).

Table 1. Effect of Tyrosine kinase inhibitors (40 μM) and vehicle (DMSO) on the maximal contraction to 5-HT (10μM), expressed as a percentage of the maximal contraction to phenylepherine ± s.e.mean (n=4).

Compound	% Phenylephrine Contraction
Control (DMSO)	65.43 ± 1.83
Genistein	28.59 ± 7.32
Daidzein	62.86 ± 5.28
Tyrphostin A23	28.78 ± 4.78
Tyrphostin A47	43.26 ± 9.16
Tyrphostin A1	65.63 ± 10.61

Both the active and inactive analogues of the tyrosine kinase inhibitors depressed contraction to 60 mM potassium Krebs buffer. However, contraction to 5-HT was depressed selectively by the active analogues. These results suggest that the contractile response mediated by 5-HT$_{1D}$-like receptors in the rabbit isolated renal artery involves activation of a tyrosine kinase-dependent pathway.

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25P IDENTIFICATION OF A NOVEL FUNCTION FOR THE α_{2A}-ADRENOCEPTOR SUBTYPE IN THE RAT KIDNEY: MEDIATION OF OSMOLAR CLEARANCE

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Background: Clonidine (α_2-adrenoceptor agonist) increases free water (prazosin-sensitive) and osmolar clearance (naltrexone-sensitive) in the rat. This pharmacological dissociation suggested that two sites mediated these effects (Intengan and Smyth, 1994). Prazosin has selectivity for the α_{2b}- over other α_2-subtypes. Thus, the prazosin-insensitive osmolar response did not involve the α_{2b}-subtype. The α_{2a}- and α_{2b}-subtypes predominate in the rat kidney. The osmolar effect, independently of the α_{2b}-subtype, may involve the α_{2a}-subtype.

Aim: We postulated that stimulation of renal α_{2a}-adrenoceptors with an α_{2a}-selective agonist (guanfacine) would increase osmolar clearance in a naltrexone-sensitive/prazosin-insensitive manner. Moreover, the selective α_{2a}-subtype antagonist, RX-821002, would attenuate the response to guanfacine.

Methods: Seven days following unilateral nephrectomy under ether anaesthesia, male Sprague-Dawley rats (300±10g) were anaesthetised (pentobarbitone, 50 mg/kg, i.p.) and surgically prepared for urine collection (Blandford and Smyth, 1991). The response to an intrarenal infusion (3.4 µl/min) of 0.9% saline (vehicle) or guanfacine (3.0 nmol/kg/min) was determined. The ability of pretreatment (i.v.) with naltrexone (3.0 mg/kg), prazosin (0.15 mg/kg) or RX-821002 (3.0 mg/kg) to attenuate the response to guanfacine was determined. Six rats were used per drug treatment group. Data are presented as the mean ± S.E.M. * denotes $p<0.05$ versus control.

Results: Guanfacine increased urine flow (table 1) by increasing osmolar clearance. These responses were naltrexone-sensitive, prazosin-insensitive, and RX-821002-sensitive.

	C	G	G/N	G/P
UV	33 ± 7	85 ± 8*	61 ± 14	112 ± 8*
C_{OSM}	72 ± 14	116 ± 7*	85 ± 16	156 ± 12*
C_{H2O}	-40 ± 11	-31 ± 8	-24 ± 9	-44 ± 8

	C	G	G/R
UV	36 ± 7	104 ± 10*	36 ± 7
C_{OSM}	79 ± 10	136 ± 7*	101 ± 5
C_{H2O}	-43 ± 4	-32 ± 13	-65 ± 7

Table 1. Effects of saline (C) and guanfacine (G) in the presence and absence of naltrexone (N), prazosin (P), or RX-821002 (R) on UV (urine flow), C_{OSM} (osmolar clearance) and C_{H2O} (free water clearance). All units are µl/min.

Conclusion: α_{2a}-Adrenoceptor stimulation increased osmolar clearance in a naltrexone-sensitive manner. This response was blocked by α_{2a}-subtype antagonism. These data suggest that the α_{2a}-adrenoceptor subtype mediates solute handling.

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26P DO α_{1B}-ADRENOCEPTORS PLAY A ROLE IN THE EXAGGERATED VASCULAR RESPONSE TO α-AGONISTS IN EXPERIMENTAL HEART FAILURE?

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Responses of the dorsal pedal artery (DPA) and the saphenous vein (SV) to α_1-adrenoceptor (AR) stimulation are enhanced following pacing-induced heart failure in the dog and it was proposed that this was due to activation of differing populations of α_1-AR subtypes (Forster & Armstrong, 1990). Therefore, in this study, the effect of the α_{1B}-AR alkylating agent, chloroethylclonidine (CEC) was examined on α-AR induced contractions of DPA and SV from control, non-paced dogs and dogs which had been paced to severe heart failure.

Dorsal pedal artery and SV segments were obtained from four male mongrel control (non-paced) dogs and five dogs which were paced (right ventricle; 250bpm) to severe heart failure. Each vessel was cut into 6 rings and denuded of the endothelium before mounting in organ baths. Once equilibrated, cumulative concentration-effect curves were constructed to noradrenaline, methoxamine and phenylephrine (2 rings for each agonist). The concentration-effect curves were repeated in the presence of CEC (10^{-6}-10^{-4}M), keeping one ring of each pair as a control in which no antagonist was added. Curves were analysed by the logistic equation: $Y=\{(a+d)/[1+(X/c)^b]\}+d$ (Parker & Waud, 1971) and expressed as % maximum response in the absence of antagonist. The table shows % maximum response for noradrenaline (mean ± s.e.mean) where * $P<0.05$ versus non-paced and ^ $P<0.05$ versus no CEC.

The results for CEC against methoxamine and phenylephrine followed a similar profile to that seen with noradrenaline. These data show that, in the DPA and SV from dogs without heart failure, CEC antagonised contractions to the α-agonists. Moreover, the potency of CEC was greater in the DPA compared to the SV. In contrast, in vessels from dogs with severe heart failure, CEC did not inhibit contractions to α-agonists in the DPA, whereas it only antagonised the response in the SV at 10^{-4}M. Furthermore, at heart failure, lower concentrations of CEC potentiated the response to α-agonists.

Table 1 Effect of CEC on noradrenaline (NA) contraction.

	DORSAL PEDAL ARTERY		SAPHENOUS VEIN	
	non-paced	*paced*	*non-paced*	*paced*
NA no CEC	100	100	100	100
10^{-6}M CEC	80 ± 4^	125 ± 9*^	99 ± 13	145 ± 8*^
10^{-5}M CEC	68 ± 8^	124 ± 6*^	104 ± 9	147 ± 9*^
10^{-4}M CEC	33 ± 3^	82 ± 18*	60 ± 5^	68 ± 13^

These results indicate that, under normal circumstances, the DPA has a significant population of α_{1B}-AR compared to the SV. At heart failure, the population of α_{1B}-AR, in both DPA and SV, is negligible implying that α_{1B}-AR do not play a role in the exaggerated response to α-agonists. The potentiation of the α-agonist response possibly involves an atypical AR which has been described recently (Daniel *et al.*, 1996).

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27P THE α_1-ADRENOCEPTORS MEDIATING CONTRACTION OF RAT SMALL MESENTERIC ARTERY ARE DIFFERENT FROM THOSE MEDIATING PRESSOR RESPONSES IN RAT PERFUSED MESENTERY

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α_1-Adrenoceptors can be divided into the α_{1H} (α_{1A}, α_{1B} and α_{1D}-adrenoceptors) and α_{1L} classes, which display high and low affinity, respectively, for prazosin (Ford *et al.*, 1994). The pressor response to noradrenaline (NA) in the rat isolated perfused mesentery is mediated by α_{1A}-adrenoceptors (Ford *et al.*, 1996), but in contrast, it was recently suggested that an α_{1L}-adrenoceptor mediates contraction of the rat isolated small mesenteric artery (SMA, Van der Graaf *et al.*, 1996). We now present further evidence that none of the three currently recognised α_{1H}-adrenoceptors play a significant role in the contraction of rat SMA. SMA's (internal diameter 100-300μm) from male Wistar rats (225-300g) were mounted as ring segments in a myograph as described before (Van der Graaf *et al.*, 1996). Tissues were calibrated with a single contraction to 30μM 5-hydroxytryptamine (5-HT). The presence of the endothelium was then confirmed by the relaxant response to 10μM methacholine. After a 30min washout period, tissues were incubated for 60min with antagonist or vehicle and with 30μM cocaine, 6μM timolol and 10nM SCH-23390 to block neuronal uptake, ß-adrenoceptors and D_1 receptors, respectively. Single NA concentration-effect (E/[A]) curves (n=3-6) were then obtained by cumulative dosing.

NA produced concentration-dependent contractions of SMA's and the individual E/[A] curves were fitted to the Hill equation to provide estimates of the midpoint location ($pEC_{50}=5.92\pm0.11$), midpoint slope ($n_H=3.14\pm0.5$) and upper asymptote ($\alpha=162\pm16\%$ compared to the 5-HT sighter; n=4). Pretreatment (30min incubation, 30min washout) of the tissues with 10μM chloroethylclonidine (CEC), a ligand known to inactivate irreversibly α_{1B}-adrenoceptors (see Ford *et al.*, 1994), had no significant effect on the Hill parameters ($pEC_{50}=5.71\pm0.09$, $n_H=2.51\pm0.38$, $\alpha=162\pm13\%$, n=3). The α_{1D}-adrenoceptor antagonist, BMY 7378 (Ford *et al.*, 1996), produced parallel rightward shift of the NA curve only at the two highest concentrations tested (1 and 10μM). Schild analysis yielded a slope parameter not different from unity (0.72 ± 0.16, d.f.=12), thus allowing for the estimation of a pK_B value (6.16 ± 0.13). The selective α_{1A}-adrenoceptor antagonist, RS-17053 (10-300nM; Ford *et al.*, 1996), also produced concentration-dependent, parallel, rightward shifts of the NA E/[A] curve. Again, the Schild plot slope parameter was not significantly different from unity (1.14 ± 0.11, d.f.=21) and a pK_B of 8.35 ± 0.10 was estimated.

The lack of effect of CEC and the relatively low potency of BMY 7378 (pK_i for rat cloned α_{1d}-adrenoceptors=8.2; Ford *et al.*; 1996) and RS-17053 (pK_i for α_{1A}-adrenoceptors in rat submaxillary gland=9.1; Ford *et al.*; 1996) indicate that none of the three α_{1H}-adrenoceptors play a significant role in mediating contraction of rat SMA. Furthermore, the potency of RS-17053 in our assay was 35-fold lower than that reported by Ford *et al.* (1996) for antagonising pressor responses to NA in the perfused mesenteric assay ($pA_2=9.9$). Therefore, our results strongly suggest that the α_1-adrenoceptors operating in the rat SMA and perfused mesentery are not identical and call into question the reliability of the SMA assay as a predictor for α_1-adrenoceptor-mediated pressor activity.

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28P WB4101 DISCRIMINATES BETWEEN SUBTYPES OF α_1-ADRENOCEPTOR WITH A LOW AFFINITY FOR PRAZOSIN

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Three subtypes of α_1-adrenoceptor with a high affinity for prazosin have been cloned ($\alpha_{1a,b,d}$) and the existence of another receptor with a relatively lower affinity for prazosin has been proposed (review: Michel et al., 1995). The present study examines the affinity of prazosin at functional α_1-adrenoceptors in a variety of tissues.

Rats were pretreated with reserpine (5mgKg^{-1}, 24hr). Rat tissues and guinea-pig aorta were set up in gassed Krebs-bicarbonate solution at 37°C. Concentration-response curves to phenylephrine were constructed in the absence and presence of prazosin or WB4101 with a 30 min antagonist equilibration period. All experiments were performed in the presence of cocaine (10μM), corticosterone (10μM) and propranolol (1μM). Control experiments were used to correct for time-dependent changes in tissue sensitivity. Affinity values were compared using Students Newman-Keuls test following ANOVA.

Prazosin had a high affinity (Table 1) on the rat aorta, spleen and perfused mesenteric bed, the aorta and mesenteric bed also having a high affinity for WB4101 compared with the spleen.

The other tissues examined had a relatively lower affinity for prazosin. Amongst these tissues, the affinity of WB4101 varied, being highest on the rat vas deferens, urethra and prostate which had affinities significantly greater than the value obtained on the guinea-pig aorta.

Table 1: pK_B values (from individual shifts of curves) and Schild plot slopes for antagonism by prazosin and WB4101 ($n\geq13$).

	PRAZOSIN		WB4101	
Tissue	pK_B	slope	pK_B	slope
Mesenteric bed	9.4±0.1	1.11	10.1±0.1	0.93
Rat spleen	9.4±0.1	1.19	8.4±0.1	0.91
Rat aorta	9.7±0.1	1.00	9.2±0.1	1.25
Vas deferens	8.6±0.1	0.95	9.4±0.1	1.12
Rat urethra	8.4±0.1	1.05	9.0±0.1	1.08
Rat Prostate	8.7±0.1	0.75	9.2±0.1	0.95
Anococcygeus	8.9±0.1	0.80	8.8±0.1	0.93
Portal vein	8.5±0.1	0.68	8.8±0.1	1.50
Left atria	8.4±0.1*	---	8.5±0.1*	0.70
Guinea-pig aorta	8.2±0.1	0.95	8.4±0.1	1.03

*apparent pK_B, Schild slope not unity ($P<0.05$)

These results suggest that two receptors with a low affinity for prazosin can be identified using WB4101.

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29P EFFECTS OF CENTRAL AND PERIPHERAL CHOLINERGIC DRUGS ON LOCOMOTOR ACTIVITY AND L-DOPA-INDUCED DYSKINESIA IN MPTP-TREATED COMMON MARMOSETS (*CALLITHRIX JACCHUS*)

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The common marmoset shows parkinsonian motor disability following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure and develops dyskinesias during chronic L-DOPA dosing (Pearce *et al.*, 1995). We have previously reported the effects of cholinergic drugs, alone and in combination with L-DOPA on locomotor activity, parkinsonian disability and dyskinesias. We now use cholinergic drugs which only act peripherally to distinguish between the central and peripheral effects of these drugs.

Animals (n=12) were divided into 3 groups: naive controls; MPTP-treated; MPTP-treated and L-DOPA-primed for 26 days to induce dyskinesias. Each group received scopolamine and the peripherally acting methylscopolamine at 0.1 and 0.3 mg/kg sc. and physostigmine at 0.2 mg/kg sc and the peripherally acting neostigmine at 0.05, 0.07 and 0.1 mg/kg sc. consecutively. The L-DOPA-primed group also received a combination of each experimental drug with carbidopa and L-DOPA each at 12.5 mg/kg po.. Activity levels were monitored in locomotor cages. Disability was scored (0=normal to 10=severely parkinsonian) and dyskinesias were rated as previously described (Pearce *et al.*, 1995).

L-DOPA was highly effective and scopolamine moderately effective in reversing MPTP-induced akinesia. Physostigmine reduced locomotor activity below baseline levels. Neostigmine and methylscopolamine had no effects on basal activity. Physostigmine administered with L-DOPA almost abolished the locomotor effects of L-DOPA whereas neostigmine when administered with L-DOPA had no effect (Figure). Scopolamine and methylscopolamine both antagonised the peak effect of L-DOPA, causing a substantial reduction in maximal activity while prolonging the period of enhanced locomotor activity. The cholinergic drugs alone had no effect on parkinsonian disability in MPTP-treated animals. L-DOPA, and each of the cholinergic drugs, alone and in combination with L-DOPA reduced parkinsonian disability in the L-DOPA-primed group. There was no additive effect of coadministering cholinergic drugs with L-DOPA. In this group, L-DOPA administration produced marked dyskinesia whereas physostigmine and neostigmine produced negligible levels of dyskinesia. Scopolamine alone induced high levels of predominantly choreiform dyskinesia while methylscopolamine produced none. In combination with L-DOPA, neostigmine and methylscopolamine had no effect on type or intensity of L-DOPA-induced dyskinesia but physostigmine caused a reduction in the proportion of chorea and scopolamine tended to exacerbate chorea.

These results suggest that procholinergic drugs abolish the locomotor effects of L-DOPA by a central mechanism. In contrast, anticholinergic drugs probably antagonise the action of L-DOPA by a peripheral action. Cholinergic manipulation of dyskinesia must be centrally mediated as peripherally acting agents have no effect.

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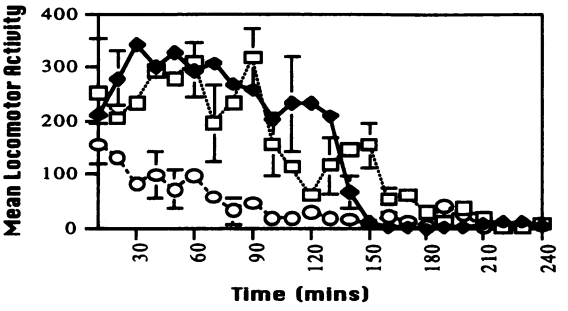

Figure. Mean locomotor activity in MPTP-treated, L-DOPA-primed marmosets after L-DOPA (12.5 mg/kg) —◆—, L-DOPA plus neostigmine (0.07 mg/kg) ·····□·····and L-DOPA plus physostigmine (0.2 mg/kg) ----○----

30P L-745,870 (3-{[4-(4-CHLORO-PHENYL)PIPERAZIN-1-YL]METHYL}-1H-PYRROLO[2,3-B]PYRIDINE): A HIGH AFFINITY AND SELECTIVE DOPAMINE D4 RECEPTOR ANTAGONIST

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The atypical antipsychotic clozapine shows 15 fold selectivity for the human D4 over D2 dopamine receptors (Van Tol et al, 1991) implicating that D4 rather than D2 receptor antagonism results in its antipsychotic effect. Until recently there has been a lack of highly selective D4 compounds with which to evaluate the role of D4 receptors in the brain. We here report the biological profile of L-745,870 which displays high affinity and selectivity for the dopamine D4 receptor.

[^{3}H] Spiperone binding and adenylate cyclase studies in chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells stably expressing human dopamine receptors were performed according to Freedman et al, 1994. For *in vivo* sigma binding, mice were injected with drug/saline 40min prior to i.v. administration of [^{3}H] SKF 10,047. 20min later, mice were killed and brain membranes prepared using Tris HCl (pH7.4, 0°C) and an aliquot filtered through GF/B filters to determine the bound fraction. Non specific binding was determined using 3mg/kg haloperidol s.c. Dopamine metabolism studies were performed according to Bristow et al, 1993. The effect of L-745,870 (0.01-10mg/kg) on rat plasma prolactin levels was determined by radioimmunoassay (TRK432, Amersham,U.K.) 30min following oral administration.

L-745,870 displayed an improved D4 receptor binding profile compared to clozapine and haloperidol with subnanomolar affinity and >2,000 fold selectivity over hD2 and hD3 receptors (Table1). In adenylate cyclase studies dopamine (1μM) inhibited forskolin (10μM) stimulated cAMP levels (from 0.45 to 10.8±0.81 pmoles cAMP per well) by 46%. L-745,870 (1μM) had no agonist activity but exhibited D4 receptor antagonist activity by antagonising the dopamine response at 100nM . The weak affinity of L-745,870 for sigma receptors (IC_{50} 0.15μM) was used to determine its brain penetration and estimate occupancy of central D4 receptors. L-745,870 (0.1-10mg/kg p.o.) inhibited in vivo binding of [^{3}H] SKF 10,047 to mouse brain (ED_{50} 3 mg/kg p.o.). Since brain concentration of L-745,870 by HPLC analysis was proportional over the dose range 30-10000μg/kg p.o, the dose required to provide 50% occupancy of central D4 receptors was projected to be 8.6μg/kg p.o. L-745,870 (10mg/kg p.o.)had no effect on rat plasma prolactin levels and had no effect (30mg/kg p.o.) on dopamine metabolism in the mouse striatum or nucleus accumbens.

In conclusion, the lack of effect with L-745,870 on dopamine metabolism or plasma prolactin levels at doses which exceeded the estimated dose required for D4 receptor occupancy, suggest that the efffect of neuroleptics on these neurochemical responses results from D2 and/or D3 receptor antagonism. Indeed, D4 receptor antagonism in man is unlikely to result in galactorrhea. Compounds such as L-745,870 will prove useful in identifying the role of D4 receptors in brain function.

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Table 1. Dopamine receptor selectivity of L-745,870.

Ki (nM) values are geometric means of at least 4 separate experiments and values in parentheses are the low and high errors of this mean.

Compound	hD2CHO Ki nM	hD3 HEK Ki nM	hD4 HEK Ki nM	D2/D4 selectivity
L-745,870	860 (680;1100)	2,300 (1800;2800)	0.43 (0.28;0.66)	2,000
Clozapine	74 (65;83)	200 (180;220)	10(5.8;19)	7.4
Haloperidol	1.4 (1.1;1.8)	2.0 (0.95;4.2)	2.3 (1.9;2.9)	0.61

31P INFLUENCE OF CANNABINOIDS ON DOPAMINE RELEASE AND cAMP GENERATION IN THE RAT STRIATUM

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Previous pharmacological studies have established not only the presence of central cannabinoid receptors (CB1), in areas such as the cerebellum and the striatum, but also their functional coupling to the inhibition of adenylyl cyclase and the isolation of anandamide as a putative endogenous ligand for the CB1 receptor (Howlett, 1995). Inhibition of adenylyl cyclase and neurotransmitter release has been difficult to demonstrate in some brain areas (Cadogan *et al.*, 1995). But in the striatum there is evidence to suggest that the CB1 receptors are associated with dopamine receptors and may exert an influence over dopamine release via inhibition of adenylyl cyclase (Bidaut-Russell & Howlett, 1991). Therefore in the present study, the effects of cannabinoid ligands on forskolin-stimulated cAMP levels in rat striatal slices were investigated. Subsequently the effects of these agents on direct dopamine release in rat striatal slices were also investigated .

Accumulation of forskolin-stimulated [^{3}H]cAMP was monitored in rat striatal slices labelled with [^{3}H]adenine as previously described (Alexander, 1995). Data (means ± s.e.mean) were expressed as a percentage conversion from the total [^{3}H] adenine from at least three separate experiments. Using a superfusion apparatus [^{3}H]dopamine pre-labelled rat striatal slices underwent two periods of electrical stimulation (biphasic pulses of 100mA at 0.5 Hz for 3 mins) with the results expressed as a ratio of the two stimulations (S2/S1).

Basal accumulation of [^{3}H]cAMP in rat striatal slices was 0.19 ± 0.01 %conversion. Forskolin (10μM) stimulated a significant increase in [^{3}H]cAMP accumulation (1.9 ± 0.18% $p<0.01$ Student's t-test n=4) which was signifcantly reduced in the presence of both the cannabinoid agonists, CP 55,940 (1μM) and anandamide (100μM) (1.2 ± 0.06, 1.2 ± 0.08 respectively $p<0.05$ Student's t-test n=4) (Howlett, 1995). The significant reductions in the forskolin-stimulated [^{3}H]cAMP accumulation seen with both CP 55,940 and anandamide were reversed in the presence of the the cannabinoid antagonist, SR141716 (1μM) (1.6 ± 0.19 and 1.8 ± 0.12 respectively) (Howlett, 1995).

Electrical stimulation of [^{3}H]dopamine pre-labelled striatal slices gave a S2/S1 ratio of 87.2 ± 9.5% (n=5) which was significantly reduced in the presence of CP 55,940 (1μM) (45.8 ± 6.2 % $p<0.05$ Student's t-test n=5) and reversed in the presence of SR 141716 (79.4 ± 7.2% n=5). SR 141716 had no effect on electrically-evoked dopamine overflow in striatal slices as compared to control conditions (control, 105 ± 12% and SR 141716, 91 ± 18 % n=3).

These data indicate that the CB1 receptors in rat striatum are negatively linked to adenylyl cyclase and dopamine release. The observation that the CB1 receptor may influence dopamine release in the striatum suggests that cannabinoids play a modulatory role in dopaminergic neuronal pathways.

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32P SH-SY5Y CELLS EXPRESS sst_2 SOMATOSTATIN RECEPTORS

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We have previously demonstrated that in the human neuroblastoma cell line SH-SY5Y, in the presence of carbachol, δ- and μ-opioid receptor agonists mobilize Ca^{2+} from thapsigargin-sensitive intracellular stores in a pertussis toxin-sensitive manner (Connor & Henderson 1996). Somatostatin also evokes a PTX-sensitive elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells in the presence of carbachol (Connor *et al.*, 1996). Here we have characterized the type of somatostatin receptor present on these cells, and examined the mechanism by which somatostatin elevates $[Ca^{2+}]_i$.

Intracellular $[Ca^{2+}]_i$ was measured in confluent monolayers of undifferentiated SH-SY5Y using Fura 2 as described previously (Connor & Henderson 1996). Data are presented as mean ± s.e.m. from 3-11 experiments.

Carbachol (1μM) caused an elevation of $[Ca^{2+}]_i$ which then declined to a plateau level in the continuous presence of the drug. In the presence of carbachol somatostatin (0.1-300nM), BIM-23027 (0.01-1000nM) a sst_2 receptor preferring agonist and L-362855 (0.01-10μM) a sst_5 receptor preferring agonist, all increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The concentration response curves were approximately parallel with the same maximum elevations (Connor *et al.*, 1996). EC_{50} values are given in Table 1. BIM-23056 (0.01-10μM), a sst_3 receptor selective agonist, did not elevate $[Ca^{2+}]_i$ in the presence of carbachol. In addition, at 1μM BIM-23056 did not antagonise somatostatin (10nM). The relative agonist potencies compare well with the values obtained for these compounds using recombinant sst_2 receptors (Castro *et al.*, 1996; Raynor *et al.*, 1993), and suggest that SH-SY5Y cells express sst_2 receptors.

Table 1. Relative potencies of somatostatin analogues to elevate $[Ca^{2+}]_i$ in SH-SY5Y cells

	EC50 (nM)	relative potency
Somatostatin$^+$	6 ± 2	1.0
BIM-23027	2.4 ± 0.7	0.4
L-362855	452 ± 198	75.3
BIM-23056	-	>1000

$^+$ From Connor *et al* 1996.

The elevation of $[Ca^{2+}]_i$ by somatostatin (100nM) in the absence of extracellular Ca^{2+} was 98 ± 9.8% of that seen in the presence of 2.5mM extracellular Ca^{2+}. Thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, promotes the emptying of intracellular Ca^{2+} stores. When cells were exposed to 100nM thapsigargin there was a gradual increase of $[Ca^{2+}]_i$ from 85 ± 9nM to 375 ± 84nM. After 10 min exposure to thapsigargin, somatostatin (100nM) in the presence of carbachol (1mM) did not evoke a further elevation of $[Ca^{2+}]_i$. Application of maitotoxin (2ng.ml^{-1}), an agent which promotes Ca^{2+} influx across the plasma membrane, elevated $[Ca^{2+}]_i$ from 71 ± 12nM to 288 ± 18nM. Application of somatostatin (100nM) in the presence of maitotoxin failed to evoke a further rise in $[Ca^{2+}]_i$. These data suggest that the elevation of $[Ca^{2+}]_i$ by somatostatin may be due to Ca^{2+} release from thapsigargin-sensitive intracellular stores, not Ca^{2+} entry across the plasma membrane.

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33P INHIBITION OF NITRIC OXIDE SYNTHASE POTENTIATES NMDA-EVOKED DOPAMINE RELEASE IN RAT STRIATUM BOTH *IN VITRO* AND *IN VIVO*

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The role of nitric oxide (NO), formed following N-methyl-D-aspartate (NMDA) receptor stimulation (Garthwaite et al, 1989), in the striatum is controversial. *In vitro* evidence suggests that inhibition of NO synthase (NOS) inhibits NMDA-evoked dopamine (DA) release (Hanbauer et al, 1992), however, *in vivo* experiments find the opposite to be true (Rose et al, 1994). To clarify this controversy, we report the effect of inhibition of NOS on NMDA-evoked DA release both *in vitro* and *in vivo*.

In vitro experiments: Striatal slices (300µm) were prepared and incubated with ^{3}H-DA (12.5µM for 30 min) in Krebs bicarbonate buffer containing pargyline (25µM). Slices were washed in Krebs and superfused at 250µl/min in chambers at 37°C. After 1h, fractions of superfusate were collected every 5min. Following determination of basal ^{3}H-DA release, NMDA (100µM) was incorporated in the Krebs for 2 fractions (S1 stimulation) followed by 4 fractions of Krebs. N^G-nitro-L-arginine methyl ester (L-NAME; 100-500µM) or N^G-nitro-D-arginine methyl ester (D-NAME; 100-500µM) were then incorporated for 6 fractions alone, for 2 fractions in combination with NMDA (100µM; S2 stimulation) followed by a further 4 fractions alone. ^{3}H-DA was measured in the fractions, and in the tissue at the end of the experiment by liquid scintillation spectroscopy. *In vivo experiments:* Microdialysis probes were implanted in the striatum of chloral hydrate anaesthetised male Wistar rats and perfused and stimulated with NMDA as previously described (Rose et al, 1994). L-NAME, 7-nitro indazole Na^+ (7-NINA), D-NAME, L-NAME + L-arginine (L-ARG), 7-NINA+ L-ARG or L-ARG alone (all at 1mM) were included in the perfusate for 1h before, during and for 40min after the S2 NMDA stimulation. DA levels were measured by h.p.l.c. The effect of inhibition of NOS was determined by comparing the S2/S1 ratio to NMDA alone or in the presence of drug.

NMDA increased DA efflux both *in vivo* and *in vitro*. L-NAME and 7-NINA increased the S2/S1 ratio *in vivo* (Figure). These effects were not seen with D-NAME, and were prevented by co-perfusion with L-ARG (Figure). L-ARG alone had no effect on the S2/S1 ratio. L-NAME (500µM but not 100µM) increased the S2/S1 ratio *in vitro* (0.87±0.04 and 0.77±0.04 compared to 0.61±0.04 and 0.76±0.08 for D-NAME, 500 and 100µM respectively; $p<0.05$ paired t-test), $n=6$

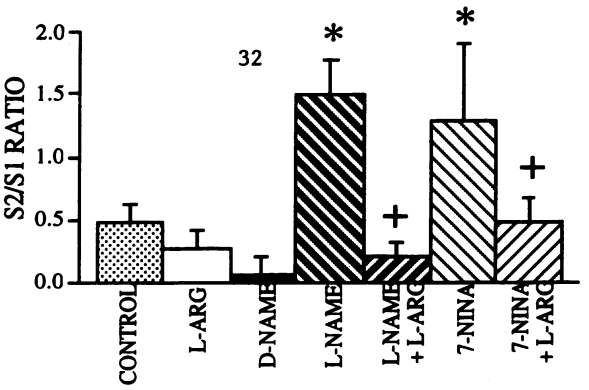

Figure The effect of 7-NINA and L-NAME on NMDA-evoked DA release in the rat striatum. * $p< 0.05$ compared to control; + $p<0.05$ compared to NOS inhibitor alone (Newman-Keul's test; n=6-9).

Inhibition of NOS potentiates NMDA-evoked dopamine release both *in vivo* and *in vitro* in the rat striatum, suggesting that NO has an inhibitory effect on NMDA-evoked DA release. This inhibition may be due to a negative feedback effect of NO on the NMDA-receptor (Lei *et al.*, 1992). Methodological differences may explain the different effect seen *in vitro* in this study compared to that of Hanbauer *et al* (1992).

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34P DO DIFFERING LEVELS OF THE VOLTAGE DEPENDENCE OF NMDA OPEN CHANNEL-BLOCKERS AFFECT THEIR *IN VIVO* ACTIONS ON SPINAL NEURONES IN ANAESTHETISED RATS?

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Uncompetitive NMDA antagonists show differing degrees of voltage dependence *in vitro*, but the extent to which this affects their actions *in vivo* is not established. We have now compared the effects of 3 channel-blocking NMDA antagonists, ketamine (KET), memantine (MEM) and MK801, on responses of spinal neurones.

In α-chloralose anaesthetised male Wistar rats (280-350g), extracellular action potentials were recorded from dorsal horn neurones (in 24 spinalised rats) or from hindlimb flexor muscle single motor units (SMU; in 9 spinalised and 22 sham-spinalised rats). Responses were elicited in 3-4min cycles of microiontophoretic NMDA/AMPA (40 s) or pinch (15 s, 1.1-2.5N over 16mm^2). Ejection/stimulus intensities were chosen to evoke paired 'lower' and 'higher' spike discharge frequency responses in each cycle. Antagonists were given in cumulative doses i.v. Effects are expressed as % of the last three pre-drug control responses (mean ± s.e.m.); statistical analysis was by the Wilcoxon matched pairs test where $n\geq6$ or the Mann-Whitney U test where $n<6$.

In all cases, lower frequency responses were reduced more than higher frequency responses, but the magnitude of the difference varied between antagonists. With NMDA responses, doses of MEM (6.3±2.0, n=6), KET (1.3±0.3, n=12) and MK801 (0.1±0.0 mg/kg, n=5) that reduced lower frequency responses (17±1 spikes/s) to similar levels (to 21±4%, 20±4% and 23±4% control) were less effective on higher frequency responses (51±1 spikes/s; reduced to 67±7%, 55±4% and 39±9% control); the differences were significant ($p<0.05$) for MEM and KET but not for MK801.

With SMU nociceptive reflex responses in sham spinalised animals, doses of MEM (32±0.0, n=6), KET (3.4±0.1, n=11) and MK801 (1.5±0.2 mg/kg i.v., n=5) that reduced lower frequency (17±1 spikes/s) responses to about 10% control (7.3±2, 9.9±2, and 12±1%) again had significantly less ($p<0.05$) effect on higher frequency (29±1 spikes/s) responses (reductions to 76±7, 64±6 and 40±9% control). The reductions by MEM and KET were more dependent on response amplitude than were those by MK801.

With SMU responses in spinalised animals, doses of MEM (19±5, n=6) and KET (4.1±0.8 mg/kg i.v., n=9) that reduced lower frequency (17±2 spikes/s) responses to about 10% control (8±1 and 10±2%) again had significantly less ($p<0.05$) effect on higher frequency (27±2 spikes/s) nociceptive responses (reductions to 60±10 and 64±3% control).

The results show that MEM, KET and MK801 were variably dependent on response firing frequency. The reduction of responses by MEM was most, and that by MK801 least affected by increasing spike discharge rate during responses. This matches with the known greater voltage dependence of MEM and KET *vs.* MK801 channel block (Parsons *et al.*, 1993). We consequently suggest that the varied *in vitro* voltage dependence of these 3 NMDA channel-blockers does have correlates *in vivo*. This may in turn contribute to the clinical advantages of memantine over drugs like ketamine.

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35P CHARACTERISATION OF THE BINDING OF [^{3}H]-NOCICEPTIN IN THE GUINEA-PIG

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A novel basic peptide, isolated from rat brain, termed nociceptin or orphanin FQ (Meunier *et al.*, 1995; Reinscheid *et al.*, 1985) has been found to be an endogenous ligand for the ORL_1 receptor which displays a high level of homology to the cloned opioid receptors. [^{3}H]-nociceptin is now available and we have investigated the binding of [^{3}H]-nociceptin in the guinea-pig and compared the binding with that found with ligands selective for each of the μ, δ and κ-opioid binding sites.

Washed membranes were prepared from the brain, cerebellum and a number of other tissues from male Dunkin-Hartley guinea-pigs in 50mM Tris.HCl(pH 7.4)(Kosterlitz *et al.*, 1981). Homogenates were incubated with tritiated ligands for 60 min at 25°C in a final volume of 1ml. Samples were filtered over GF/B filter which were pre-soaked with 1% polyethyleneimine and washed with ice-cold Tris.HCl. Non-specific binding was determined with 100nM unlabelled nociceptin. [^{3}H]-Bremazocine, [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO), [^{3}H]-naltrindole and [^{3}H]-CI-977 were used to label the opioid sites and non-specific binding was defined with 1μM naloxone.

In homogenates of guinea-pig brain, [^{3}H]-nociceptin labels a single population of binding sites with a K_D of 0.134±0.03nM and a binding capacity of 9.78±1.9 pmol.g^{-1} (n=3). The binding capacity of the universal opioid ligand [^{3}H]-bremazocine was 14.2±1.51 pmol.g^{-1} whereas those of [^{3}H]-DAGO (μ), [^{3}H]-naltrindole (δ) and [^{3}H]-CI-977 (κ) were between 3.3 and 4.94 pmol.g^{-1}. Binding of [^{3}H]-nociceptin was also found in the cerebellum, retina, spinal cord and myenteric plexus but not in the kidney, lungs, adrenals and spleen.

Although nociceptin has some affinity for opioid binding sites, [^{3}H]-nociceptin was not labelling opioid sites as the binding of 0.1nM [^{3}H]-nociceptin was not displaced by the opioid antagonists diprenorphine, naloxone or naltrexone at a concentration of 1μM.

The results indicate that [^{3}H]-nociceptin labels a unique population of binding sites in tissues of neural origin. Despite the similarity in the structure of the ORL_1 receptor and the cloned opioid receptors, it is unlikely that the sites labelled by nociceptin represent a subtype of opioid receptor as the binding is not displaced by classical opioid ligands and the binding capacity is greater than that of any of the μ–, δ– or κ-opioid sites.

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36P CHARACTERISATION OF THE RESPONSE IN THE RAT VAS DEFERENS TO THE ORL1 AGONIST NOCICEPTIN

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Nociceptin (or Orphanin FQ) is the recently isolated endogenous ligand of the ORL_1 receptor. ORL_1 transcripts are present in various regions of the CNS, and are also found in some peripheral tissues including the rat vas deferens (RVD) (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

We have tested for the presence of a functional ORL_1 receptor in the RVD using preparations from Hooded Lister rats (~250g) mounted in 3 ml silanised glass organ baths containing modified Krebs solution (1.25 mM $CaCl_2$, 0.6 mM $MgSO_4$) at 37°C. The tissues were electrically stimulated with 5 × 0.5 ms pulses at 10 Hz every 20 seconds at supramaximal voltage (~70 mA). Values for IC_{50} are presented as mean (with range); those for apparent pK_B and pK_i are given as mean ± s.e.mean.

Cumulative administration of nociceptin to electrically stimulated RVD produced a concentration-dependent decrease in tissue contraction with an IC_{50} of 18.21 nM (13.58-20.48, n=6) and an E_{max} of 92.44±1.16%. This was qualitatively similar to the response obtained with β_{rat}-endorphin, where the IC_{50} was 7.88 nM (6.99-9.24, n=6) and an E_{max} of 94.94±0.87%. The response to nociceptin could not be antagonised by naloxone up to 3 μM. As expected, the response to β-endorphin was competitively antagonised by naloxone with an apparent pK_B of 8.28±0.01 (n=3), suggesting an action at the μ-opioid receptor.

Addition of peptidase inhibitors (30 μM amastatin, bestatin, captopril and phosphoramidon) increased the potency of nociceptin as an agonist 4-fold (IC_{50} 4.90 nM, 3.48-7.29, n=6), indicating that nociceptin was sensitive to peptidases in the RVD. The β-endorphin response, however, was unaffected by the peptidase inhibitors.

The response to nociceptin could not be antagonised by diprenorphine, CI-977, meptazinol, naltrexone or naltrindole, all at 3 μM, or the irreversible opioid receptor alkylating agent β-CNA (30, 100 or 300 nM, using incubation periods up to 90 minutes). However, addition of the opioid ligands bremazocine, Win 44441, MR 2266 and naloxone benzoylhydrazone at high concentrations (1-15 μM) was able to antagonise the nociceptin-induced response (apparent pK_B values of 5.69±0.06, 5.75±0.24, 5.16±0.05 and 5.77±0.28 respectively, n=4-6).

Electrically-evoked contractions of rabbit vas deferens were tested in a similar manner and were also found to be inhibited by nociceptin; in the presence of peptidase inhibitors the IC_{50} was 3.37 nM (1.2-7.5, n=3). This response was not antagonised by 3 μM naltrexone or 300 nM of the classical κ-opioid receptor antagonist norbinaltorphimine.

In binding experiments in guinea-pig brain membranes (Kosterlitz *et al.* 1981) nociceptin had some affinity for μ- and κ-binding sites (pK_i 6.59±0.051 and 6.55±0.156 against 1 nM [^{3}H]-DAGO and 0. 25nM [^{3}H]-CI-977 respectively, n=3). The lack of effect of classical opioid antagonists naloxone or naltrexone against the response to nociceptin in the vasa deferentia, however, excludes an interaction with the μ- or κ-receptor in the mediation of the opioid-like action of nociceptin we have described; rather the effect may be via an endogenously expressed ORL_1 receptor.

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Reinscheid, R.K., Nothacker, H-P., Bourson, A. *et al.* (1995) *Science* 270, 792-794.

37P INCREASED EXCITABILITY OF DAMAGED RAT SENSORY NEURONES INVOLVES ALTERED COUPLING BETWEEN CALCIUM CHANNELS AND ADRENOCEPTORS

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Adrenergic mechanisms contribute to chronic pain syndromes that follow peripheral nerve damage. Following injury, sympathetic fibres sprout into dorsal root ganglia (DRG, McLachlan *et al.* 1993) where they activate ectopic excitatory α-adrenoceptors. We sought to elucidate the mechanism(s) of noradrenaline(NA)-induced activity in axotomized rat DRG cells. The sciatic nerve of male rats (120-170g) was sectioned and a 10 mm segment removed to prevent regeneration. The extent of autotomy (foot mutilation) was scored according to Wall *et al* (1979). After 2-7 weeks, DRG cells were dissociated and A-, H- and C-cells examined by whole-cell recording. The excitability of A-, H- or C-cells from control animals was unchanged by NA (10-100μM) whereas it was increased in 2/15 A-, 6/13 H- and 11/18 C-cells from axotomized rats. In cells from rats that exhibited autotomy, excitability was increased in 10/15 A-, 9/12 H- and 14/16 C-cells. NA (10μM) potentiated I_{Ca} in control C-cells (12/14) by 34.5±2.4% and in H-cells (7/13) by 18.5±2.8%. The effect of NA on I_{Ca} in axotomized cells was opposite to that seen in controls. Thus, NA decreased the current in 3/12 A-cells by 19.9±2.6%, in 11/12 H-cells by 26.5±2.0% and in 13/13 C-cells by 29.8±2.0%. In cells from rats that exhibited autotomy, NA reduced I_{Ca} of 8/12 A-cells by 29.2±2.2%, 12/12 H-cell by 34.8±2.5% and 13/13 C-cells by 36.4±2.0%. NA (10μM) reduced *only* the Ca^{2+}-sensitive portion of outward K^+-current (n=11-14). It therefore increased excitability by affecting Ca^{2+}-activated K^+ conductance(s) as a consequence of its action on I_{Ca}. Differences between the control and axotomy groups were significant ($P<0.01$; χ^2 or Student's t-test). In control cells, the NA-induced potentiation of I_{Ca} was occluded by 2μM nifedipine (n=5 or 9), blocked by 1μM propranolol (n>3) and mimicked by 10μM isoprenaline (n>4). α-adrenoceptor agonists and antagonists were ineffective. By contrast, the effects of NA on repetitive discharge and I_{Ca} in cells from axotomized rats were blocked by 1μM yohimbine (n=2-4) and were mimicked by 10μM clonidine (in 2/6 C- and 1/4 H-cells) and by 10μM U.K.14,304 (in 3/6 C-, 2/4 H- and 1/3 A-cells). The effects of NA in cells from axotomized rats were insensitive to prazosin and propranolol (up to 10 μM) and were not mimicked by isoprenaline. Suppression of I_{Ca} was occluded by 1μM ω-conotoxin GVIA (n=3-10). NA therefore affects β-adrenoceptors to increase L-type I_{Ca} in control neurones and acts on α_2-adrenoceptors to suppress N-type I_{Ca} and increase excitability in axotomized neurones. This change in coupling between Ca^{2+}-channels and adrenoceptors may underlie the ectopic activity in injured sensory nerves that may contribute to chronic pain. This is supported by the observation that NA is more effective in animals that exhibit autotomy and in C-cells that normally transmit nociceptive information.

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38P SYNERGISTIC SUPPRESSION OF EXTRACELLULAR NORADRENALINE CONTENT IN THE RAT FOREBRAIN BY SODIUM PENTOBARBITONE AND α_2-ADRENOCEPTOR AGONISTS

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Selective α_2-adrenoceptor agonists produce a marked reduction in barbiturate (Mason & Angel, 1983) and volatile (Devcic et al., 1994) anaesthetic requirement. It is unclear whether these effects are mediated *via* activation of presynaptic or postsynaptic α_2-adrenoceptors in the CNS. To resolve this we have examined the combined effects of sodium pentobarbitone (PB), and two α_2-agonists, clonidine and mivazerol (Noyer *et al.*, 1994), on extracellular levels of noradrenaline (NA) in the rat forebrain. This approach should establish whether barbiturates and α_2-agonists act in a concerted manner at the presynaptic α_2-adrenoceptor.

Dialysis probes were vertically implanted in the frontal cortex (AP+3.0; L±3.2; V-4.0 *cf* bregma) of male Wistar rats (245-285 g) under PB anaesthesia (96 $mg.kg^{-1}$ i.p.) and, 24 h later, perfused with phosphate-buffered medium (pH 7.4) at 2.0 $\mu l.min^{-1}$. Basal NA levels were determined over 6x10 mins by HPLC-ECD before rats were either anaesthetised with PB (96 $mg.kg^{-1}$ i.p.) and placed on a heating blanket set at 37°C, or injected with saline and maintained in their home-cage. Thereafter, either clonidine hydrochloride (n=6), mivazerol hydrochloride (n=6) or saline (n=6) were administered by i.v. infusion (each at 2.05$\mu g.kg^{-1}.h^{-1}$;1.25 $ml.h^{-1}$). Each drug solution also contained 4.78 $\mu Ci.ml^{-1}$ of respective tritiated ligand (12% of total dose). 2 h later, rats were killed by PB overdose and cervical dislocation. Several brain regions were then dissected out, dissolved in soluene (20 $\mu l.mg^{-1}$) and analysed by scintillation counting. Data were either analysed by ANOVA and *post hoc* Dunnett test (*f*moles NA.10 min^{-1}) or by a Mann-Whitney U test (d.p.m.mg^{-1} tissue).

Acute PB administration transiently reduced NA levels in cortical dialysates. This reduction was potentiated by doses of clonidine and mivazerol which alone produced no significant effect on spontaneous overflow (Table 1). PB did not alter tritium accumulation in any brain region examined including the cortex after [^{3}H]-mivazerol or [^{3}H]-clonidine administration (Table 1).

Table 1. Effects of single or combined treatment with PB, clonidine (CLN) and mivazerol (MIV) on dialysate levels of NA (*f*moles.10 min^{-1} ± s.e.mean) and tritium content (d.p.m.mg^{-1}) in the rat cortex. * $P<0.01$ *c.f.* basal levels (time 0 min) within each treatment group.

treatment	0 min	40 min	80 min	120 min	d.p.m.mg^{-1}
saline	15.9±0.7	14.2±0.8	11.3±1.0	15.3±1.6	-
PB	15.9±1.2	2.0±1.1*	8.8±1.5*	15.7±2.1	-
CLN	18.2±2.4	17.2±1.7	15.5±1.1	15.4±1.6	103.2±9.1
PB+CLN	15.1±0.9	0.0±0.0*	0.3±0.3*	0.8±0.4*	86.7±26
MIV	15.6±1.9	17.7±2.1	14.9±0.8	18.4±1.7	28.5±2.0
PB+MIV	14.0±0.9	0.0±0.0*	2.0±0.7*	2.7±1.2*	27.9±6.7

The results suggest that PB and α_2-agonists act synergistically at presynaptic α_2-adrenoceptors to reduce extracellular levels of NA in the rat forebrain. This reduction may contribute to the synergistic effects of α_2-agonists on barbiturate-induced anaesthesia in rodents. The mechanism underlying this interaction is unknown but appears not to involve any change in the CNS penetration of α_2-agonists.

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39P THE IDENTIFICATION OF PUTATIVE IMIDAZOLINE$_2$ BINDING SITES IN FROG BRAIN

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Imidazoline-2 (I_2) binding sites are present in the brains of a range of mammalian species. Their function is unclear but appears linked physically and functionaly to monoamine oxidase. Their density is changed in a number of disease states and agmatine has been proposed as the endogenous neuromodulator (for review see Parini *et al.*, 1996). We have now examined whether I_2-sites exist in a lower species, the frog, and investigated their pharmacology using [^{3}H]2-(2-benzofuranyl)-2-imidazoline ([^{3}H]2BFI a radioligand specific for mammalian I_2 sites (Lione *et al.*, 1995).

Brains were removed from *Rana Temporaria* (15-25g, of either sex), homogenised (10vol 50mM Tris-HCl buffer, pH 7.4) and pelleted by centrifugation (32,000g). Membranes were washed twice by centrifugation and frozen (-70°C) until use. Aliquots of thawed membrane (30μg) were incubated with either 0-30 nM [^{3}H]2-BFI for saturation binding studies or 2nM for competition assays, for 1h (22°C, final vol 0.5ml). Bound ligand was separated by filtration and determined by scintillation counting. Results were analysed by Prism (GraphPAD Software).
Saturation binding studies yielded a Bmax of 13709·3 ± 1946·2 fmol mg^{-1} protein and a K_D of 8·6 ± 2·5 nM (mean ± s.e.m, n=4), with the specific component of binding defined by the I_2 ligand BU224 (2-(4,5-dihydroimidaz-2-yl)quinoline, 10μM). In competition experiments non-specific binding accounted for 10% of total ligand bound. The imidazoline compounds BU224 and 2-BFI were found to have high affinity for sites labelled by [^{3}H]2-BFI with Ki values of 39·4 ± 5·4 nM and 52·1 ± 12·1nM respectively (mean ± s.e.m, n=3-4, Fig 1). Amiloride was also of moderate affinity (Ki 122·6 ± 30·5 nM). In contrast, the I_1-site ligand rilmenidine and α_2-antagonist rauwolscine were of very low affinity. Idazoxan and agmatine, were found to have comparable affinity (respective Ki values 6·7 ± 1·1; 6·2 ± 2·1μM)

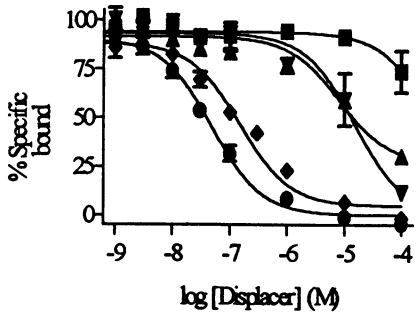

Figure 1. Inhibition of [^{3}H]2-BFI binding in frog brain by BU224 (●), amiloride (◆), idazoxan (▲), agmatine (▼), and rauwolscine (■).

These data show that frog brain contains a high density of sites that recognises the I_2 ligands [^{3}H]2-BFI and BU224. They are amiloride-sensitive indicating they are of the I_{2A} subtype and demonstrate low affinity for an I_1 ligand. It is interesting that agmatine has comparable affinity to idazoxan albeit in the low μM range. This novel pharmacology of the frog putative I_2-binding site may be of use to understanding their function in different species.

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40P PRIMARY CULTURES OF PARASYMPATHETIC NERVES FROM GUINEA-PIG TRACHEA CONTAIN FUNCTIONAL M2 MUSCARINIC RECEPTORS

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In the lungs, release of acetylcholine from the parasympathetic nerves is limited by inhibitory,, muscarinic receptors located on the nerves. These inhibitory receptors have been classified as M2 receptors since gallamine is an antagonist in vivo (Fryer & Maclagan, 1984). However, other pharmacological data suggest that the neuronal receptor does not strictly fit the profile of an M2 receptor and thus may be an M4 muscarinic receptor (Kilbinger et al, 1991).. Because the lungs contain a heterogeneous population of cells and of muscarinic receptor subtypes, the parasympathetic nerves were isolated in culture, and the subtype of muscarinic receptors on the nerves determined.

Guinea-pig tracheas were removed, minced and incubated with 0.2% collagenase overnight at 4°C then at 37°C for 30 min. Cells were washed, and plated and grown in serum-free medium with 100ng/ml nerve growth factor and 1.0μM cytosine arabinoside. After 7 days in culture, the majority of cells were confirmed to be nerves by immunofluorescence with Neurotag® (Boehringer) and by immunocytochemistry with an antibody selective for a nerve protein (PGP9.5;; Gulbenkian et al, 1987).

Immunocytochemistry using antibodies which are selective for the different muscarinic receptor subtypes (Levey, 1993) demonstrated that M2 muscarinic receptors are presented on the cultured nerves. In contrast, no positive staining for either M1 or M4 muscarinic receptors was detected. In addition, using polymerase chain reaction, M2 receptor mRNA was present in cultured nerves, while mRNA for M1 or M4 receptors was not present. Electrical stimulation (2 or 5 Hz, 0.2ms, 4 or 10 sec, 30V) of the nerve cultures caused release of acetylcholine measured by chemiluminescense (Israel & Lesbats, 1987). Acetylcholine release was significantly increased by 10μM atropine (incub. 1 min; from 25±5% to 80±4% of total acetylcholine; total determined by release with 3mM KCl; n=3), and significantly decreased by 0.1μM methacholine (incub. 1 min; from 34±4% to 20±3% of total acetylcholine; n=3).

Thus, it appears that the parasympathetic nerves in the guinea-pig trachea express only M2 muscarinic receptors, and that these receptors function to inhibit ACh release.

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41P VIRAL INFECTION OF CULTURED AIRWAY PARASYMPATHETIC NERVES INCREASES ACETYLCHOLINE RELEASE AND DECREASES M_2 MUSCARINIC RECEPTOR EXPRESSION

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Viral infections increase vagally-mediated bronchoconstriction in guinea pigs (Buckner, 1985) and humans (Empey, 1976). Inhibitory m_2 muscarinic receptors (m_2R) on the vagal nerve endings, which normally provide a negative feedback limiting the release of acetylcholine, are dysfunctional during parainfluenza infections (Fryer and Jacoby 1991). We investigated whether viral infection of cultured airway parasympathetic neurons, decreases the function and expression of inhibitory m_2R.

Cultured guinea pig airway parasympathetic neurons were infected with parainfluenza type 1 (Sendai virus). Infection was demonstrated by adding erythrocytes and observing them adhering to the neurons. This results from the expression of viral hemagglutinin on the infected cells' surface, and is not seen in uninfected cells.

Twenty-four hours after infection, cultures were stimulated electrically using a 4 sec pulse train (5 Hz, 0.2 msec pulse duration, 30 V). Stimulation increased acetylcholine release by 107±9% (n=3) over baseline in uninfected cells, and by 192±30% in virus-infected cells.

To determine the effect of viral infection on m_2R gene expression, we developed a competitive reverse transcription-polymerase chain reaction (RT-PCR) method. We excised a 43-base portion of our m_2R PCR product to create a template of lower molecular weight. This was transcribed in vitro to synthesize cRNA that was 43-bases smaller than the original m_2R RNA. RT-PCR using new primers based on the guinea pig sequence yielded a product of 559 bases using guinea pig m_2 mRNA, and 516 bases using the truncated internal standard cRNA. We extracted RNA from control nerve cells, nerve cells that were infected with parainfluenza virus for 24h, and nerve cells that were exposed to interferon-γ (300 U/ml) for 24h. Five hundred nanograms of RNA were added to internal standard cRNA (0.01-10 pg/sample). RT-PCR was carried out on these mixtures, and the PCR products were quantified by electrophoresis and densitometry. Competition for primers between the internal standard and the neuronal RNA yields bands of equal density when the input amount of m_2R mRNA is equal to the input amount of internal standard. Viral infection decreased nerve cell m_2R mRNA levels by 73% and interferon-γ (300 units/ml) decreased it by 82%.

Thus viral infection of cultured airway parasympathetic neurons increases acetylcholine release and decreases expression of the gene for inhibitory m_2 receptors.

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42P ROLE OF LARGE-CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS IN THE REGULATION OF ACETYLCHOLINE RELEASE BY PRE-JUNCTIONAL M_2-MUSCARINIC RECEPTORS

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Cholinergic, parasympathetic nerves play a dominant role in the control and regulation of airway tone in animals and humans. Indirect functional studies have suggested that airway cholinergic neural responses are modulated pre-junctionally by various agents such as μ-opioids, NPY and clonidine, and further, that the inhibitory neuromodulation of acetylcholine (ACh) release from cholinergic nerve terminals may be via a common mechanism i.e. activation of large-conductance calcium (Ca^{2+}) activated potassium channels (BK_{Ca}) (Muira *et al*, 1992). Studies involving the direct measurement of ACh release have identified pre-junctional muscarinic M_2 autoinhibitory receptors on cholinergic nerve terminals in guinea- pig and human trachea (Patel *et al*, 1995). We have studied whether BK_{Ca} channels are involved in muscarinic autoinhibition of ACh release by investigating whether iberiotoxin (IbTX), a selective BK_{Ca} channel blocker antagonises the inhibitory action of the muscarinic agonist, oxotremorine M (oxo M) on ACh release. This is determined by measuring [^{3}H]-overflow evoked by electrical field stimulation (EFS: 40V, 4Hz, 0.5ms for 1min) from epithelium-denuded guinea-pig trachea pretreated with [^{3}H]-choline. In addition, we have determined the effect of the putative BK_{Ca} channel opener, NS 1619 (Olesen *et al*, 1994) on cholinergic neurotransmission by studying its effects on cholinergic contractile responses evoked by EFS (40V,0.5ms, 4Hz for 15s every 4 min) and by the application of exogenous ACh (30μM). We have also examined the ability of NS1619 to inhibit ACh release and whether this effect is reversed by IbTX.

Oxo M inhibited EFS-induced ACh release (30nM, 50.0 ± 4.1% inhibition, n=7, $p<0.01$), however addition of 100nM IbTX for 30 min did not reverse the inhibitory effect of oxo M (48.5% inhibition after IbTX, n=7, NS compared with before IbTX). NS1619 inhibited EFS-induced cholinergic contractile responses (1μM, 12.6 ± 4.0% inhibition, n=5, $p<0.01$; 10μM, 40.3 ± 8.1% inhibition, n=5, $p<0.05$; 30μM, 60.0 ± 7.3 % inhibition, n=5, $p<0.01$) compared to that of time-matched vehicle control experiments (18.6 ± 6.6% inhibition, n=5, NS). Addition of IbTX (100nM) significantly, but not completely, reversed the inhibitory effect of NS1619 (30μM) on cholinergic, contractile responses (40.6 ± 7.4% reversal, n=5, $p<0.01$), however, IbTX alone reproducibly increased smooth muscle tone by 1525 ± 791.3 mg tension. In contrast, NS1619 (1μM-30μM) had no significant effect on contractile responses produced by exogenous ACh (30μM) indicating a pre-junctional effect of NS1619. Further, NS1619 (30μM) significantly inhibited EFS-induced [^{3}H]ACh release (33.9± 9.1% inhibition, n=8, $p<0.05$) compared to that of time-matched, vehicle-treated control tissues (3.9 ± 5.1% inhibition, n=7, NS) however IbTX (100nM) did not significantly reverse the inhibitory effect of NS1619 (30μM, 33.9 ± 3.9% inhibition before IbTX, n=7, $p<0.01$; 13.8 ± 20.96% inhibition after IbTX, n=7, NS). IbTX alone had no effect on ACh release (1.95 ± 14.7 % facilitation, n=6, NS).

Our functional data suggests that NS1619 is inhibiting EFS-induced cholinergic contractile responses at the pre-junctional level which is confirmed by its inhibitory effect on ACh release. However the inhibitory action of NS1619 and oxo M on ACh release is not reversed by IbTX, suggesting that activation of the muscarinic autoinhibitory receptor does not involve IbTX-sensitive BK_{Ca} channels. Furthermore, NS1619 may be inhibiting ACh release via interaction with other types of channels such as voltage-gated potassium channels and calcium channels (Edwards *et al*,1994).

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43P INCREASED FUNCTION OF NEURONAL M_2 MUSCARINIC RECEPTORS IN DIABETIC RAT LUNGS IS ASSOCIATED WITH INCREASED AGONIST AFFINITY

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In the lungs, neuronal M2 muscarinic receptors (nM2Rs) inhibit vagally induced bronchoconstriction (VIBr) by limiting acetylcholine (ACh) release from the parasympathetic nerves. A cause of asthma may be dysfunction of nM2Rs [Minette, et al., 1989]. Since M2Rs are hyperfunctional in many diabetic tissues, we tested whether the protection from asthma seen in diabetics [Abrahamson, 1941] is due to increased function of the nM2Rs, and thus decreased VIBr, in the diabetic rat.

Sprague Dawley rats were made diabetic with streptozotocin (65mg/kg iv). After 7-14 days rats were anesthetized with urethane (1.5g/kg ip), ventilated and paralyzed with suxamethonium (30 mg/kg iv). Distal electrical stimulation of cut vagi produced VIBr, measured as an increase in inflation pressure. Some 7 day diabetic animals were treated with low doses of NPH insulin (2 iu/day sc for 7days). Blood glucose levels were significantly greater in both diabetic (476±25.1 mg/dL) and insulin-treated rats (419±41.2 mg/dL) vs controls (162±19.8 mg/dL).

Electrical stimulation of the vagi (5-70Hz, 40V, 0.4ms, 6s) produced significantly less VIBr in diabetic rats compared to controls (max response at 70Hz, 19±4 vs 120±35 mmH_2O in controls). In diabetic rats, nM2R function (as measured by the ability of pilocarpine (pilo), 0.01-100μg/kg iv to inhibit VIBr) was increased 25 fold (pilo ED_{40}=0.27±0.13μg/kg vs 7.6±1.7μg/kg for controls; n=6). Both the frequency response and nM_2R function were restored to control levels by insulin treatment (max response 70Hz 99 ± 26 mmH2O; pilo ED_{40} was 16.7±14, n=3).

Competitive binding studies were performed using rat heart membranes. Carbachol displaced [^{3}H]QNB from M2Rs in membranes from all groups with both high (70%) and low (30%) binding affinity. Diabetic rat heart muscarinic receptors displayed an increase in agonist binding at both the high (Ki=0.06 ± 0.02 uM) and low (Ki=20.6 ± 2.9 uM) affinity sites vs controls (Ki=0.24 ± 0.06 and 97.2 ± 27 uM). M2R from insulin treated diabetic heart membranes showed no significant difference in binding from controls.

These data suggest that the hyperfunctional nM2R seen in the diabetic rat lung is associated with an increase in agonist binding affinity at the M2 receptor. Insulin treatment restored these changes to near control values without restoring diabetic blood glucose to control levels. Hence these changes can not be ascribed solely to hyperglycemic effects. Thus, there may be a role for insulin in the modulation nM2R function and expression.

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44P MONOCLONAL ANTIBODY TO VERY LATE ACTIVATION ANTIGEN-4 PROTECTS THE NEURONAL M_2 MUSCARINIC RECEPTORS FROM ANTIGEN CHALLENGE IN THE GUINEA-PIG

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Release of acetylcholine from parasympathetic nerves in the lungs is controlled by inhibitory M_2 muscarinic autoreceptors (Fryer & Maclagan 1984). Antigen challenge of sensitized guinea-pigs decreases M_2 receptor function thus potentiating vagally-induced bronchoconstriction (VIBr) by increasing release of acetylcholine. Loss of M_2 receptor function is associated with the accumulation of eosinophils (Eos) around the airway nerves. Eos release major basic protein which is an endogenous antagonist of inhibitory neuronal M_2 muscarinic receptors (Jacoby et al, 1993). Thus Eos products may increase acetylcholine release from the parasympathetic nerves, by acting an endogenous antagonists of the inhibitory M_2 receptors. To determine whether recruitment of Eos via very late activation antigen-4 (VLA-4) or L-selectin is critical for loss of M_2 receptor function, sensitized guinea pigs were pretreated with mAbs to VLA-4 (HP1/2) or L-selectin (LAM1-116) 60 min prior to antigen challenge (4mg/kg/day i.p.).

Guinea-pigs (300g) were sensitized to ovalbumin (10mg/kg i.p.) and 3 weeks later challenged by aerosol with 5% ovalbumin (5 min/day x 4 days). 24 hours later, guinea-pigs were anesthetized with urethane (1.5g/kg ip), ventilated and paralyzed with suxamethonium (10 μg/kg/min iv). Electrical stimulation of the distal ends of the cut vagi produced VIBr, measured as increases in lung inflation pressure.

In non-sensitized, non-challenged controls, blockade of neuronal M_2 muscarinic receptors by gallamine (0.1-10mg/kg i.v.) potentiated VIBr (max response 508 ± 0.5%; n=5) while in challenged animals this effect was significantly reduced (214±0.3%; n=5), confirming M_2 receptor dysfunction. Pretreatment with HP1/2, but not with LAM1-116, protected M_2 receptors in antigen challenged animals (max responses to gallamine were 490±0.8 and 239±0.1% respectively; n=5). HP1/2 also significantly inhibited accumulation of Eos in the lungs as measured by histology and by lavage (total million Eos recovered: controls, 2.7±.6; challenged 19.7±2.9; +HP1/2, 6.7±1.4; +LAM1-116 17,4±5.8). Recruitment of other inflammatory cells was not altered by HP1/2.

Thus, inhibition of Eo influx into the lungs is associated with protection of neuronal M_2 muscarinic receptor function in antigen challenged guinea-pigs. Since antigen induced hyperresponsiveness is also inhibited by HP1/2 (Pretolani, et al 1994), protection of neuronal M_2 muscarinic receptor function may be a mechanism for inhibition of hyperreactivity.

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45P CHARACTERISATION OF FUNCTIONAL MUSCARINIC RECEPTORS IN HUMAN BLADDER

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Muscarinic receptors are know to play an important role in the control of bladder contraction during micturition. This is particularly the case in humans where virtually all of the bladder contraction is mediated via cholinergic neurotransmission in normal tissue (Sibley, 1984). Despite this observation, the functional muscarinic receptor sub-types on bladder smooth muscle have not been extensively characterised. Five human muscarinic receptors have been cloned and four physiological receptor subtypes have been identified in functional studies (Caulfield, 1993). Studies on guinea-pig and rat bladder smooth muscle have suggested that functional responses to cholinergic agonists in these tissues are mediated by the M_3 muscarinic receptor subtype (Noronha-Blob *et al.*, 1989; Longhurst *et al.*, 1995). The aim of the present studies was to characterise functional muscarinic receptor sub-types on human bladder smooth muscle.

Strips of normal human bladder (supplied by the International Institute for the Advancement of Medicine) were mounted for isometric recording in tissue baths containing Krebs-Henseleit solution (37°C, gassed with 95%O_2/5%CO_2). Cumulative concentration-response curves to acetylcholine were carried out in the presence and absence of subtype-selective muscarinic receptor antagonists (20 min pre-equilibration). Apparent pA_2 values for single antagonists concentrations were determined according to the Gaddum-Schild equation (Table 1).

Table 1 Apparent pA_2 values for muscarinic antagonists on human bladder (mean ± s.e. mean, n = 4)

Compound (selectivity)	Antagonist potency (Apparent pA_2)
Atropine (none)	9.32 ± 0.11
Darifenacin (M_3)	8.72 ± 0.19
Methoctramine (M_2/M_4)	<5
Pirenzepine (M_1/M_4)	6.61 ± 0.11

The M_3 selective antagonist, darifenacin (Wallis *et al.*, 1995) had an apparent pA_2 value on human bladder similar to that reported for guinea-pig bladder (Newgreen and Naylor, 1996), consistent with a response mediated through M_3 receptors. The relatively low potencies of the M_1/M_4 selective antagonist, pirenzepine and the M_2/M_4 selective antagonist, methoctramine suggest that these receptors are not functionally active in human bladder. Thus, it can be concluded from the present study that the functional responses to acetylcholine in human bladder smooth muscle are mediated by M_3 receptors.

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46P EXACERBATION OF THE RELEASE OF PROSTAGLANDIN E_2 BY BRADYKININ AFTER COX-2 INDUCTION IN HUMAN AIRWAY EPITHELIAL CELLS

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Bradykinin and lysyl-bradykinin (kallidin) are biologically active peptides which are formed during inflammation from protein kininogens by the enzymes tissue and plasma kallikrein. They mediate their effects through two cell surface receptors defined pharmacologically as B_1 and B_2. B_1 receptors are highly inducible in response to inflammatory stimuli, whereas B_2 receptors are considered to be constitutively expressed. Bradykinin has been implicated in the pathophysiology of a number of inflammatory diseases including asthma. As bradykinin stimulates the enzyme phospholipase A_2 (PLA_2), many of its inflammatory effects are believed to be mediated, at least in part, via release of lipid mediators, in particular cyclo-oxygenase (COX) metabolites. COX exists in at least two distinct isoforms, constitutive (COX-1) and inducible (COX-2). (Mitchell *et al.*, 1995) COX-2 is induced in response to a number of inflammatory stimuli and we hypothesise that increased expression of this isoform will exacerbate the release of prostanoids on exposure to bradykinin. Therefore, we have looked at the effect of bradykinin and a B_1 agonist [$desArg^{10}$] kallidin on the release of PGE_2 from a human epithelial cell line (A549) which is known to express COX-2 in response to the cytokine IL-1β (Mitchell *et al.*, 1994).

A549 cells were cultured in 96 well plates as previously described (Mitchell *et al.*,1994.). Untreated cells or those treated with IL-1β (10ng/ml) for 24hr, to induce COX-2, were exposed to fresh medium containing bradykinin or [$desArg^{10}$]Kallidin (10^{-12} to 10^{-4} M) for 15min. The medium was then removed and analysed for PGE_2 by radioimmunoassay (limit of detection ≤ 0.4ng/ml) In separate experiments the B_2 receptor antagonist Hoe140 (1μM) was added 30 min prior to treatment with bradykinin followed by a 15min incubation period before the medium was removed and analysed as outlined above.

In untreated cells the release of PGE_2 in response to either bradykinin or [$desArg^{10}$] Kallidin was below the level of detection for the radioimmunoassay (limit of detection ≤ 0.4ng/ml). In IL-1β stimulated cells there was a limited release of PGE_2 in response to [$desArg^{10}$] kallidin reaching a maximum at 10^{-6}M (2.68±1.11 ng/ml) (Fig.1). However, in stimulated cells treated with bradykinin there was a dose dependent release of PGE_2 (Fig.1). This release was blocked in the presence of the B_2 receptor antagonist with an apparent pK_B value of 7.86±0.245 (n=3) (Fig.2)

Fig 1

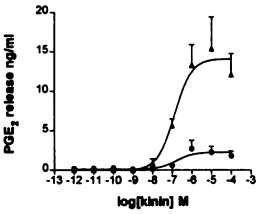

Fig 2

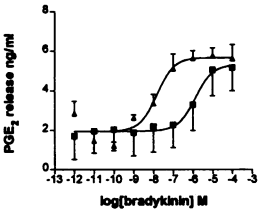

Fig 1 demonstrates the release of PGE_2 in IL-1β treated cells stimulated with bradykinin (filled triangle) and [$desArg^{10}$] Kallidin (filled circle). Fig 2 demonstrates the release of PGE_2 in IL-1β treated cells stimulated with bradykinin (filled triangle) and bradykinin in the presence of the B_2 receptor antagonist Hoe140 (1μM) (filled square). n=9 determinations from three experimental days

This data shows that airway epithelial cells, expressing COX-2, release exaggerated levels of PGE_2 in response to bradykinin mediated via activation of the B_2 receptor. Thus it is tempting to suggest that COX-2 induction in human airway is an important component of inflammatory diseases such as asthma. Furthermore our observations may help to explain the bronchoconstriction caused by bradykinin in asthmatic subjects which is absent in healthy volunteers

This work was supported by the British Lung Foundation, British Heart Foundation and the Wellcome Trust.

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47P GUINEA-PIG TUMOUR NECROSIS FACTOR-INDUCED AIRWAY INFLAMMATION: INHIBITION BY INTERLEUKIN-13

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Cytokines may have pro- and anti-inflammatory activity in the lung. We have demonstrated the presence of tumour necrosis factor in guinea-pig allergic airway inflammation (Watson *et al.*, 1993). In this study we report the *in vivo* airway inflammation induced by guinea-pig tumour necrosis factor-α (gpTNF; White *et al.*, 1996), and its suppression by human interleukin (IL) -13.

Guinea-pigs (300-400g) were anaesthetised (ketamine 40mg/kg, xylazine 5mg/kg, i.m.) and test agents, in a total volume of 50µl, were administered by tracheal instillation with the aid of a laryngoscope. Animals were sacrificed by pentobarbitone overdose 6-72h following instillation and the lungs lavaged with 4x10ml PBS/ 1mM EDTA/ 0.1% BSA. Total and differential counts of bronchoalveolar lavage (BAL) leukocytes made.

Following instillation of gpTNF (50ng), an eosinophilic leukocyte accumulation occurred peaking at 24h, and this time point was used for subsequent studies. 15, 50 and 150 ng gpTNF induced BAL accumulation of 8.8±2.2, 18.8±1.4 and 19.3±1.9 million eosinophils respectively, compared with 5.1±1.6 million in vehicle treated animals (mean ± s.e.mean, n=3). Pre-incubation of 50ng gpTNF with neutralising rabbit anti-murine TNF sera (1/100 final dilution) prior to instillation reduced the gpTNF induced eosinophil infiltration by 79.2 ± 9.4 % ($p<0.01$, n=3).

Co-administration of recombinant human IL-13 with 50ng gpTNF caused a dose-dependant reduction in the BAL eosinophil and monocyte/macrophage infiltration (Table 1).

Table 1: IL-13 suppresses gpTNFα - induced cell accumulation

	monocyte/ macrophage	eosinophil	neutrophil
vehicle (saline/0.1% BSA)	10.5±1.2	2.7±0.8	0.9±0.4
IL-13 (100ng) alone	11.6±1.3	3.6±0.7	4.3±2.1
gpTNF (50ng) alone	21.6±1.7 †††	15.4±1.6 †††	6.3±2.4 †
gpTNF + IL-13 (100ng)	11.3±1.0 ***	4.4±0.7 **	4.2±3.4
gpTNF + IL-13 (10ng)	15.2±0.9 *	7.4±2.3 *	5.4±2.8
gpTNF + IL-13 (1ng)	20.2±1.4	10.8±3.5	2.1±0.4

Data are mean ± s.e.mean BAL cells (millions), n=5-6. Statistical analysis was performed on log data using one-way ANOVA followed by Dunnett's test. †, $P<0.05$; †††, $P<0.001$ compared with vehicle treated group; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared with animals treated with gpTNF alone.

Hence, a pronounced leukocyte accumulation in the guinea-pig airways is induced by tracheal administration of gpTNF. This *in vivo* response can be suppressed by co-administration of IL-13.

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48P IL-8 PRODUCTION BY HUMAN CULTURED AIRWAY SMOOTH MUSCLE

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Leukocyte accumulation and activation are key events in the pathogenesis of inflammatory lung disease. This migration is mediated by a series of co-ordinated signals, probably including the production of chemotactic factors by lung tissue. We have investigated the ability of human airway smooth muscle (HASM) stimulated with interleukin (IL-) 1α or tumour necrosis factor α (TNFα) to produce the neutrophil and T-cell chemokine chemoattractant, IL-8.

Bronchial tissue was obtained from patients who had undergone surgery for lung carcinoma. Minced muscle tissue was digested with collagenase and elastase (Panettieri *et al.*, 1989). All experiments were done using 4th or 5th passage HASM cells that stained positively for anti-α-smooth muscle actin. Cells were grown until confluent ($1x10^5$ cells/well) in 24 well plates. The culture medium was replaced with 0.5 ml DMEM+5% FBS. IL-1α or TNFα were added to duplicate wells and cultures were incubated for 24h before collection of supernatants. IL-8 levels were assessed by specific ELISA (Ceska *et al.*, 1989).

In the absence of cytokine stimuli, low IL-8 levels were measured (0.31±0.14 pmol/well, mean ± s.e.mean in 6 experiments). Following 24h stimulation with IL-1α (0.01-1 ng/ml) or TNFα (0.3-30ng/ml), high concentrations of IL-8 immunoreactivity were detected in the supernatant. IL-1α (1ng/ml) induced 18.3±5.8 pmol/well IL-8 compared with 30ng/ml TNFα which induced 6.4±0.5 pmol/well IL-8. Hence, IL-1α was both more potent and more effective as a stimulus of IL-8 release compared with TNFα. IL-8 like bioactivity was assessed by the ability of supernatants to elevate $[Ca^{2+}]_i$ in fura-2 loaded human neutrophils (Watson *et al.*, 1988). Control supernatants from unstimulated HASM cells did not elevate $[Ca^{2+}]_i$ in neutrophils, whereas supernatants from HASM stimulated with IL-1α or TNFα for 24h resulted in concentration related elevations of neutrophil $[Ca^{2+}]_i$. For example, elevation in response to supernatants from IL-1α and TNFα (both at 0.3ng/ml) treated HASM were 171nM and 34nM respectively. Following pre-incubation with murine monoclonal anti-IL-8, the Ca^{2+} elevating activity of a maximal concentration of recombinant IL-8 (3nM) was totally eliminated, while the antibody was only able to reduce the HASM cell supernatant-stimulated $[Ca^{2+}]_i$ elevation by 40%. Hence there appears to be at least two neutrophil activating agents released from IL-1α treated HASM cells.

Northern analysis was used to investigate chemokine mRNA expression in HASM cells (Jordan *et al.*, 1995). In unstimulated cells, there was no detectable expression of mRNA for IL-8 whereas expression was upregulated following 6h stimulation with IL-1 (3ng/ml) or TNFα (30ng/ml).

These data indicate that HASM may constitute an important source of leukocyte attractants in the inflamed lung, where the inducing stimuli IL-1α and TNFα are also likely to be present.

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49P SULPHIDE-INDUCED APNOEA: PERIPHERAL OR CENTRAL MECHANISM AND ITS PREVENTION

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Fatal H_2S intoxication is caused by apnoea that has been attributed to inhibition of the respiratory centre in the brain stem (Haggard, 1925), but direct evidence for this is lacking. Inhibition of some cellular functions by NaHS have been shown in regions of the rat brain (Kombian *et al.*, 1993) but *in vitro* rhythm generation from the brain stem, though reduced in frequency, was not abolished (Greer *et al.*, 1995); therefore a direct inhibition of the rhythm centre may not explain the apnoea that causes death. We hypothesize that a sulphide insult to the lungs mediates a sensory signal for respiratory arrest. Sprague Dawley male rats, 300-350 g, were anaesthetized with urethane (1.5 g kg^{-1}, i.p.). Spontaneous respiration was recorded through a tracheal cannula or with a whole body plethysmographic chamber, linked to a Validyne conditioner and transducer. Either the femoral vein or the carotid artery was cannulated for the administration of drugs, and the animal then allowed to stabilize for 30 min.

With apnoea as an end-point, the effectiveness of NaHS as a respiratory depressant was determined. Incremental single doses in the range 0.1 mg kg^{-1} to 2.0 mg kg^{-1} were given as a bolus of 5-50 µl followed by 0.5 ml saline. Recovery time, 30 min, was allowed between doses. The effects of femoral vein and carotid artery administration were compared. The minimum effective dose for 100% apnoea with i.v. administration was 0.6 mg kg^{-1} (n=4). In contrast, by the carotid route the minimum effective dose was 2.5 mg kg^{-1} (n=3). This higher dose suggests that the lungs are more sensitive to NaHS than the brain and may be implicated in the respiratory depressant action of NaHS.

The known inhibition of carbonic anhydrase by mercaptans (Swenson *et al.*, 1993) suggested a mechanism for further investigation namely the effects of supplementary bicarbonate (parenteral solution, 1.4%) on NaHS intoxication. A lethal dose of NaHS, 30 mg kg^{-1} (Warenycia *et al.*, 1990) given i.p. to anaesthetized rats was fatal to 83% of untreated animals (n=6), but caused no fatalities in rats that had been pretreated (10 min) with bicarbonate 2 ml i.p. (333 µmoles, n=4, $P < 0.005$), or in rats infused i.v. with bicarbonate solution 0.1 ml min^{-1} (17 µmoles min^{-1}, n=4, $P < 0.02$). We conclude that apnoea induced by NaHS has a peripheral rather than a central mechanism. In addition, apnoea and lethality can both be prevented by the administration of bicarbonate.

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50P GUINEA-PIG RANTES ACTIVATES HUMAN, BUT NOT GUINEA-PIG, EOSINOPHILS

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In our search to identify key mediators of allergic inflammation, we have expressed and purified recombinant guinea pig (gp) RANTES, a member of the C-C chemokine family. RANTES has previously been shown to attract a particular profile of leukocytes, namely eosinophils, memory CD45RO T lymphocytes and monocytes, cells which comprise the cellular infiltrate in many allergic sites (Kameyoshi *et al.*, 1992; Schall *et al.*, 1990). To investigate the role of RANTES, we have characterised the protein by assessing *in vitro* activity using guinea pig peritoneal eosinophils and macrophages, as well as human peripheral blood eosinophils.

We obtained the gpRANTES cDNA from a concanavalin A-stimulated gp spleen cell cDNA library. However we were unable to obtain high expression levels of the protein in *E. coli* systems. Therefore we mutated the construct expressing human (h) RANTES at 6 positions, such that the protein produced was identical to the predicted gpRANTES amino acid sequence. Since gpRANTES has not yet been identified from natural sources, the amino terminus was expressed with the equivalent amino terminus as the human protein. The protein was purified from cell extracts using gel filtration, cation exchange FPLC and reverse phase HPLC. *In vitro* activity of the protein was assessed by chemotaxis (modified Boyden chamber, membrane pore size 5µm), and changes in intracellular free concentration ($[Ca^{2+}]_i$) in fura-2 loaded cells (Bourne *et al.* 1995).

gpRANTES and hRANTES had a similar potency on human eosinophils as chemoattractants; however neither of these proteins was able to stimulate gp eosinophil chemotaxis (Table 1).

Table 1. Human and gp eosinophil chemotaxis in response to RANTES.

		hRANTES		gpRANTES		C5a
species	basal	3nM	30nM	3nM	30nM	100nM
human	12±4	53±21	96±10	23±5	80±6	122±13
gp	19±6	36±5	30±4	27±7	32±9	101±6

Data are mean ± s.e.mean eosinophils per high powered field, n=3.

Furthermore gp chemotaxis was not observed in the presence of the priming agent IL-5, which caused a one fold increase in response to IL-8. Similarly, gpRANTES elevated $[Ca^{2+}]_i$ in human but not gp eosinophils. For example 30nM gpRANTES caused changes in $[Ca^{2+}]_i$ of 108nM in human eosinophils compared with 7nM in gp eosinophils. Although the distinct activity profile of the two eosinophil populations may reflect differences in blood derived cells versus cells from inflammatory sites, gp peritoneal macrophages from the same animals were strongly activated by gpRANTES (for example, 30nM gpRANTES caused a change in $[Ca^{2+}]_i$ of 330nM).

These results indicate that RANTES has differing cellular selectivity in the guinea-pig compared with the human, which may be important in the use of animal models in the analysis of allergic disorders. Moreover, these selectivities do not appear to be accounted for by differences in guinea-pig and human protein sequences.

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51P MONOCYTE CHEMOTACTIC PEPTIDE-1: SIGNALLING STUDIES IN THP-1 CELLS

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Monocyte chemotactic peptide-1 (MCP-1) is a member of the C-C chemokine group and is a potent activator of human monocytes (Yoshimura *et al.*, 1989). In this study we were interested in characterising the signal transduction pathways activated in THP-1 cells following MCP-1 binding to its receptor. Firstly, we utilised ^{125}I-MCP-1 to confirm MCP-1 binding to these cells. Scatchard analysis identified that MCP-1 bound with a Kd of 4.71 ± 0.91 nM to 19 979 ± 3 508 receptors per cell (mean ± s.e.mean, n=4) with a slope of 1.0 indicating a single class of high affinity receptors. This data correlates with previous data (Van Riper *et al.*, 1993; Wang *et al.*, 1993)

We have previously identified an increase in the intracellular free calcium concentration in response to MCP-1 in these cells (Turner and Westwick, 1995), but, as yet, no group has detected an increase in the levels of IP_3, a potent calcium mobilising agent produced by the breakdown of $PI(4,5)P_2$ by phospholipase C. Using an IP_3 competition binding detection system we identified a significant increase in the levels of IP_3 in response to 12.5nM MCP-1, with a control IP_3 level of 8.0 ± 4.25 pmol/10^7 cells, increasing to a maximum of 29.25 ± 7.3 pmol/10^7 cells at 5s after MCP-1 stimulation (mean ± s.e.mean, n=3; $p<0.01$). This rise preceded the intracellular calcium rise, suggesting calcium mobilisation induced by IP_3. The IP_3 concentration was almost back to basal levels (12.35 ± 4.85 pmol/10^7 cells;mean ± s.e.mean, n=3) by 60s.

We have previously demonstrated that MCP-1 induces, in THP-1 cells, an increase in phosphatidylinositol-(3,4)-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol-(3,4,5)-trisphosphate ($PI(3,4,5)P_3$) both products of phosphoinositide 3-kinase (PI 3-kinase) (Turner *et al*, 1996), but we had not identified the type of PI 3-kinase activated, i.e. G-protein-linked or tyrosine kinase-linked. By labelling the cells with [^{32}P]-orthophosphate, extracting, deacylating and HPLC analysis as described by Ward *et al* (1995), we found that 12.5nM MCP-1 induced a 364.1 ± 35.3% (mean ± s.e.mean, n=4) increase in $PI(3,4,5)P_3$ levels at 30s, which was completely abrogated by pretreating the THP-1 cells (10^6/ml) with 100ng/ml pertussis toxin for 16 h (n=4) as compared to vehicle treated cells. However, we identified the activation of a second PI 3-kinase by using an antibody specific to the p85 subunit of the phosphotyrosine-coupled PI 3-kinase, which is distinct from the recently identified G-protein linked PI 3-kinase (Stephens *et al.*, 1994). Cell lysates were immunoprecipitated using this antibody and then subjected to an *in vitro* lipid kinase assay (Ward *et al*, 1995). The results showed a dose- (1.25-300nM) and time-dependent (5-300s) increase in tyrosine kinase-linked PI 3-kinase activity following MCP-1 stimulation.

This study has determined PLC mediated calcium mobilisation in these cells following MCP-1 binding as well as an increase in $PI(3,4)P_2$ and $PI(3,4,5)P_3$ by both a G-protein-linked and a tyrosine kinase-linked PI 3-kinase.

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52P RECRUITMENT OF EOSINOPHILS BY CHEMOKINES AND OTHER CHEMOATTRACTANTS IN AN *IN VIVO* MOUSE MODEL

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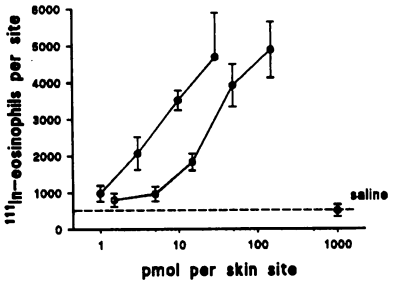
There has been considerable interest in chemokines as potent and specific eosinophil chemoattractants. With the exception of eotaxin (Jose *et al*, 1995), the activity of the majority of eosinophil-active chemokines has been only characterized *in vitro*. In this study, we describe the development of an *in vivo* mouse model to compare the effects and potency of eosinophil-active mediators.

Blood was obtained by cardiac puncture of CBA/Ca mice overexpressing the mouse interleukin (IL)-5 gene (Dent *et al*, 1990). These animals have a high blood level of eosinophils (60-70% of circulating leukocytes). After dextran sedimentation to separate leukocytes from red blood cells, eosinophils were purified on a discontinuous Percoll gradient followed by immunomagnetic negative selection with anti-CD2 and anti-B cell (B220) monoclonal antibodies (mAb). The purified blood eosinophils (>95% pure) were labelled with ^{111}In and injected i.v. into non-transgenic CBA/Ca mice (10^6 eosinophils per animal), followed 5 min later by i.d. injection of PAF (1.5 to 500 pmol per site), LTB_4 (1.5 to 150 pmol per site), hC5a (1.5 to 50 pmol per site) and the chemokines mEotaxin (0.1 to 30 pmol per site), mKC (1 to 30 pmol per site) and mMIP-2 (1 to 30 pmol per site). Each animal received up to 6 injections and ^{111}In-eosinophil accumulation per skin site was assessed after 2 h. The Figure shows the effects of i.d. administration of mEotaxin and LTB_4. mEotaxin was approximately 15 times more potent than LTB_4 but induced similar maximal responses at the top dose tested. PAF was very potent (EC_{50} ~ 2.0 pmol) at inducing ^{111}In-eosinophil accumulation but it was less effective than mEotaxin (eg. PAF 500 pmol/site, 3045 ± 377 ^{111}In-eosinophils/site; mEotaxin 10 pmol/site, 6148 ± 1540; n= 4). Similarly, a maximally effective dose of hC5a induced only half the ^{111}In-eosinophil accumulation seen in response to rmEotaxin (hC5a 50 pmol, 2010 ± 227 ^{111}In-eosinophils; mEotaxin 10 pmol, 4328 ± 740; n= 6). In contrast, i.d. injection of mKC or mMIP-2 had no significant effect on ^{111}In-eosinophil accumulation.



Effect of mEotaxin (closed circles) and LTB_4 (open circles) on ^{111}In-eosinophil accumulation in mouse skin. Results are mean ± s.e.mean for n= 5-6 animals.

In conclusion, we have shown that mEotaxin is a potent and effective inducer of eosinophil recruitment into mouse skin. In contrast, similar doses of the C-X-C chemokines KC and MIP-2 were ineffective at inducing eosinophil recruitment in the same model. This model should prove useful in comparing the *in vivo* chemoattractant effects of eosinophil-active C-C chemokine such as RANTES, MCP-3 and MIP-1α.

We are grateful to Sandoz, Switzerland and the National Asthma Campaign for support.

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53P THE ROLE OF LTB4 AND LTD4 IN SUBSTANCE P-INDUCED EOSINOPHIL ACCUMULATION IN GUINEA-PIG SKIN AS DETERMINED BY NOVEL AND SPECIFIC ANTAGONISTS

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The neuropeptide substance P (SP), released from sensory nerves, is a major mediator of neurogenic inflammation. Previously, we have demonstrated that SP induces oedema formation and the accumulation of ^{111}In-eosinophils in guinea pig skin and that the 5-lipoxygenase (5-LO) inhibitor, ZM-230,487 significantly inhibited SP-induced eosinophil accumulation (Walsh *et al.*, 1995). The aim of the present study was to identify the 5-LO products involved in this response. For this purpose, we have investigated the effects of a novel leukotriene (LT) B_4 antagonist, Ro 25-4094 (2-[(5-carboxypentyl)oxy]-6-[6-[(3,4-dihydro-4-oxo-8-propyl-2H-1-benzopyran-7-yl)oxy]hexyl] benzenepropanoic acid) and a novel LTD_4 antagonist, Ro 24-5913 ((E)-4-[3-[2-(4-cyclobutyl-2-thiazolyl)ethenyl]phenylamino] 2,2-diethyl-4-oxobutanoic acid) on SP-induced inflammatory responses.

The local accumulation of ^{111}In-eosinophils and ^{125}I-albumin were measured in guinea pig skin as previously described (Faccioli *et al.*, 1991). Using a 2 hour accumulation period, i.d. SP (10^{-10}-10^{-9} mol/site) induced significant ^{111}In-eosinophil accumulation and oedema formation above levels detected in sites injected with saline (10^{-9} mol/site induced 3808±579 ^{111}In-eosinophils/site and 44.2±5.4µl plasma compared to 489±207 cells/site and 9.7±1.8µl plasma seen with i.d. saline, $p<0.01$ n=5). The LTB_4 antagonist, Ro 25-4094 (0.5 mg/kg i.v.), abolished the ^{111}In-eosinophil accumulation induced by LTB_4 (10^{-10} mol/site elicited 6506±1480 cells/site and 683±60 cells/site in control and drug treated animals respectively, $p<0.01$ n=6 pairs) and significantly attenuated the ^{111}In-eosinophil accumulation induced by SP (10^{-9} mol/site elicited 3808±579 cells/site and 2003±150 cells/site in control and drug treated guinea pigs respectively, 47% inhibition, $p<0.05$ n=6 pairs). As we have previously found with a 5-LO inhibitor (Walsh *et al.*, 1995), the LTB_4 receptor antagonist had no effect on the SP-induced oedema formation. Further, this antagonist had no effect on the inflammatory responses elicited by LTD_4, PAF or histamine.

The LTD_4 antagonist, Ro 24-5913 (0.5 mg/kg i.v.), whilst abolishing the LTD_4-induced oedema formation (LTD_4 does not induce significant accumulation of ^{111}In-eosinophils) had no significant effect on the ^{111}In-eosinophil accumulation or oedema formation induced by SP. Similarly, this antagonist had no effect on the responses elicited by other inflammatory stimuli including LTB_4. The co-administration of the two receptor antagonists resulted in a greater level of inhibition of the SP-induced ^{111}In-eosinophil accumulation than that seen with either of the drugs on their own (10^{-9} mol/site induced 3217±507 cells/site and 923±117 cells/site in control and drug-treated animals respectively, 71% inhibition, $p<0.01$ n=5 pairs). The combination of Ro 25-4094 and Ro 24-5913 again had no effect on the oedema induced by SP.

These results demonstrate that both Ro 25-4094 and Ro 24-5913 are effective antagonists of LTB_4- and LTD_4-induced responses in this model and that LTB_4 is involved in SP-induced ^{111}In-eosinophil accumulation. In addition, our results suggest that SP-induced endogenous generation of LTB_4 and LTD_4 may act synergistically in eliciting the accumulation of eosinophils in this reaction.

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54P SUBSTANCE P INDUCES EQUINE EOSINOPHIL SUPEROXIDE ANION GENERATION VIA NK1 RECEPTOR ACTIVATION

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Lesions of horses with the intensely pruritic allergic skin disease, sweet itch, are characterised by eosinophil infiltration and oedema. Moreover, eosinophil numbers have been shown to correlate with the size of the response to intradermally injected antigen (Foster *et al*, 1995). Histamine, the involvement of which has been reported in sweet itch (Reik, 1955), induced superoxide anion (O_2^-) production by equine eosinophils (Foster & Cunningham, 1995). Since histamine may induce substance P (SP) release from cutaneous sensory nerve endings, the present study has examined the effect of SP on O_2^- generation by eosinophils from normal horses.

Production of O_2^- by equine eosinophils was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c. Eosinophils were purified from normal horses and resuspended in medium containing 2.5 mg ml^{-1} cytochrome c at $1x10^6$ cells ml^{-1}. Cells ($5x10^5$) were added in triplicate to tubes with or without NK_1 receptor antagonist (CP96345; $3x10^{-7}$-10^{-4}M), inactive enantiomer (CP96344; 10^{-5}-10^{-4}M) or vehicle treatment for 10 min at 37°C. SP, 1-7 amino acid SP fragment (SP 1-7) or 7-11 amino acid SP fragment (SP 7-11) were then added at final concentrations of $3x10^{-6}$-$3x10^{-4}$M and incubated for 30 min at 37°C. Histamine (10^{-5} or 10^{-4}M) and phorbol myristate acetate (PMA; 10^{-7}M) were used as positive controls. For comparison, neutrophils purified from the same horses were also incubated with SP ($3x10^{-6}$-$3x10^{-4}$M). After centrifugation supernatant (250µl) from each tube was transferred to 96-well microtitre plates and the reduction of cytochrome c measured colorimetrically at 550 nm. Blank values were subtracted from each test sample and the results expressed as nmol of reduced cytochrome c/10^6 cells.

SP ($3x10^{-6}$-$3x10^{-4}$M) induced a significant dose-related increase in SOD-inhibitable O_2^- production by eosinophils ($P<0.01$; n=4), but not neutrophils. The maximal response was observed at $3x10^{-4}$M SP and was greater than that induced by 10^{-4}M histamine (48±7 vs 16±1 nmol/10^6 cells; n=4; $P<0.05$). SP 7-11 ($3x10^{-6}$-$2x10^{-4}$M), but not SP 1-7, induced a significant dose-related increase in O_2^- production by eosinophils ($P<0.01$; n=5). Pre-incubation of eosinophils with CP96345 (10^{-4}M), but not CP96344, inhibited SP (10^{-4}M)-induced O_2^- production (13±2 vs 4±1 nmol/10^6 cells for SP alone and SP+CP96345, respectively; n=7; $P<0.05$). In contrast, histamine (10^{-5}M)-induced eosinophil O_2^- production was not inhibited by CP96345 (10^{-4}M).

These findings show that SP, if released from cutaneous sensory nerves in equine skin following antigen exposure, could selectively activate equine eosinophils. Moreover, this response appears to be mediated via NK_1 receptor activation.

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55P NERVE GROWTH FACTOR INDUCES AN IMMEDIATE AND ALSO A LATE PHASE OF OEDEMA FORMATION IN RAT SKIN

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Nerve growth factor (NGF) is known to regulate neuropeptide levels in inflammation. However, intradermal (i.d.) NGF also induces a mast cell-dependent oedema in rat skin (Otten *et al*, 1985). We have investigated the time dependent effect of NGF on oedema formation in rat skin.

Male Wistar rats (200-350g) were anaesthetized with sodium pentobarbitone (50mg/kg, i.p.), the dorsal skin shaved and test agents (0.1 ml in Tyrode) injected intradermally (i.d.) in a balanced, randomized pattern. Oedema formation was measured by the extravascular accumulation of ^{125}I-albumin (100 kBq, i.v.), administered at the start of the appropriate accumulation period. (Brain & Williams, 1985). Statistical comparisons between treatments were made using ANOVA followed by Bonferroni's modified t-test.

7S NGF, the NK_1 agonist GR73632 and the mast cell degranulating agent compound 48/80 all caused significant plasma extravasation between 0 and 30 min post injection, as expected (see Table 1). The early (0-30 min) NGF-induced oedema was significantly abolished ($p<0.001$) by co-administration of the H_1 antagonist mepyramine (1.9nmol/site) and the 5-HT antagonist methysergide (2.8nmol/site).

In a series of time course experiments, NGF was seen to produce a further phase of extravasation between 3-5h post-injection. This extravasation was not seen in sites which received i.d. GR73632 or compound 48/80 (see Table 1). In contrast to the earlier response, extravasation 3-5h after injection of 8pmol NGF (at 0h) was unaffected by coinjection of mepyramine and methysergide, either at 0h or at the start of the accumulation period (i.e. 3h). Results as follows: NGF 45.2±4.8µl, NGF+mepyramine and methysergide (given at 0h) 44.2±3.9µl, NGF+mepyramine and methysergide (given at 3h) 42.1±4.5µl (mean±s.e.mean., n=9).

NGF therefore seems to be causing an initial plasma extravasation as a consequence of mast cell degranulation. This is followed by a later phase of extravasation which is not due to release of mast cell amines, or dependent on the effects of their earlier release.

Gavin Bennett is the recipient of a BHF PhD studentship.

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Table 1 Plasma extravasation induced by NGF, GR73632 and compound 48/80 over various time periods post-injection. Results in table are mean±s.e.mean, µl plasma/skin site, n=number of animals used.* $p<0.05$; **$p<0.01$; ***$p,<0.001$ compared with Tyrode sites.

Accumulation Period	n	Tyrode	GR73632 (30pmol)	Compound 48/80 (100ng)	7S NGF (8pmol)
0-30min	8	18.7±1.8	57.4±5.7**	100.9±10.3***	57.6±5.8***
30min-3h	10	12.7±1.0	14.2±3.1	14.2±2.6	17.3±2.5
3-5hr	5	13.8±1.9	14.3±0.8	12.9±0.6	26.8±3.0*

56P PEROXYNITRITE INDUCES PLASMA EXTRAVASATION IN RAT DORSAL SKIN

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Nitric oxide (NO) plays an essential role in the maintainance of vasodilator tone. NO synthase .inhibitors attenuate both short and long term plasma extravasation in a number of different models (Hughes *et al.*, 1990; Boughton-Smith *et al.*, 1993) and this can be secondary to inhibition of microvascular blood flow. NO is a free radical which can react rapidly with the superoxide radical ($O_2^{\cdot}$) to form peroxynitrite anion ($ONOO^-$). Due to its ability to interact with lipids, thiols and proteins, peroxynitrite is a potent tissue damaging species (see Darley-Usmar *et al.*, 1995). In this study we have investigated the effect of peroxynitrite on plasma extravasation in the rat cutaneous microvasculature.

The dorsal skin of anaesthetised (pentobarbitone, 50 mg kg^{-1}, i.p.) male Wistar rats (220-250 g) was shaved. Peroxynitrite was injected intradermally (i.d.; 8.75-340 nmol $site^{-1}$) 60 s after addition to buffer. Concentration was calculated by measuring absorbance at 302 nm. Plasma extravasation was measured over 0-45 min by the accumulation of i.v. ^{125}I-albumin. Peroxynitrite induced a dose-related increase in plasma extravastion whilst control (Tyrode solution, 0.1ml $site^{-1}$), vehicle control (NaOH in Tyrode 300 nmol $site^{-1}$) and the negative control (340 nmol equivalent site), a decomposed form of peroxynitrite, were without effect. These data illustrate that peroxynitrite produces an increase in cutaneous microvascular plasma extravasation. Time course studies indicate that this is rapid in onset but is not ongoing, suggesting that the response is not due to irreversible tissue damage. We suggest that the plasma extravasation produced by peroxynitrite could be involved in inflammatory diseases where NO is produced and peroxynitrite formed.

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Table 1. Peroxynitrite-induced plasma extravasation. Results are expressed as µl plasma $site^{-1}$, mean ± s.e.mean. ***$p<0.001$ peroxynitrite vs control; ANOVA, followed by Bonferroni's modified t test.

Test agent ($site^{-1}$)	µl $site^{-1}$		n
Control	20.1±1.5		6
Vehicle control	27.2±4.4		3
Negative control	22.4±1		6
peroxynitrite 8.75 nmol	25.1±3.6		3
30 nmol	24.5±1.7		3
87.5 nmol	99.5±8.3	***	6
175 nmol	157±31.8	***	3
340 nmol	143.3±9.2	***	3

57P STRICT RELATIONSHIP BETWEEN MCP-1 AND MONOCYTE RECRUITMENT IN THE MOUSE PERITONEAL CAVITY

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Chemokines such as monocyte-chemotactic protein-1 (MCP-1) are proposed to control the selective recruitment of leucocytes to sites of inflammation. In this study we assessed the effects of human recombinant MCP-1 (hrMCP-1) in the mouse and measured the formation of endogenous MCP-1 in response to an inflammatory stimulus *in vivo*.

In vitro, up-regulation of CD11b or CD11a antigen on mouse or human monocytes, respectively, in response to 100 ng/ml hrMCP-1 (R&D, Abingdon, Oxon) was measured by flow cytometry according to a published protocol (Harris *et al.*, 1995). *In vivo*, male Swiss Albino mice (28-32 g) were treated with 0.25 ml sterile saline, hrMCP-1 (0.1-1 μg) or zymosan (1 mg) i.p. At various times after injection, peritoneal cavities were washed with 3 ml of PBS containing 3 mM EDTA. Cells were stained with Turk's solution (1:10) and differential cell counts performed using a a Neubauer hæmatocytometer. Lavage fluids from zymosan treated mice were centrifuged (1200 r.p.m. for 10 min) and endogenous MCP-1 in the supernatants measured by ELISA (Cambridge Bioscience, Oxford). In some experiments mice were pre-treated with an anti-CD11b mAb (0.4 mg i.v.; clone 5C6; Serotec, Oxford, UK) 16 h prior to zymosan injection, and peritoneal cavities washed 4h later. In some experiments dexamethasone (Dex; 30 and 100 μg s.c.) was given 1 h prior to the zymosan. Monocyte or polymorphonuclear cell (PMN) influx are reported as 10^6 cells per mouse. Data are shown as mean ± s.e.mean, and statistical differences were assessed by ANOVA.

HrMCP-1 up-regulated CD11b antigen on mouse monocytes (from 10899 ± 1460 to 16470 ± 76 molecules per cell) and CD11a on human monocytes (23918 ± 1130 to 34456 ± 2456 molecules per cell) (n=4 experiments; $P < 0.05$ in both cases). *In vivo*, i.p. injection of hrMCP-1 caused a time and dose-related increase in monocyte counts, peaking at 4 h post-injection, with an ED_{50} of 0.24±0.10 μg per cavity, and a maximum net increase of 4.53±0.51 x10^6 monocytes, n=11 mice, at 1 μg dose. PMN counts (very low) were unaffected by any dose of hrMCP-1. Zymosan injection caused PMN accumulation, which peaked at 4 h, followed by monocyte infiltration (which was maximal at 24 h). Endogenous MCP-1 was detected in the lavage fluids at 2, 4 and 6 h post-zymosan (3.37 ± 0.3, 6.17 ± 0.32 and 3.46 ± 0.56 ng MCP-1 per mouse, respectively, n=6-8) and returned to baseline (≈ 30 pg) at later time-points. Pretreatment of mice with mAb anti-CD11b reduced (>80%) PMN migration without altering endogenous MCP-1 levels in the 4 h exudates (7.88 ± 0.79 ng per mouse, n=7). In contrast, administration of Dex attenuated both 4 h cell influx and MCP-1 release in dose-related manner: 47% and 58% reduction of PMN accumulation and 38% and 78% reduction in MCP-1 levels in the lavage fluids at 30 μg and 100 μg Dex, respectively (n=5; $P < 0.05$ in all cases).

In conclusion, i) hrMCP-1 up-regulated β_2-integrin expression in both mouse and human monocytes; ii) hrMCP-1 caused a time and dose-related increase in peritoneal monocyte, but not PMN, counts; and iii) this novel finding was substantiated by detecting endogenous MCP-1 release (which was not due to infiltrating PMN) prior to monocyte recruitment in a murine model of acute inflammation.

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58P MODULATION OF ALLERGEN-INDUCED MOUSE EOSINOPHIL MIGRATION BY DEXAMETHASONE IN A NOVEL MODEL

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Eosinophilia is a characteristic feature of a number of allergic disease states, including asthma and atopic dermatitis, which can be modulated by glucocorticoid treatment. Since some actions of these hormones on cell recruitment occur *via* the mediator lipocortin 1 (LC1) [1], we have investigated the effect of dexamethasone (Dex) in a novel model of allergen-induced eosinophil (EØ) infiltration into murine air-pouches and the role played by endogenous LC1.

Balb/c mice (20-22 g) were sensitised to ovalbumin (OA) by sub-cutaneous injection (100 μg adsorbed to 3.3 mg aluminium hydroxide gel) on day 0 and 7. On day 8 the development of air-pouches was initiated by injection of 2.5 ml air [2]. Mice were challenged locally with either saline or 10 μg OA on day 15 and sacrificed at various times. Dex was given s.c. 1 h prior to challenge, whereas an anti-LC1 polyclonal serum (termed LCS3) or non-immune sheep serum (NSS) were given s.c. 24 h prior to Dex. In all cases air-pouches were washed with 1 ml PBS + EDTA (10mM) and total and differential cell counts performed on cytospin preparations. Blood cell numbers were determined from samples collected by cardiac puncture. Bone marrow (BM) EØ numbers were counted from cell suspensions obtained by flushing femurs. Kimura positive cells were counted in a haematocytometer and accepted as EØs. Flow cytometry analysis allowed quantification of LC1 levels in migrated cells following permeabilisation with 0.015 % saponin and staining with LCS3 or NSS (1:100 final dilution). Results are shown as net mean flourescence intensity (MFI). All other data is presented as the mean ± sem of n animals, with statistical differences being assessed by Mann-Whitney U test (two groups) or Kruskal-Wallis test (with more than two groups).

A significant cell infiltration into the air-pouch was measured after OA challenge, from as early as 6 h, peaking at 24 h (Table 1) and persisting up to 48 h.

Table 1. Leucocyte infiltration 24 h post- OA challenge

	Cells per mouse (x10^5)			
Treatment	Total cells	EØs	PMN	Monos
Saline (n=5)	2.5±0.6	0.02±0.01	0.05±0.02	2.4±0.6
OA (n=24)	13.8±1.9 *	5.1±0.7 *	2.5±0.5 *	6.2±0.8 *

* $P<0.05$

An increase in blood EØ numbers was seen in sensitised mice when compared to naive mice (1.5±0.3 x10^5 vs 0.5± 0.1x10^5, n=15; $P<0.05$) with no further increase 24 h after OA challenge (1.4±0.3 x10^5, n=13). In contrast, BM EØ numbers dropped from 2.0±0.1 x10^6 to 0.7±0.2 x10^6 24 h post OA challenge (n=6; $P<0.05$). Infiltrating EØs were positive for LC1 with a net MFI value of 53. Treatment of mice with Dex (50 μg) reduced EØ infiltration following OA challenge both in NSS and LCS3 pre-treated animals (EØs per mouse, x10^5): 5.0±0.6 in controls and 2.0±0.4 after Dex in the NSS group (n=8-15); and 6.0±0.5 vs. 3.0±0.5 in the LCS3 group (n=16-21).

In conclusion, we have established a novel model of OA-induced EØ migration into the air-pouch of sensitised mice, and demonstrated for the first time the presence of LC1 in murine eosinophils. Although Dex produced a strong inhibition of EØ influx, its effect was not mediated by endogenous LC1.

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59P INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) EXPRESSION ON RAT MONOCYTES AND MACROPHAGES IS CONTROLLED BY DEXAMETHASONE

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The *in vivo* effect of glucocorticoid hormones on leucocyte adhesion molecule expression has not been investigated in detail. Here we report that prolonged exposure to dexamethasone (Dex) inhibited basal expression of ICAM-1, but not basal or up-regulated expression of CD11b, on rat peripheral blood circulating monocytes and peritoneal macrophages (Mø).

Male Sprague-Dawley rats (250-300 g body weight) received Dex (0.1 mg/kg s.c.) or saline (1 ml/kg) for 5 days. Blood was collected by cardiac puncture under halothane anæsthesia, and peritoneal cavitities were washed with 10 ml of PBS + 3 mM EDTA. CD11b and ICAM-1 expression on rat peripheral blood leucocytes was monitored using a whole blood protocol recently described (Harris *et al.*, 1995). Blood aliquots (200 µl) were incubated at room temperature with or without 1 µM platelet-activating factor (PAF) for 30 min. After washing, cells were stained with a specific anti-rat CD11b mAb (5 µg/ml; Serotec, Oxford), anti-rat ICAM-1 mAb (12.5 µg/ml; Serotec) or control mouse IgG (5 or 12.5 µg/ml) for 60 min (4°C) and labelled with FITC-conjugated anti-mouse IgG Ab (5 min, room temperature) prior to lysis of red blood cells. Monocytes, lymphocytes and polymorphonuclear leucocyte (PMN) populations were identified by FACS analysis and cell-associated fluorescence was measured in the FL1 channel. Similarly, peritoneal cells (>80% Mø) were stained with the anti-ICAM-1 mAb (1 h at 4°C) prior to labeling with the FITC-conjugated second antibody and FACS analysis. Data (mean ± s.e.mean of n animals per group) are reported as number of antigen molecules per cell. ANOVA and Bonferroni tests were used to detect statistical differences.

Treatment of rats with the 5-day protocol with this low dose of Dex did not alter basal expression of CD11b either on monocytes (12625±1349 and 16741±1614 CD11b molecules per monocyte were measured in control and Dex-treated rats, respectively, n=8) or on PMN (18653±1709 and 19531±939 CD11b molecules per cell, n=9). Following incubation of monocytes with 1 µM PAF, CD11b levels were increased to 18217±1623 and 18856±1839 molecules per cell in saline- and Dex-treated rats, respectively (n=8, not significant). A similar result was obtained with PAF-stimulated PMN (not shown). In contrast to CD11b, 5-day treatment with Dex significantly reduced ICAM-1 expression on circulating monocytes, from 8419±1614 to 3744±737 ICAM-1 molecules per cell (n=8, $P<0.05$). Circulating PMN had little ICAM-1 expression even in the absence of steroid treatment (678±145 and 454±129 molecules per cell, in control and Dex-treated rats, n=8). Peritoneal Mø had higher ICAM-1 expression than circulating monocytes (18049±510 molecules per cell, n=7), and again the levels of this adhesion molecules were reduced following treatment with the steroid (14361±833 ICAM-1 molecules per cell, n=6; $P< 0.05$).

In conclusion, we found that prolonged exposure of animals to Dex selectively modified adhesion molecule expression on leucocytes of the mono-myelocytic lineage. We have identified ICAM-1 as a molecular target the levels of which can be inhibited by these potent hormones *in vivo*. No effect was detected on basal and PAF-stimulated expression of the β_2-integrin CD11b.

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Harris JG *et al.*, (1995) Eur. J. Pharmacol. 279, 149-157.

60P THE LIPOCORTIN-1 BINDING SITE ON HUMAN MONOCYTES IS SENSITIVE TO PROTEOLYTIC ENZYMES

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Lipocortin-1 (LC1) has been implicated in mediating some of the antiinflammatory effects of glucocorticoids. Monocytes bind LC1 in a saturable, Ca^{2+}-dependent manner via 14 and 18 kDa surface proteins (Goulding et al., 1996). Impaired LC1 binding has been observed in leucocytes obtained from air pouch or peritonitis models of inflammation (Perretti *et al.*, 1993) and in rheumatoid arthritis (RA) (Goulding *et al.*, 1992). Since proteolytic enzymes are present at sites of inflammation and in the sera of RA patients, we have investigated the role of proteases in modulating LC1 binding to monocytes by flow cytometry and confocal microscopy.

Peripheral blood mononuclear cells (MNC) were prepared from healthy volunteers by density centrifugation over histopaque. MNC were washed in phosphate-buffered saline (PBS) and $1x10^6$ cells suspended in RPMI 1640 + 0.2% bovine serum albumin + 1.8mM calcium (RPMI++) in the presence or absence of either collagenase, elastase, trypsin or cathepsin G. and incubated at 37°C for 30 min. In 4 experiments, a specific inhibitor was added to block elastase activity. At the end of the incubation period MNC were washed twice with RPMI++ to remove exogenous enzyme and incubated at $1x10^6$ cells per well in 96 well plates with human recombinant LC1 (10µg/ml.) for 1 h at 4°C, followed by reaction with an anti-LC1 mAb (1h 4°C). After further washing, cells were stained with a FITC- goat anti-mouse IgG for 30 min. at 4°C. Following washes in PBS++, cells were fixed in 1% paraformaldehyde. MNC LC1 binding was analysed by confocal microscopy (Bio-Rad MRC 1024) and flow cytometry (FACScan, Becton Dickinson). Monocytes and lymphocyte were identified by the forward and side scatter characteristics and LC1 binding sites expressed as FITC molecules bound per cell, standardised against FITC-coated beads.

Monocyte LC1 binding was substantially reduced in the presence of each of these proteolytic enzymes (Table 1). Lymphocytes were consistently negative for LC1 binding even in the absence of protease treatment.

Table 1. Protease inhibition of LC1 binding to human monocytes (Mean FITC equiv. LC1 binding sites per cell ± s.e.)

protease	conc.	n	control	+ enzyme
trypsin	250 µg.ml^{-1}	12	38765±4642	12770±2542 **
collagenase	5 U.ml^{-1}	7	86071±8957	7443±957 *
	0.5 U.ml^{-1}	7	"	29794±5485 *
	0.05 U.ml^{-1}	7	"	59216±7456
elastase	100 µg.ml^{-1}	2	43501±21832	10814±8088
	10 µg.ml^{-1}	6	50360±16964	21251±6916 *
	1 µg.ml^{-1}	2	43501±21832	24493±6653
	10 µg.ml^{-1}	4	33340±11666	41658±9782 †
cathepsin G	100 µg.ml^{-1}	2	96006±3234	26038±1629

** $p<0.05$, ** $p<0.001$ Wilcoxon signed rank test*
† In the presence of specific elastase inhibitor ONO 5046 (10µM)

Confocal microscopy revealed discrete clusters of LC1 bound to the monocyte surface which were diminished in size and intensity following protease treatment.

In conclusion, LC1 binding sites on monocytes are reduced when exposed to proteases. Levels of collagenase, cathepsin G and elastase are elevated at sites of inflammation and recognised as significant cartilage-degrading enzymes in RA and other arthritides. Thus, these data may indicate an important mechanism by which LC1 binding capacity is lost at sites of inflammation.

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61P DETECTION OF LIPOCORTIN 1 (LC1) AND LC1 BINDING SITES IN THE RAT ANTERIOR PITUITARY GLAND BY FLUORESCENT ACTIVATED CELL ANALYSIS/SORTING (FACS)

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Lipocortin 1 (LC1) is an important protein mediator of glucocorticoid action on the anterior pituitary gland (1). Its molecular mode of action is unknown but data from our functional studies suggest that the protein is exported by target cells in response to a steroid challenge and subsequently binds to cell-surface 'receptors', thereby serving as an autocrine or paracrine agent (1). To investigate this further we have used a combination of fluorescence activated cell sorting (FACS) and electron microscopy (a) to detect and characterise LC1 binding sites on anterior pituitary cells and (b) to identify the pituitary cell subtypes which express LC1 protein.

Anterior pituitary cells (collected post mortem from adult male rats) were dispersed into a single cell population (0.2% collagenase, 0.002% DNAse) and incubated with human recombinant LC1 (hrLC1, 0.5nM-0.5μM). LC1 molecules bound to cell surfaces were detected by sequential incubation with a monoclonal anti-LC1 antibody (Ab, Biogen Research Corp.) and a fluorescent conjugated (FITC) second Ab. Cells displaying positive fluorescence for surface LC1 binding sites were subsequently quantified and separated by FACS and identified by electron microscopy. For measurement of intracellular LC1 freshly dispersed cells were fixed with paraformaldehyde (2%, 1h) and permeabilised with Triton (0.4%, 10min) prior to detection with a specific anti-LC1 monoclonal Ab (Zymed) and FITC-conjugated second Ab. Cells expressing LC1 were characterised by FACS and electron microscopy as above.

Concentration dependent, saturable binding of hrLC1 to the heterogenous anterior pituitary cell population was observed (estimate Kd 13.65±1.8nM; n=4). LC1 binding was Ca^{2+}- and temperature dependent; binding was also abolished by treatment of the cells with trypsin (0.05%, 10min) but reappeared following 24h in culture (sites/cell at saturation 138,650±15,170, vs non-trypsinised 131,900±16,920, estimate Kd 14.2±2.6nM; n=4). Regeneration of the protein binding sites was blocked by cycloheximide (0.5μg/ml) but not by actinomycin D (0.1μg/ml) and was therefore dependent on the translation but not the transcription of new protein. Electron microscopical analysis showed that the proportions of the various pituitary cell subtypes in the dispersed cell population were similar to the intact tissue and that corticotrophs, lactotrophs, gonadotrophs and somatotrophs were all included in the population of cells expressing LC1 binding sites.

Electron microscopical examination of the permeabilised cells prior to FACS showed that the cells were well preserved and that all the various pituitary secretory cell subtypes were present in the dispersed cell preparation. Intracellular measurements on this heterogeneous cell population revealed an average of 46,200±7,200 LC1 molecules per cell (vs isotype matched negative control antibody 1270±450; n=4). Corticotrophs, lactotrophs, somatotrophs and gonadotrophs were all found to express LC1. In conclusion these data show that a number of anterior pituitary secretory cell types express both intracellular LC1 and surface LC1 binding protein. Further studies are required to investigate the transduction pathways used by the 'receptors' leading to the inhibition of hormone release by these cells.

The Wellcome Trust (grant no. 041943/Z/94/Z) and Isle of Man Education Authority generously supported this study.

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62P IMPORTANCE OF LIPOTEICHOIC ACID AND PEPTIDOGLYCAN IN THE INDUCTION OF NITRIC OXIDE SYNTHASE IN MURINE MACROPHAGES BY GRAM-POSITIVE ORGANISMS

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Peptidoglycan (PepG) and lipoteichoic acid (LTA) are major cell wall components of pathogenic Gram-positive organisms such as *Staphylococcus aureus* (*S.aureus*). A moiety of PepG, N-acetyl-D-glucosaminyl-β-[1→4]-N-acetylmuramyl-L-ala-D-isoglutamine (NAG-AP) accounts for the ability of PepG to synergise with LTA to induce nitric oxide synthase (iNOS) in murine macrophages (Kengatharan *et al.*, 1996). However, this moiety is also present in the PepG structure of the non-pathogenic Gram-positive bacterium, *Bacillus subtilis* (*B.subtilis*). Here, we show that PepG from either bacterium synergises with *S.aureus* LTA but not *B.subtilis* LTA to induce iNOS activity in macrophages.

Murine macrophage cells (J774.2) were cultured in 96- or 6-well plates with culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4 mM) until cells reached confluence. PepG (from *S. aureus* or *B.subtilis*) either alone or in combination with LTA (from *S. aureus* or *B.subtilis*) was added to the cells. In a separate experiment, LTA (from *S. aureus* or *B.subtilis*) was incubated with NAG-AP. Nitrite accumulation, an indicator of NO formation, was measured 24h later in the supernatant of J774.2 cells by the Griess method. In one experiment, the cells were extracted 24h after the addition of LTAs and NAG-AP and the expression of iNOS protein was determined by the Western (immuno) blot analysis.

The LTA from *S.aureus*, but not the LTA from *B.subtilis*, caused increases in nitrite accumulation in the supernatant of J774.2 cells from 2±1μM (baseline) to 57±2μM (at 30μg.ml^{-1}, $p<0.05$, n=12). Interestingly, *B.subtilis* PepG, but not *S.aureus* PepG, caused a small but significant rise in nitrite formation at 30μg.ml^{-1} (Table 1). When *S.aureus* LTA was co-incubated with PepG from either *S.aureus* or *B.subtilis*, significant increases in nitrite formation were observed at 24h (Table 1). This rise in nitrite formation was greater than the sum of nitrite formed by either the LTA or PepGs alone. In contrast, co-incubation of *B.subtilis* LTA with *S.aureus* PepG did not produce significant increases in nitrite accumulation over 24h (Table 1). However, the increase in nitrite due to *B.subtilis* LTA and *B.subtilis* PepG was not greater than the sum of nitrite formed by the individual components. When co-incubated with NAG-AP, *S.aureus* LTA, but not *B.subtilis* LTA, caused a significant increase in the nitrite formation as well as the expression of iNOS protein compared to LTAs alone.

Table 1. Effect of bacterial cell wall components on nitrite formation.

Treatment	Nitrite (μM)
baseline	2±1
LTA (*B.subtilis*, 10μg.ml^{-1})	6±1
PepG (*B.subtilis*, 30μg.ml^{-1})	17±1*
LTA (*B.subtilis*, 10μg.ml^{-1}) + PepG (*B.subtilis*, 30μg.ml^{-1})	22±6*
LTA (*S.aureus*, 0.1μg.ml^{-1})	14±2*
PepG (*S.aureus*, 30μg.ml^{-1})	3±1
LTA (*S.aureus*, 0.1μg.ml^{-1}) + PepG (*S.aureus*, 30μg.ml^{-1})	37±5*
LTA (*S.aureus*, 0.1μg.ml^{-1}) + PepG (*B.subtilis*, 30μg.ml^{-1})	52±6*
LTA (*B.subtilis*, 10μg.ml^{-1}) + PepG (*S.aureus*, 30μg.ml^{-1})	4±1
NAG-AP (10μg.ml^{-1})	3±1
LTA (*S.aureus*, 0.1μg.ml^{-1}) + NAG-AP (10μg.ml^{-1})	59±1*
LTA (*B.subtilis*, 10μg.ml^{-1}) + NAG-AP (10μg.ml^{-1})	3±2

Values are given as mean ± s.e.mean (n=9-12). *$P<0.05$ *vs* baseline (unpaired *Student's* t-test).

These results show that PepG from either bacterium synergises with *S.aureus* LTA but not *B.subtilis* LTA to induce iNOS activity in J774.2 macrophages. Furthermore, NAG-AP synergised with *S.aureus* LTA but not *B.subtilis* LTA to induce the expression and activity of iNOS protein. As NAG-AP is a moiety that is conserved in the structure of PepG from a wide range of pathogenic and non-pathogenic bacteria, differences in the structure of LTA (rather than PepG) may determine whether LTA and PepG synergise to induce iNOS in J774.2 macrophages.

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63P THE EFFECT OF CHRONIC DIAZEPAM OR ZOLPIDEM TREATMENT ON THE LEVELS OF GABA$_A$ RECEPTOR SUBUNIT mRNAs IN RAT CORTEX

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Diazepam and zolpidem produce their overt effects by interaction with the GABA$_A$ receptor. In comparison with the classical benzodiazepines, zolpidem shows reduced tolerance and dependence liability (1,2). The present study investigates the effects of chronic diazepam and zolpidem treatment on expression of the GABA$_A$ receptor α1-6, β1-3 and γ1-3 subunit mRNAs in rat cortex. Male Sprague-Dawley rats were injected sub-cutaneously once daily for 7 or 14 days with 15 mg/kg diazepam or 15 mg/kg zolpidem in sesame oil vehicle. *In vivo* binding studies have indicated that these doses give comparable levels of receptor occupancy in rat cortex (3). Steady-state levels of GABA$_A$ receptor subunit mRNAs were quantified by solution hybridization (4), with results summarized in Table 1. Briefly, the levels of α4- and β1-subunit mRNAs were significantly increased after 7 days of both diazepam and zolpidem treatment. These effects were reduced at 14 days, at which point significance was maintained for diazepam only. Significant changes specific to diazepam included increased levels of α3-, α5- and γ3-subunit mRNAs and reduced amount of γ2-subunit mRNA. A significant decrease in the level of α1-subunit mRNA after two weeks of treatment was the only zolpidem-specific effect observed. The genes which encode the GABA$_A$ receptor subunits exist in the genome as α/β/γ clusters (5). Our data indicate a degree of association between a particular drug treatment and changes in the levels of mRNAs arising from a given gene cluster. The results of this study are consistent with a model of diazepam dependence based on GABA$_A$ receptor subunit isoform switching.

	Diazepam		Zolpidem	
	7 days	14 days	7 days	14 days
α1	82.4 +/- 3.8	83.3 +/- 12.1	94.6 +/- 11.0	*72.6 +/- 11.1
α2	100.9 +/- 8.4	120.1 +/- 11.5	125.7 +/- 12.9	111.03 +/- 8.9
α3	101.1 +/- 8.3	*136.8 +/- 1.0	110.9 +/- 11.6	88.6 +/- 15.0
α4	*181.7 +/- 19.2	*150.4 +/- 12.9	*170.8 +/- 10.3	110.1 +/- 9.8
α5	136.8 +/- 10.3	*141.6 +/- 10.9	95.2 +/- 11.3	102.0 +/- 4.3
α6	ND	ND	ND	ND
β1	*153.6 +/- 14.5	*131.9 +/- 7.9	*149.1 +/- 5.5	118.8 +/- 6.5
β2	90.6 +/- 9.1	72.8 +/- 16.1	99.9 +/- 9.4	92.4 +/- 7.4
β3	96.6 +/- 25.0	106.2 +/- 2.8	90.0 +/- 23.0	100.5 +/- 13.3
γ1	134 +/- 18.8	100.7 +/- 21.9	116.5 +/- 11.2	120.1 +/- 7.6
γ2	93.1 +/- 9.5	*73.7 +/- 6.5	95.1 +/- 3.5	86.14 +/- 5.3
γ3	*161.3 +/- 27.8	*156.7 +/- 9.2	106.6 +/- 8.9	84.8 +/- 6.2

Table 1. The levels of GABA$_A$ receptor subunit mRNAs expressed as a percentage of untreated control values with the standard error as a percentage of the mean (n= 4 to 6; *p<0.05). There were no significant effects of vehicle treatment.

We thank Hoffman LaRoche and Synthelabo Recherche for generously providing diazepam and zolpidem, respectively. RAH is in receipt of an Alberta Mental Health Studentship.

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64P EFFECTS OF DIAZEPAM ON GABA$_A$ RECEPTOR SUBUNIT mRNA LEVELS: DRUG DELIVERY VIA OSMOTIC MINIPUMPS

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The anxiolytic diazepam produces its overt effects by interaction with GABA$_A$ receptors of the mammalian central nervous system. We show here that continuous delivery of diazepam from implanted osmotic minipumps does not produce the same changes in GABA$_A$ receptor subunit gene expression as those we have reported previously using the equivalent doses injected daily (1).

Male Sprague-Dawley rats (180 to 200 g) were subcutaneously implanted with Alzet osmotic minipumps containing diazepam in a solution of dimethylsulphoxide/propylene glycol (1:1, v/v) to deliver 15 mg/kg/day. We have previously shown that under the *in vivo* experimental conditions used here, these pumps will deliver diazepam at a relatively constant rate over a period of four weeks *in vitro* (2). Groups of rats, treated in this manner with either the vehicle or vehicle containing diazepam were killed after 7 or 14 days. Steady-state levels of GABA$_A$ receptor subunit mRNAs were determined by solution hybridization (3) and quantified by densitometric analysis.

Diazepam steady-state levels, determined by HPLC analysis of rat brain cortex were 189 ± 55 ng/g (mean ± S.E.M.; n=8) after 7 days and 144 ± 21 ng/g (n=12) after 14 days. Only the α4-subunit mRNA level changed significantly expressed as a percentage of vehicle-treated animals (52±7; mean ± S.D.; n=13; p>0.001) after 7 days. No significant changes were found after 14 days. In contrast, we have previously demonstrated significant changes in the levels of α3-, α4-, α5-, ß1-, γ2- and γ3-subunit mRNAs after daily injections of the same dose (1).

It is possible that the vehicle required to allow the use of osmotic minipumps compromised the effects of diazepam on GABA$_A$ receptor gene expression. Alternatively, the repeated decreases in receptor occupancy (mini-withdrawals) experienced by animals dosed by multiple single daily injections is responsible for the induction of changes in GABA$_A$ receptor gene expression. Clearly the animals treated via osmotic minipumps will not suffer this repeated withdrawal. These results suggest that benzodiazepine receptor occupancy *per se* may not be the primary cue for the induction of changes in GABA$_A$ receptor gene expression.

Table 1. Mean GABA$_A$ receptor subunit mRNA levels, relative to vehicle-treated controls, as a consequence of diazepam treatment via osmotic minipumps. Data are expressed as a percentage change together with the coefficient of variation for multiple determinations in parentheses.

	7 days	14 days
α1	101 (4.9)	98 (6.1)
α2	101 (13.4)	114 (18.1)
α3	103 (3.9)	100 (14.6)
α4	51.5 (8.7)	86.3 (6.3)
α5	100 (17.2)	110 (3.8)
ß2	91.7 (25.7)	102 (10.8)
γ2	98.3 (5.4)	94 (12.6)

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65P THE EFFECT OF SOCIAL COMPETITION AND EXPOSURE TO AN OPEN FIELD ON PLASMA CORTICOSTERONE LEVELS IN THE RAT

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Social hierarchies formed between animals have been proposed as models of both psychosocial stress and anxiety (Fuchs *et al.* 1993, Joly and Sanger 1991) Stable hierarchies determined by agonistic interactions between rats have shown different levels of plasma corticosterone depending on the relative rank order of the rat (Blanchard *et al.* 1993). As social competition for sweetened milk has been proposed to be based on different levels of fear it is possible that animals with different rank orders may also have different levels of plasma corticosterone. The purpose of this study was to determine whether there are different corticosterone levels between dominant, intermediate and subordinate rats following a social competition trial and exposure to a novel environment. In addition, the behaviour of the rats in the novel environment was observed to assess any behavioural differences between each animal in the social hierarchy.

Baseline levels of competition were established for a 5 week period during 5 min access to sweetened milk in triads (n=13) of male Lister Hooded rats weighing 256.2±5.1g at the start of the study. For each group the social hierarchy was established by observing which animal had access to the spout at 5s intervals throughout the testing period (e.g. 60 observations for one 5 min trial). All groups were tested twice a week. Trunk blood was obtained from dominant, intermediate and subordinate rats 10 min after social competition (n=6) and also 10 min following exposure to an open field (n=7). The open field was 122cm in diameter and the rats were exposed for a 10 minute test period during which time the distance travelled (m) was measured by a computer tracking system. Blood samples were centrifuged and the plasma analysed for corticosterone by radioimmunoassay (Gamma-B I^{125} corticosterone, Immuno Diagnostic Systems Limited). Data are expressed as mean ± s.e. mean and statistical analysis was performed using two way ANOVA followed by Dunnett's t-test.

During the initial 5 week study the triads of rats developed stable hierarchies consisting of dominant, intermediate and subordinate animals which had access to the drinking spout for 40.7±2.1%, 27.8±2.7% and 22.0±1.2% of the 5 min testing period respectively. Following social competition there were no significant differences in plasma corticosterone levels between dominant, intermediate and subordinate rats, 73.7±11.7ng/ml, 67.2±11.0ng/ml and 53.7±5.6ng/ml respectively. Following exposure to an open field, dominant rats showed a significant increase in plasma corticosterone levels compared to the levels following social competition, 73.7±11.7ng/ml compared to 150.2±20.4ng/ml, ($p<0.05$), in addition there was also a significant difference between dominant and subordinate animals 150.2±20.4ng/ml compared to 63.2±15.3ng/ml respectively ($p<0.01$). There was no significant difference between the distance travelled in the open field by dominant, intermediate and subordinate rats.

These results show that social competition in groups of rats competing for sweetened milk does not create a situation which leads to increased plasma corticosterone in any subgroup of rats within the social hierarchy. However, although no differences were observed between the animals in terms of distance travelled in an open field, dominant rats exhibited an increase in corticosterone which was not seen in intermediate or subordinate rats.

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66P CENTRAL BENZODIAZEPINE RECEPTOR AUTORADIOGRAPHY IN HUMAN RESECTED EPILEPTIC TEMPORAL LOBE: CHANGES IN RECEPTOR DENSITY AND AFFINITY

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Central BZ receptor binding, determined by [^{3}H]-flumazenil autoradiography at a single concentration, is reduced in specific regions of hippocampi resected from patients with unilateral mesial temporal lobe epilepsy (TLE) (Burdette *et al.*, 1995). This decrease has been correlated with changes in neuronal densities due to hippocampal sclerosis (HS). We have performed saturation autoradiographic studies using [^{3}H]-flumazenil, to determine the binding parameters and investigate relationships with neuronal density, in hippocampi resected from 8 patients (average age 32 years) with intractable TLE due to HS and 6 neurologically normal controls (average age 69 years).

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, mounted onto charged microscope slides and stored at -80°C until assayed. Total binding was assessed in at least 2 sections per concentration. Sections were pre-incubated (2 x 30 minutes) in buffer (0.17M Tris-HCl, pH 7.4). Binding incubation was for 60 mins at 0-4°C using fresh buffer containing 0.25-20nM of [^{3}H]-flumazenil. Non-specific binding (1-2 sections) was determined in the presence of 2µM clonazepam. Slides were washed (2 x 1 minute) in fresh buffer, briefly dipped in distilled water, dried with cold air and apposed to [^{3}H]-sensitive film for 21 days at room temperature. Film optical densities (converted to fmol/mg protein) were measured in six hippocampal subregions identified by cresyl violet-staining following autoradiography. Mean optical density values for each region were obtained by outlining the entire region. Kd and Bmax were determined by computer analysis (Graph Pad Prism). 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.

Bmax values for [^{3}H]-flumazenil binding, in all six hippocampal areas measured in HS (31-352 fmol/mg protein), were significantly reduced compared with controls ($p<0.05$, Student's *t*-test - Control range 217-600 fmol/mg protein). The largest reductions in Bmax were observed in the CA1 (-94 ± 0.6%, $p<0.001$), hilus (-63 ± 6%, $p<0.001$), CA2 (-46 ± 6%, $p<0.001$) and dentate gyrus (-45 ± 5%, $p<0.001$) regions. Significant increases in affinity compared with control (Kd values 2.7-5.2 nM), were observed in three of the six subregions measured: dentate gyrus (+40 ± 8%, $p<0.01$); hilus (+42 ± 8%, $p<0.01$) and subiculum (+43 ± 6%, $p<0.01$). Neuronal densities available in five subregions indicated changes which paralleled the decreases in Bmax values in all areas except for CA1 where the Bmax was reduced to a significantly greater extent ($p<0.05$) than the neuronal density (-94 ±0.6% and -87 ±1.2% respectively).

Whilst these data would support the view that the decrease in BZ receptor binding is principally due to neuronal loss, in regions other than CA1, this may not be definitive and ongoing *in situ* hybridization studies may provide a clearer interpretation.

Control autopsy brain tissue was provided by the Parkinson's Disease Society Brain Bank, Institute of Neurology, London WC1N 1PJ.

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67P THE DEPRESSANT ACTIONS OF CARBAMAZEPINE, LAMOTRIGINE AND PHENYTOIN ON THE RAT SPINAL CORD *IN VITRO*

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The aim of the present experiments was to identify a stimulus/recording protocol that would detect therapeutic concentrations of anticonvulsant drugs. Hemisected spinal cord preparations from 3 to 9 day old Wistar rats of either sex were bathed in medium containing 1.5 mM Ca^{2+} and 1.25 mM Mg^{2+} at 25 ^{o}C. MSRs measured as the amplitude of the initial monosynaptic population spikes of motoneurones were recorded from the L5 ventral root proximal to the spinal cord. The L5 dorsal root was stimulated proximally every 20 s with paired cathodal pulses of 0.5 ms duration at different intensities and 50 to 100 ms apart such that the first pulse, at intensities below twice threshold, produced a monosynaptic population spike (MSR1) of less than half maximum amplitude and the second pulse at intensities of three times threshold or higher, produced a maximum response (MSR2).

Carbamazepine 10 and 20 µM depressed the mean peak amplitude of MSR1 by 12% (n=2) and 31% ±9 s.e.mean (n=3) respectively. Lamotrigine (5, 10 and 20 µM) produced corresponding values of 16 ±7% (n=2), 36 ±18% (n=4) and 36 ±19% (n=2) respectively and on one preparation phenytoin produced 26% depression of MSR1 at 10 µM and 49% depression at 20 µM. The depressant effect of lamotrigine on MSR1 was significant at $P<0.002$ (sign test). All three drugs produced no significant depression of MSR2. The amplitude of MSR1 reflects the number of motoneurones which are activated by the submaximal afferent input. The susceptibility of MSR1 to the anticonvulsants can be explained through a postjunctional depression of the excitability of motoneurones by the drugs. MSR2 is the response of all available motoneurones activated supramaximally. Under this condition the anticonvulsants are unable to reduce excitability sufficiently to cause failure in a significant number of the motoneurones. This situation contrasts with the action of baclofen which depresses transmitter release from primary afferent terminals (Edwards et al 1989). On the present preparation baclofen produces marked depression of the MSR elicited following supramaximal shocks (Siarey et al, 1992).

The experiments were carried out during an award under the Senior Academics in Industry Scheme between Tocris Cookson Ltd and the Department of Trade and Industry.

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68P THE INTRACELLULAR MECHANISMS UNDERLYING PARALLEL FIBRE-INDUCED, HETEROSYNAPTIC LONG-TERM DEPRESSION IN THE CEREBELLUM

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Simultaneous and repetitive activation of the two excitatory inputs to cerebellar Purkinje cells, parallel fibres (PF) and climbing fibres (CF), leads to a long-term depression (LTD) of transmission at the PF-Purkinje cells synapse (Ito *et al.*, 1982). Three elements are necessary and sufficient for LTD. Activation of i) AMPA receptors, ii) metabotropic glutamate receptors (MGluRs) and iii) calcium influx through voltage gated calcium channels. The first two elements are generally attributed to the PF input and the third to the CF. Recent evidence, however, indicates that moderately strong PF stimulation is sufficient to raise calcium levels in isolated spiny dendritic branchlets and induce LTD (Hartell, 1996). Although the calcium influx is spatially confined, LTD spreads to distant synapses on the same cell through a mechanism that does not require postsynaptic calcium but is mediated through the diffusible messenger nitric oxide (NO). The aim of this study was to further characterize the receptor subtypes and second messenger pathways involved in each of these two, pharmacologically distinct mechanisms of LTD.

2-3 week old male Wistar rats were decapitated under ether anaesthesia and 200 µm thick sagittal slices of cerebellum prepared. Whole cell current clamp techniques were used to record responses to alternate (0.2Hz) activation of two separate PF inputs to a single Purkinje cell. Stimulus strengths were minimized to ensure epsp amplitudes did not exceed 5 mV to limit calcium entry. After a 10 minute control period, the intensity of stimulation to one of the two PF inputs (test input) was raised to evoke epsps of amplitudes greater than 9-10 mV and the frequency of stimulation was raised to 1 Hz. No changes were made to the control input. After 5 minutes, stimulus parameters to the test input were returned to control values. The initial slopes of epsps to stimulation of each input were expressed as a percentages of mean control levels.

The extent of depression at test and control inputs, 30 minutes after raised PF stimulation was 54 $\pm$ 10 and 55 $\pm$ 10 % of control respectively (mean $\pm$ sem, n=6). Inclusion of the specific guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 5 µM) to the bathing media completely prevented depression at the control site (101 $\pm$ 2 %, $p<0.05$ Mann-Whitney U test, n=5) but had no effect at the test site (48 $\pm$ 2 %). Similar effects were observed at test and control sites in the presence of the protein kinase G (PKG) inhibitor KT5823 (0.1 µM; 40 $\pm$ 12 % and 92 $\pm$ 3 % of control respectively, n=5). Inclusion of the selective PKC inhibitor, chelerythrine (n=5) into the intracellular patch solution completely prevented LTD of PF responses at both the test (106 $\pm$ 21) and control sites (101 $\pm$ 19, n=6). Pre-incubation of slices with the mGluR antagonist MCPG (500 µM) for 15 minutes prior to and during the control period also significantly reduced the extent of depression to both test (80 $\pm$ 8) and control inputs (74 $\pm$ 16%).

These data indicate that mGluRs and PKC are essential elements for both of these separate pathways of LTD induction. The calcium-independent NO/cGMP/PKG cascade may serve to spatially reinforce LTD or provide a neuroprotective mechanism following excessive stimulation.

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69P MODULATION OF CHLORIDE RESPONSES TO GLYCINE BY 5-HT$_3$ RECEPTOR LIGANDS

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Modulations of Cl^- responses to glycine by various ligands of 5-HT receptors were studied at room temperature by whole-cell recording of rat ventral spinal neurones in culture. The initial observation of a reduction of glycine responses by 100 µM 5-HT (by 16±3% (5)) led to two unexpected results.

First, mCPBG (1-(m-chlorophenyl)-biguanide), known as a potent and selective 5-HT$_3$ agonist, reduced responses to 20 µM glycine by 30±7% (5) and 59±15% (8), at 5 and 20 µM, respectively. This effect was not associated with any change of the resting conductance (in zero glycine), and was not mimicked by other 5-HT$_3$ agonists (phenylbiguanide, 2-methyl-5-HT, up to 20 µM, n=4).

The second, more interesting, observation was the reversible potentiation of Cl^- responses to glycine by three chemically related 5-HT$_3$ antagonists: LY-278,584 (1-methyl-N-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1H-indazole-3-carboxamide maleate), at 1-10 µM, MDL-72222 (3-tropanyl-3,5-dichlorobenzoate) and ICS-205,930 (3-tropanyl-indole-3-carboxylate hydrochloride), in a low concentration range, 10 nM - 1 µM. The potentiations were observed over the whole voltage range, without any change either in the reversal potential of glycine responses or in the resting conductance. The degree of potentiation depended on glycine concentration, potentiation resulting from an increased affinity of the receptors for glycine: 200 nM ICS-205,930 reduced the EC_{50} (34±11 µM (8)) by 25±4% (8) without changing either the maximum response or n_H. The responses to 20 µM glycine were enhanced by 73±30% (11), 60±23% (11) and 49±21% (10), by 10 µM LY-278,584, 100 nM MDL-72222 and 100 nM ICS-205,930, respectively. The potentiations persisted after glycine uptake blockade (by Na removal), and in excised membrane patches. In contrast, at 10 µM, both MDL-72222 and ICS-205,930 reduced glycine responses (by 41±0.5% (3) and 14±3% (4), respectively, for 80 µM glycine, at -30 mV).

In conclusion, LY-278,584, MDL-72222 and ICS-205,930 potentiate glycine responses independently of their known 5-HT$_3$ antagonist properties. Furthermore, neither LY-278,584 (10 µM, n=3) nor MDL-72222 (100 nM, n=6) potentiated Cl^- responses to 2 µM GABA (see also Klein *et al.*, 1994). These results may allow the development of a new class of neuromodulators: selective and high-affinity potentiators of Cl^- glycine responses.

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70P EFFECTS OF CHRONIC PAROXETINE ON 5-HT$_{1A}$ AUTORECEPTORS CONTROLLING DORSAL RAPHE CELL FIRING AND 5-HT RELEASE

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Current thinking suggests that the selective serotonin reuptake inhibitors (SSRIs) exert their antidepressant effect by desensitising somatodendritic 5-HT$_{1A}$ autoreceptors in the dorsal raphe nucleus (DR) and/or the 5-HT$_{1B/D}$ terminal autoreceptors (Blier & De Montigny, 1994). To test the role of the 5-HT$_{1A}$ receptors we looked at the effect of chronic SSRI treatment on two *functional* assays of 5-HT$_{1A}$ sensitivity.

Male Wistar rats (125-175g at start of dosing) were treated with paroxetine (10mg/kg/day p.o.) or water for 21 days. Twenty-four hours after the last dose, two DR brain slices (350µm) were taken. One, used for electrophysiological recording, was superfused (2ml/min) with warm (32°C) oxygenated (95%O_2/5%CO_2) artificial cerebrospinal fluid (ACSF) containing 5µM phenylephrine throughout to maintain cell firing (Sprouse and Aghajanian, 1987) while in the other, kept at 31°C, voltammetry was used to measure electrically stimulated (10 pulses at 200 Hz) 5-HT release (Davidson & Stamford, 1995).

The selective 5-HT$_{1A}$ agonist (+)-8-hydroxy-DPAT inhibited 5-HT release and DR cell firing. Chronic paroxetine treatment caused a rightward shift of the 8-OH-DPAT concentration-response curve (CRC) on DR cell firing ($p<0.01$, 2-Way ANOVA), but did not shift the 8-OH-DPAT CRC on 5-HT release (Figure 1). Paroxetine treatment increased the 8-OH-DPAT E_{max} (75.2±1.4% inhibition) on 5-HT release compared to controls (52.0±5.0%), ($p<0.01$, t-test).

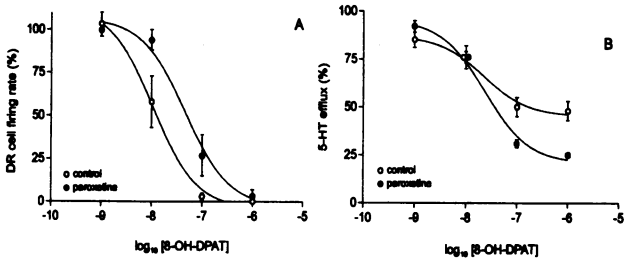

Fig. 1 The effect of 8-OH-DPAT on (A) DR cell firing and (B) stimulated 5-HT release in slices from rats chronically treated with paroxetine (10mg/kg/day p.o. ●) or water (O). All values are means ± SEM (n=3 to 8) expressed as a percentage of the pre-8-OH-PAT values.

These data suggest that there may be two distinct subgroups or populations of 5-HT$_{1A}$ receptors, one of which controls 5-HT release and another which influences firing. Furthermore chronic paroxetine treatment appears able to differentially modulate these different populations.

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71P PROLONGED ETHANOL INTAKE ALTERS THE EFFECTS OF REPEATED ADMINISTRATION OF NICOTINE ON LOCOMOTOR ACTIVITY

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Dependence on ethanol is almost always accompanied by dependence on nicotine; about 95% of alcoholics smoke cigarettes. Some interactions have been demonstrated experimentally between these two drugs, for example nicotine increased voluntary ethanol intake (Potthoff et al., 1983). In this study we examined the effects of nicotine on locomotor activity in mice after chronic ethanol treatment. The time of the first tests was chosen to be after the acute behavioural signs of ethanol abstinence had subsided, as we wished to investigate prolonged changes which might be involved in the relapse back into drug taking which is characteristic of dependence on these drugs. The effects of repeated nicotine administration were then studied.

Male TO mice (35-40g) were administered ethanol by liquid diet. Ethanol-treated mice first received control diet for 3 days, followed by 2 days 3.5% v/v ethanol in the diet, then 9 days 5% ethanol, followed by 9 days 8% ethanol (daily intake was 24-28 g/kg). Control groups were pair-fed control diet. There were 18 mice per treatment group. Mobile and static counts of locomotor activity and rearing activity were measured, by breaking of infra-red beams, after the nicotine administration, starting 6 days after the cessation of the ethanol treatment. Animals were placed in the test cages for 30 min, to acclimatise, then nicotine was given subcutaneously at 0.4 mg/kg, and the activity measured over the next 30 min. Nicotine was then given at this dose, once daily for 28 days and the effects on activity measured, as above, on the 28th day.

The activity behaviour after nicotine administration was not significantly altered by prior ethanol treatment when this was tested on the first day of nicotine administration. By the 28th day, however, the mobile activity counts and the rearing activity, in response to acute injection of nicotine, were significantly higher ($P < 0.05$, Student's t-test) in mice that had previously received chronic ethanol treatment compared with those given the control diet. No differences in any activity were seen after saline administration, on either testing day.

These results show that prolonged alcohol intake can alter the effects of repeated nicotine administration. We have previously shown that the locomotor stimulant actions of amphetamine and of cocaine, after repeated administration, are also increased by this ethanol treatment schedule (Manley and Little, 1996). These results indicate that chronic ethanol intake may cause prolonged neurochemical changes which outlast the acute withdrawal phase which follows cessation of ethanol intake, and which alter the effects of repeated nicotine administration.

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Table 1. Effects of nicotine on activity, over 30 min period. Results are mean ± s.e.m. * $P < 0.05$, compared with ethanol diet+nicotine †† $P<0.01$ compared with control diet + saline; † $P = 0.06$ compared with ethanol diet + saline

Diet/drug	Day 1	static counts	mobile counts	rears	Day 28	static counts	mobile counts	rears
Control/saline		2133 ± 236	3361 ± 680	348 ± 98		1847 ± 271	1644 ± 413	208 ± 99
Control/nicotine		1316 ± 183††	3580 ± 764	427 ± 175		1640 ± 142	2221 ± 534	190 ± 79
Ethanol/saline		2270 ± 268	2864 ± 484	685 ± 175		1742 ± 200	1516 ± 337	151 ± 42
Ethanol/nicotine		1623 ± 201†	2835 ± 561	369 ± 126		1529 ± 128	3247 ± 787*	408 ± 121*

72P BLOOD PRESSURE AND VASCULAR ENDOTHELIAL RESPONSE IN ALCOHOL-PREFERRING RATS

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Hypertension is recognised as a risk factor for stroke, heart and kidney disease and excessive alcohol intake is known to elevate blood pressure. However, the mechanisms by which alcohol increases blood pressure are not completely known. The present study assesses changes in vascular sensitivity and the role of the endothelium in response to vasoconstrictors such as angiotensin.

Two lines of genetically selected rats (N=7 per line) were obtained from the colony at the Addiction Research Foundation of Ontario. The HARF line (mean body weight 164±8g) prefer alcohol while the LARF line (mean body weight 151±5g) do not. Male rats were offered a brief daily access to increasing concentrations (3%, 6% and 12%) of alcohol and water as described previously (MacDonall & Marcucella, 1979). Following cessation of alcohol drinking, mean blood pressure was measured in triplicate in both groups via the tail-cuff method. Animals were subsequently euthanised and the descending thoracic aorta was carefully dissected. Accordingly, we explored the responsiveness of both endothelium-intact and -denuded aorta to angiotensin I, II and III. Cumulative concentration effect curves were constructed to the angiotensins on pairs of aortic rings (one endothelium-intact and one -denuded). Curves were analysed by the logistic equation: $Y=\{(a+d)/[1+(X/c)^b]\}+d$ (Parker & Waud, 1971). Efficacy data are summarised in the table below where * represents $P<0.05$. for LARF vs HARF.

The drinking and mean blood pressure characteristics of the 2 lines of rats were as follows. Based on the final 6 days of the 12% alcohol challenge, the mean alcohol consumption ($g.Kg^{-1}$) was 0.96±0.11 and 0.47±0.09 ($P<0.01$) for HARF and LARF respectively. The mean blood pressure was significantly ($P<0.01$) less in HARF compared to LARF (109±3mmHg and 140±3mmHg, respectively).

These results indicate that the HARF line exhibit a significantly lower blood pressure despite a higher alcohol intake compared to the LARF line. The high blood pressure in the latter group may be a reflection of an enhanced vascular response to angiotensins which is particularly evident in endothelium-denuded preparations.

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Table I. Efficacy for angiotensins ($g.mm^{-2}$)

	ENDOTHELIUM-INTACT		
	angiotensin 1	angiotensin II	angiotensin III
HARF	1.9 ± 0.6	1.7 ± 0.5	1.7 ± 0.5
LARF	3.2 ± 0.6*	1.9 ± 0.4	2.3 ± 0.6
	ENDOTHELIUM-DENUDED		
	angiotensin I	angiotensin II	angiotensin III
HARF	1.9 ± 0.2	2.3 ± 0.4	1.1 ± 0.1
LARF	4.4 ± 0.4*	3.7 ± 0.4*	3.6 ± 0.7*

73P ADENOSINE RECEPTORS IN THE LEFT ANTERIOR DESCENDING CORONARY ARTERY OF THE RAT

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In most species adenosine dilates the coronary artery and this is thought to be mediated by adenosine A_1 (Merkel *et al.*, 1992) and A_2 receptors (Vials & Burnstock, 1993), perhaps in association with other subtypes (Abebe *et al.*, 1994). However, despite the use of rat blood vessels in many studies of the vasculature, the adenosine receptors present in the rat coronary artery have not previously been characterised.

The left anterior descending coronary artery was removed from male Wistar rats (250-500g) and segments (length 1.5-2.0 mm, normalised internal diameter 250-350 μm) were mounted on 40 μm wire in a small vessel myograph (JP Trading, Aarhus, Denmark) for isometric tension recording. The vessel was bathed with physiological salt solution (composition, mM: NaCl 115.3, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5, glucose 11.1) equilibrated with 95% O_2/5% CO_2 at 37°C. Vessels were allowed to equilibrate for 60 min before normalisation (Mulvany & Halpern, 1977). After a further 60 min period, vessels were precontracted with 3 μM 5-hydroxytryptamine (5-HT), giving ~60% of the maximum contraction, for determination of cumulative concentration-relaxation curves to adenosine receptor agonists. Responses are expressed as percentage relaxations of the 5-HT-induced tone.

5'-N-ethylcarboxamidoadenosine (NECA) was the most potent of the agonists studied with an EC_{50} of 0.33± 0.11 μM and caused 100% relaxation. N^6-cyclopentyladenosine (CPA) had a biphasic concentration-response curve, with a maximum relaxation of 77±2% (EC_{50} = 6.4±0.9 μM). 2-p-(2-carboxyethyl)phenethylaminoadenosine (CGS 21680) was less potent than CPA and caused a relaxation of 72±11% at the highest concentration used (30 μM); the mean concentration causing 50% relaxation of the 5-HT-induced tone ($EC_{50\%}$) was 26.7±6.9 μM. Like NECA, adenosine was able to cause 100% relaxation of the 5-HT-induced tone but was the least potent of the agonists tested (EC_{50} = 0.17±0.05 mM).

Addition of the A_1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 nM), the A_{2A} antagonist 8-(3-chlorostyryl)caffeine (CSC, 500 nM) or the mixed A_1/A_2 antagonist 8-phenyltheophylline (8-PT, 10 μM) 30 min through the second equilibration period had no effect on baseline tension. The concentration-response curve to CGS 21680 was unchanged in the presence of 10 nM DPCPX but showed a rightward shift of approximately 3 fold in the presence of 500 nM CSC showing that CGS 21680 was acting on A_{2A} receptors.

The concentration-response curve to CPA was unchanged in the presence of either 10 nM DPCPX or 500 nM CSC. In the presence of 10 μM 8-PT, the responses to lower concentrations of CPA (approximately 1 nM - 1 μM) were unchanged, but the responses to higher concentrations range showed a rightward shift of approximately 3 fold. This suggests that CPA is acting both on a xanthine-sensitive receptor together with a xanthine-insensitive receptor. The lack of effect of DPCPX and CSC suggest that neither of these receptors is of the A_1 or A_{2A} subtypes. The identity of the receptors mediating the response to CPA is therefore uncertain but might include the A_{2B} and A_3 receptors.

These results suggest the presence of multiple adenosine receptors, including the A_{2A} subtype, in the rat left coronary artery. It seems unlikely that the A_1 receptor is present but the identity of the other adenosine receptors remains to be elucidated.

CEO is a Medical Research Council Research Student.

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74P ACIDOSIS-INDUCED CORONARY CONSTRICTION IN THE ISOLATED RAT HEART IS SPECIFICALLY ATTENUATED BY L-TYPE CALCIUM CHANNEL BLOCKERS

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Nifedipine causes coronary dilation and attenuates acidosis-induced coronary constriction in the isolated rat heart (Wilson & Woodward 1995). In the present investigations we examined the effects of other coronary dilator agents and hypoxia on the response to acidosis to determine if the effect of nifedipine was due to physiological antagonism or specifically to its calcium channel blocking actions.

Hearts were removed from pentobarbital-anaesthetised male Wistar rats (300-320g) and perfused (10ml.min.$^{-1}$) with modified Krebs-Henseleit solution (37°C; pH 7.4 or 6.8 gassed with 95%O_2/5%CO_2 or 95%N_2/5%CO_2). Coronary perfusion pressure (CPP mmHg) was used as an index of coronary tone. Metabolic acidosis was applied for 5 min followed by a 10 min recovery period. Agents were applied 10 min and their effects on basal parameters noted. A further acidotic challenge was made and the effects compared to control. Data expressed as mean±s.e.m., n=4. Statistical analysis performed using paired t-test except where stated otherwise.

In control hearts, acidosis increased CPP from 90±8 to 160±5 mmHg ($p<0.05$). In calcium-free solution, basal CPP was suprisingly maintained at 87±11 mmHg, however, the acidosis-induced vasoconstriction, was significantly attenuated, CPP increasing to 94±11 mmHg ($p<0.05$ compared to control ONEWAY ANOVA).

Coronary vessels have been shown to undergo hypoxic vasodilatation (Cyrys & Daut 1994). Hypoxic perfusate significantly reduced CPP (87±2 to 58±7 mmHg ($p<0.05$). Under these conditions the response to acidosis, was not altered. Control acidotic CPP increased to 158±13 mmHg ($p<0.05$) while under hypoxic conditions acidosis increased to 167±9 mmHg ($p<0.05$ compared to basal CPP).

P1Pi, the isopropyl ester of palmitoyl carnitine, is a coronary vasodilator (Reeves *et al.* 1995). At a concentration of 1μM, P1Pi significantly reduced CPP from 88±9 to 68±8 mmHg ($p<0.05$). This agent had no significant effect on acidosis-induced constriction, with CPP increasing to 151±7 and 130±13 mmHg for control and in the presence of P1Pi respectively (increases of 64±6 and 63±7 mmHg).

100 nM nifedipine significantly reduced basal CPP from 100±9 to 49±8 mmHg ($p<0.05$). The control acidosis-induced increase in CPP (168±9 mmHg) was significantly reduced by this compound (58±9 mmHg; $p<0.05$ compared to control ONEWAY ANOVAR). Similar results were obtained with verapamil (1μM) ($p<0.05$) and amlodipine(1-100nM) ($p<0.05$). In the presence of 100nM nifedipine, 30 pmoles endothelin-1 was capable of inducing a prolonged increase in CPP from 49±8 to 85±9 mmHg ($p<0.05$) showing that the coronary vasculature was still capable of contraction.

These data suggest that acidosis-induced coronary constriction in the rat heart requires influx of extracellular calcium. Data obtained using hypoxia, P1Pi and endothelin-1 would indicate that the antagonism of the response by the calcium channel blockers is a specific calcium channel blockade and not physiological antagonism and that acidosis is activating calcium channels in the rat coronary smooth muscle.

D.A.W. is a University of Bath research student.

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75P SURAMIN MODIFIES THE CONDUCTANCE AND GATING OF RYANODINE RECEPTOR CHANNELS

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The cardiac and skeletal isoforms of the sarcoplasmic reticulum (SR) Ca^{2+}-release /ryanodine receptor channel have an adenine nucleotide binding site on the cytosolic face of the channels. Suramin has been shown to act as an antagonist at cell surface P_2 purinoceptors and it has been suggested that suramin modifies SR Ca^{2+}-release by interacting with the adenine nucleotide site on the skeletal ryanodine receptor (Emmick *et al.*, 1994). We therefore investigated the effects of suramin on the conductance and gating of the rabbit skeletal and sheep cardiac ryanodine receptors incorporated into planar phospholipid bilayers. Vesicles of heavy SR were fused with phosphatidylethanolamine lipid bilayers and single channel events were recorded under voltage-clamp conditions as previously described by Sitsapesan *et al.* (1991). The free [Ca^{2+}] was approximately 50 mM on the *trans* (luminal) side of the bilayer and 10 μM on the *cis* (cytosolic) side. Experiments were performed at 22-24 °C with all solutions at pH 7.2. The mean values ± s.e mean are given.

Suramin (100 μM), when added to the cytosolic side of the bilayer, increased single channel conductance from 103.4±1.5 pS (n=5) to 126±2.6 pS (n=4; P<0.01). No significant change to the apparent extrapolated reversal potential (41.1±2.3 mV before and 44.35±0.85 mV after suramin) was observed. In the presence of 10 μM cytosolic Ca^{2+}, cytosolic additions of suramin increased the open probability (Po) of ryanodine receptors in a concentration dependent manner. For example, 10 μM suramin increased the Po of cardiac channels from 0.037±0.018 (n=10) to 0.254±0.026 (n=6; P<0.01). The increase in Po was caused predominantly by an increase in the duration of open lifetimes which increased from 0.76±0.12 to 22.46±11.23 ms (n=4). The above effects of suramin were completely reversible and did not occur when suramin was added to the *trans* (luminal) side of the bilayer. Agents which activate the ryanodine receptor channel by binding to the adenine nucleotide binding site do so by increasing both the frequency and the duration of open lifetimes without changing single channel conductance (McGarry & Williams, 1994). Therefore it is unlikely that suramin is interacting with the adenine nucleotide site on the ryanodine receptor and we propose that suramin acts via a separate and possibly novel binding site.

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76P EFFECTS OF CICLAZINDOL ON EDHF-MEDIATED RELAXATIONS IN THE RAT HEPATIC AND GUINEA-PIG BASILAR ARTERIES

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In the rat hepatic (RHA) and guinea-pig basilar (GBA) arteries, a combination of 0.1 - 0.3 µM apamin and 0.1 - 0.3 µM charybdotoxin (but not apamin plus iberiotoxin), inhibitors of the small and large conductance calcium-sensitive potassium (K) channels, respectively, abolishes relaxations mediated by EDHF (endothelium-derived hyperpolarising factor; Petersson et al., 1996; Zygmunt & Högestätt, 1996). Since charybdotoxin (but not iberiotoxin) also inhibits delayed rectifier K-channels (K_V; Chandy & Gutman, 1995), we examined whether the K_V inhibitor ciclazindol could substitute for charybdotoxin.

In the RHA and GBA, EDHF-induced relaxations were elicited using acetylcholine (1 nM - 100 µM) in the presence of 0.3 mM N^G-nitro-L-arginine (L-NOARG) and 10 µM indomethacin. Ciclazindol (10 µM) alone reduced these relaxations in both arteries (Table 1). However, in the additional presence of 0.1 - 0.3 µM apamin (which alone had no effect: Petersson et al., 1996; Zygmunt & Högestätt, 1996), acetylcholine-induced relaxations were abolished in both preparations (Table 1).

A co-operative inhibitory action of ciclazindol and apamin was also observed in both arteries when A23187 (0.1 - 3 µM) was used instead of acetylcholine to release EDHF (Table 1). In contrast, ciclazindol (10 µM) plus apamin (0.3 µM) did not inhibit relaxations induced by acetylcholine in the absence of L-NOARG in the RHA (-log EC_{50} = 8.2 ± 0.1 and E_{max} = 99 ± 1 %, test; 8.4 ± 0.1 and 100 ± 0 %, control: n = 4). These findings are consistent with inhibitory effects on the smooth muscle rather than on the vascular endothelium.

The present study has shown that when combined with apamin, ciclazindol can substitute for charybdotoxin in antagonising the actions of EDHF in two different arteries. Further studies are in progress to determine which K-channel subtypes are sensitive to these inhibitor combinations.

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Table 1

	Rat hepatic artery					Guinea-pig basilar artery				
		-log EC_{50}		E_{max}			-log EC_{50}		E_{max}	
	n	control	treated	control	treated	n	control	treated	control	treated
Acetylcholine										
ciclazindol 10 µM	5	7.8 ± 0.2	6.6 ± 0.1*	96 ± 2	97 ± 2	7	6.4 ± 0.0	5.2 ± 0.2*	97 ± 1	38 ± 12*
ciclazindol 10 µM + apamin 0.1 µM (GBA) or 0.3 µM (RHA)	5	7.8 ± 0.2	†	96 ± 2	†	6	6.3 ± 0.0	†	97 ± 1	6 ± 4*
A23187										
ciclazindol 10 µM	4	6.0 ± 0.2	5.8 ± 0.2	84 ± 6	88 ± 8	5	6.2 ± 0.0	5.8 ± 0.0*	99 ± 1	80 ± 19
ciclazindol 10 µM + apamin 0.1 µM (GBA) or 0.3 µM (RHA)	5	6.1 ± 0.2	†	86 ± 5	†	4	6.3 ± 0.0	†	99 ± 1	3 ± 2*

*P < 0.05 compared to control (Student's *t*-test, two-tailed, unpaired). † Could not be calculated due to complete inhibition.

77P ROLES OF NITRIC OXIDE AND COX-METABOLITES IN PROLIFERATION OF RAT AORTIC SEGMENTS IN ORGAN CULTURE

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Certain cytokines or bacterial lipopolysaccharide (LPS) can induce inflammatory isoforms of nitric oxide (NO) synthase NOS (iNOS), and cyclo-oxygenase (COX; COX-2; Mitchell et al., 1995) in the rat aorta in organ culture (Bishop-Bailey et al., 1996). Mediators such as NO or prostanoids which elevate cGMP or cAMP have been suggested to have an anti-mitogenic effect of vessels in organ culture (Soyombo et al., 1995). In this study we have assessed the ability of interleukin-1β (IL-1) to induce the release of NO and COX metabolites. In addition we have investigated the roles of these mediators in the IL-1 - induced changes in vascular proliferation.

Organ culture methods and measurement of NO and prostaglandin (PG)E_2 release, were as previously described (Bishop-Bailey et al., 1996), with the following modifications. Using serum free DMEM, segments of rat aorta were incubated for 48h. Fresh medium and drugs were added at 0h and replaced at 24h. ^{3}H-thymidine (^{3}H-Tyd; 1μCi.ml^{-1}) was added at 24h, and incubated with the tissue for the next 24h period. The medium was then removed and the tissue washed twice with PBS, and blotted dry. The tissue was then weighed, and left to dissolve in soluene 350 for 24h. Total tissue ^{3}H-Tyd was then measured by liquid scintillation counting. The incorporation of ^{3}H-Tyd in segments of rat aorta over this time period was 10913±660 cpm.mg^{-1} (n=8). Using the method described by Angelini et al., (1985), we found that 68±2% (n=4) of the ^{3}H-Tyd was incorporated into the DNA fraction.

In parallel experiments performed in the absence of ^{3}H-Tyd, IL-1 (10ng.ml^{-1}) caused a co-release of NO (NOx nmols.ml^{-1}.mg^{-1}; 2.5±0.5 control ; 7.1±1.5* with IL-1) and PGE_2 (pmols.ml^{-1}.mg^{-1}; 0.04±0.04 control; 2.4±0.4* with IL-1). *=p<0.05 paired t-test. Table 1 summarises the effects of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME 1mM), the COX-1/ COX-2 inhibitor indomethacin (INDO 30μM), and the glucocorticoid steroid dexamethasone (DEX 1μM) on the incorporation of ^{3}H-Tyd in the rat aorta under control culture conditions and in the presence of IL-1. The data is expressed as percent of ^{3}H-Tyd incorporated under control culture conditions in the absence of any drugs (n=4-8).

Table1	Control	L-NAME	INDO	DEX
basal	100	89±21	125±19	34±6 *
IL-1	27±8 *	85±12	ND	28±4

*Table 1: Using one sample test, * =p<0.05 difference between basal vs treatments. ND= not determined*

Under basal conditions ^{3}H-Tyd was incorporated into the rat aorta in organ culture, probably through the proliferation of medial smooth muscle cells (Soyombo et al., 1990). This was inhibited by DEX but not by L-NAME or indomethacin. Similarly to LPS (Bishop-Bailey et al., 1996), exposure of rat aortic segments to IL-1 induced NO and PGE_2 release, presumably through iNOS and COX-2 induction. Furthermore IL-1 inhibited vascular proliferation, an effect which was reversed by L-NAME, but not DEX. These results suggest that the induction of iNOS and the subsequent release of NO directly inhibits proliferation of vascular cells and may play a protective role in the cardiovascular system.

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78P THAPSIGARGIN INHIBITS SMOOTH MUSCLE CELL PROLIFERATION AND INTIMA FORMATION BUT NOT METALLOPROTEINASE EXPRESSION IN HUMAN SAPHENOUS VEIN ORGAN CULTURE

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A principal cause of vein graft failure following coronary artery bypass grafting (CABG) is intimal thickening (IT) (Bryan and Angelini, 1994). Key events in IT are the proliferation and migration of vascular smooth muscle cells (VSMCs) of medial origin (purported to be mediated by extracellular matrix protein degradation by matrix metalloproteinases [MMPs]), to the intimal surface where they continue to proliferate and secrete matrix proteins (George et al., 1996). In turn, an increase in cytosolic calcium is believed to be an important trigger in mediating VSMC migration and proliferation. In order to investigate this area further, we studied the effect of thapsigargin (TH: elicits an increase in intracellular calcium) on neointima formation, VSMC replication and MMP expression in a human saphenous vein (SV) organ culture model.

Human SV segments were obtained from patients undergoing CABG and cultured as described previously (George et al, 1996). Intimal thickening was measured using histology and immunocytochemistry, VSMC proliferation using thymidine incorporation and autoradiography, toxicity by asessing intimal and medial VSMC cell death with in situ end labelling of fragmented DNA and lactate dehydrogenase (LDH) release and MMP (1, 2, 3 and 9) expression with zymography and immunocytochemistry. After 14 days in culture, neointimal thickness (NIT) and medial cell proliferation (MCP) was inhibited by TH in a dose dependent manner (table 1 [* $p < 0.05$]). The % of cells dying in the neointima (NCD) and the media (MCD) was not affected by 1, 5 and 10 nM TH, but were significantly greater than controls in the presence of 100 and 1000 nM TH (table 2 [* $p < 0.05$]). LDH release followed a similar pattern. These latter data indicate that the inhibitory effect of TH cannot be ascribed to cytotoxicity. At concentrations that inhibit intimal thickening, TH had no effect on the expression of MMPs .

These data demonstrate: 1) that thapsigargin is a potent inhibitor of neointima formation and medial VSMC proliferation, 2) intracellular calcium pools may play roles in controlling VSMC proliferation and migration, 3) thapsigargin has no effect on MMP expression and 4) caution should be exercised with the use and interpretation of drugs such as thapsigargin in this experimental setting (organ culture) since at higher concentrations it is cytotoxic. This, in turn could lead to spurious conclusions vis a vis inhibitory effects on parameters such as proliferation and growth.

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Table 1 Effect of TH on NIT and MCP (mean ± SEM; n =6)

TH (nM)	0	1	5	10	100	1000
NIT (μm)	22±3	19±4	14±2*	9±2*	0.1±0.1*	0.2±0.2*
MCP (%)	17±4	14±1	13±2	7±2*	2±1*	0±0*

Table 2. Effect of TH on NCD and MCD (mean ±SEM; n =6)

TH (nM)	0	1	5	10	100	1000
NCD (%)	4±1	1±0.3	5±3	5±2	30±9*	38±9*
MCD (%)	3±1	1±1	5±2	5±2	22±5*	33±9*

79P EVIDENCE FOR THE INVOLVEMENT OF POTASSIUM CHANNELS IN THE RELAXATION OF RAT ISOLATED MESENTERIC ARTERIES TO THE NO DONOR SIN-1

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Relaxation of isolated vascular preparations by nitric oxide (NO) is generally thought to be mediated by increases in cyclic GMP within the smooth muscle cells (Ignarro, 1990). However, it has recently been shown that NO can directly activate charybdotoxin (CTX)-sensitive potassium channels independently of cyclic GMP (Bolotina *et al.*, 1994). In the present study, the role of potassium channels in relaxation to the NO donor 3-morpholino-sydnonimine (SIN-1) was examined in rat isolated mesenteric arteries.

Male Wistar rats (250-300 g) were stunned and then killed by cervical dislocation. Segments of third order mesenteric artery (D_{100} 310 ± 10 μm; n=30) were mounted in a myograph under a normalised tension as previously described (Garland & McPherson, 1992). In some experiments the endothelial cell layer was removed mechanically or basal NO production was inhibited by incubating tissues with the NO synthase inhibitors, N^G-Nitro-L-arginine methyl ester (L-NAME) and N^G-Nitro-L-arginine (L-NOARG; both 100 μM; 30 mins). 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.

Application of SIN-1 (0.01-10 μM) caused concentration-dependent relaxation of arterial segments pre-constricted with phenylephrine (PE; 1-3 μM). SIN-1-evoked relaxation was not significantly different between endothelium-intact and denuded arterial segments or where basal NO production was inhibited (maximum relaxation 94.4 ± 1.3 %, 95.5 ± 0.95% and 93.5 ± 3.4 %, respectively n=8; p>0.05).

Exposure of endothelium-intact arterial segments to the potassium channel inhibitor charybdotoxin (CTX; 50 nM; 10 mins) significantly inhibited SIN-1-evoked relaxation reducing the maximum response to 9.8 ± 2.7 % (n=5; p<0.01). In contrast, in arterial segments in which either the endothelial cell layer had been removed or basal NO production inhibited, SIN-1-evoked responses were not significantly reduced in the presence of CTX (maximum relaxation 94.8 ± 3.5 % and 91.8 ± 2.3 %, respectively, n=6; p>0.05).

Pre-incubation with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10μM; 10 mins), a novel inhibitor of soluble guanylyl cyclase (Garthwaite *et al.*, 1995) or glibenclamide, did not generally alter SIN-1-evoked relaxation of PE-induced tone under any of the above conditions (n=5; p>0.05). However, in the presence of CTX and ODQ together, SIN-1-evoked relaxation of endothelium-denuded tissues and those exposed to NO synthase blockers, was abolished (n=6; p<0.01). Furthermore, in the presence of ODQ and glibenclamide together, SIN-1-evoked relaxation of intact tissues was attenuated and the maximal response reduced to 64.0 ± 6.0 % (n=6; p<0.01).

These data demonstrate that, although relaxation to the NO-donor SIN-1 is not significantly different between endothelium-intact and denuded tissues, the mechanisms mediating SIN-1-evoked relaxation of rat isolated mesenteric arteries appear to be modulated by the basal release of endothelium-derived NO. In the presence of an intact endothelial cell layer, the major mechanism for SIN-1-evoked relaxation is the activation of CTX-sensitive potassium channels. In contrast, when basal NO-production is inhibited, the SIN-1 appears to be able to cause full relaxation by both the activation of CTX-sensitive potassium channels and stimulation of soluble guanylyl cyclase.

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80P CHARACTERISATION OF POTASSIUM CURRENTS IN RAT PULMONARY ARTERIAL SMOOTH MUSCLE CELLS

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Single smooth cells were freshly isolated from segments of rat pulmonary artery using dithiothreitol and papain. When the cells were held at -90 mV under calcium (Ca)-free conditions, stepping for 500 ms to a series of test potentials (-80 mV to +50 mV) generated outward potassium (K) currents. These exhibited slow activation and inactivation kinetics characteristic of delayed rectifier currents ($I_{K(V)}$) in other smooth muscle cells. There was no evidence of a rapidly-activating and -inactivating A-type K-current.

The pharmacology of $I_{K(V)}$ was evaluated using tetraethylammonium (TEA) and 4-aminopyridine (4-AP), ciclazindol and terfenadine. From a holding potential of -90 mV, the degree of inhibition of $I_{K(V)}$ at the end of the 500 ms, 50 mV test pulse by these agents was 74 ± 6%, 48 ± 2%, 78 ± 9% and 48 ± 8% for terfenadine (500 nM), ciclazindol (10 μM), 4-AP (1mM) and TEA (1mM), respectively (n = 3 - 4).

When cells were held at -10 mV, $I_{K(V)}$ became inactivated and on stepping for 500 ms to the test potentials in the range -80 mV to +50 mV, non-inactivating currents were generated. Levcromakalim (10 μM) increased the amplitude of these currents (by 178 ± 34 pA at -10 mV, n = 4), an effect which was totally inhibited by glibenclamide (10 μM), indicating the involvement of the ATP-sensitive K-channel (K_{ATP}). Since K_{ATP} is believed to comprise an inwardly-rectifying K-channel coupled to a sulphonylurea binding site (Inagaki et al., 1995), cells were held at -10 mV and stepped to a series of potentials in the range -150 mV to -20 mV. With external K^+ concentrations of 4.7 mM and 20 mM, no evidence of any inwardly-rectifying current was obtained.

From a holding potential of -10 mV, stepping for 500 ms to test potentials more positive than 0 mV, NS1619 (Edwards et al., 1994) induced a noisy current with time-dependent activation properties. When measurements were made at the end of the 500 ms, 50 mV test pulse, NS1619 (33 μM) increased the current by 401 ± 73% (n = 3). This effect was totally inhibited by iberiotoxin (250 nM), indicating that the increased current was carried by the large conductance, Ca-sensitive K-channel, BK_{Ca}. As previously reported in rat portal vein (Edwards et al.,1994), NS1619 (33 μM) simultaneously inhibited $I_{K(V)}$ (by 71 ± 3%, n = 3, measured at +50 mV).

This study has identified the presence of $I_{K(V)}$, $I_{BK(Ca)}$ and $I_{K(ATP)}$ in the rat pulmonary artery. The sensitivity of $I_{K(V)}$ to terfenadine may suggest some similarities between K_V in the pulmonary vasculature and Kv1.5 cloned from human heart (Yang et al., 1995). In pulmonary artery, the presence of $I_{K(ATP)}$ coupled with the absence of an inwardly-rectifying K-selective conductance is in agreement with earlier studies in rat and human bladder (Green et al., 1994, 1995). If K_{ATP} in these tissues is indeed an inwardly-rectifying K-channel, its apparent absence from these rat and human smooth muscles is surprising. Further studies are underway to clarify this anomaly.

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81P PREVENTION OF SMALL BOWEL ALLOGRAFT REJECTION WITH CYCLOSPORINE AND BUDESONIDE, A LOCALLY ACTING GLUCOCORTICOID

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Potent systemic immunosuppression is required to prevent rejection of small bowel transplants (SBT), Consequently, recipients have a high rate of infection and post-transplant lymphoproliferative disease. Most cyclosporin (CsA) immunosuppression protocols for SBT include glucocorticoids which have short and long-term systemic complications. Budesonide (BUD), a locally acting steroid which is almost completely metabolised during first-pass, was used in the present study to see if it would prevent graft rejection in rats without producing undesirable systemic effects.

Materials and Methods: Orthotopic SBT with portal-venous return was performed in LEW rats with BN allografts[1]. They were grouped as follows: 1) no drug treatment; 2) BUD, 0.1 mg/kg/day, PO; 3) BUD, 1 mg/kg/day, PO; 4) CsA, 2 mg/kg, SC (a subtherapeutic dose); 5) CsA, 2 mg/kg, SC + BUD, 0.1 mg/kg/day PO and 6) CsA, 2 mg/kg, SC + BUD, 1 mg/kg/day PO. Additional non-transplanted LEW rats were given prednisolone (4 mg/kg/day) as positive controls for systemic steroid effects.
Animal survival, adrenal and spleen weights normalised to body weight, and plasma ACTH and CsA levels were compared between groups. Results are expressed as means (±SE).

Group	n	Survival (days)	Spleen (mg/g)	Adrenal (mg/g)	ACTH (pmol/L)	CsA (ng/mL)
Untreated	7	9.7±0.3	2.1±0.2	0.21±0.03	56±13.2	
BUD(0.1mg)	6	9.1±0.4	1.8±0.2	0.20±0.02	45±11.0	
BUD(1mg)	6	11±0.8	1.6±0.1	0.19±0.02	45±8.6	
CsA (2mg)	7	15±1.5*	3.1±0.8	0.22±0.03	42±8.5	20±2.0
BUD(0.1mg) +CsA(2mg)	6	28±5.3*°	5.1±0.6	0.19±0.02	27±2.2*	44±10.6°
BUD(1mg) +CsA(2mg)	6	37±7.9*°	3.1±0.6	0.18±0.03	28±10.2*	59±8.2°
Pred(4mg)	6	n/a	1.4±0.2	0.10±0.01	12±0.9*	

p<0.5 (*) vs untreated; (°) vs CsA alone

Conclusions: Budesonide with subtherapeutic doses of CsA significantly prolong small bowel allografts with fewer systemic side-effects. Graft survival may be partly due to increased plasma CsA resulting from coadministered BUD; both drugs are substrates for CYP-3A4. The results suggest that locally acting glucocorticoids have advantages over the systemic steroids currently used for clinical SBT.

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82P MODULATION OF RAT SYNOVIAL BLOOD FLOW BY THE CGRP RECEPTOR ANTAGONIST, CGRP (8-37)

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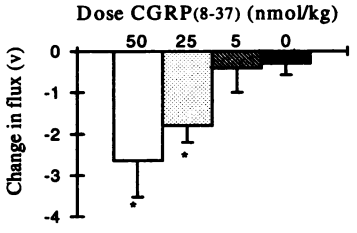
Unmyelinated afferent nerves innervating synovial joints are the primary source of neuropeptides, such as calcitonin gene-related peptide (CGRP), a potent vasodilator in several species, including man (Brain & Williams, 1985; Mapp *et al.*, 1990). Here, we investigate the effects of CGRP and the GCRP receptor antagonist, ($CGRP_{(8-37)}$) on synovial blood flow in the rat knee joint.

Male Wistar rats (250-400g) were anaesthetised with urethane (2.5g/kg, i.p.). A carotid artery was cannulated for measurement of mean arterial pressure and the skin over the knee was removed to expose the medial aspect of the joint. Drugs were applied to the surface of the joint (50 or 100 µl bolus). Changes in flux were measured by laser Doppler perfusion imaging and results expressed as changes in voltage (V).

Topical application of CGRP (0.01, 0.1 and 1 nmol/kg) induced a dose-dependent increase in synovial blood flow which, was attenuated by co-administration of the CGRP antagonist, $CGRP_{(8-37)}$ (5 nmol/kg). For example, 1 nmol/kg CGRP elicited an increase in flux of 2.11±0.5 V and 0.92±0.3 V in the absence and presence of $CGRP_{(8-37)}$, respectively (n=6-7, p<0.05). This dose of $CGRP_{(8-37)}$ had no effect on basal blood flow. In contrast, 25 or 50 nmol/kg $CGRP_{(8-37)}$ elicited a significant reduction in basal synovial blood flow (n=7-8; p<0.05) (see Figure 1).

Figure 1: $CGRP_{(8-37)}$ attenuates rat synovial blood flow



The vasoconstriction induced by 50 nmol/kg $CGRP_{(8-37)}$ was maintained for up to 60 min while, in rats receiving the lower dose (25 nmol/kg) of the antagonist blood flow had returned to resting levels after 20 min (n=7-8; p<0.05).
Thus, in the rat synovium, $CGRP_{(8-37)}$ at low doses (5 nmol/kg) attenuates the vasodilator actions of exogenously applied CGRP, without altering resting tone. However, higher doses (25 or 50 nmol/kg) of $CGRP_{(8-37)}$ elicit a marked fall in resting blood flow. These results suggest a role for CGRP in the regulation of blood flow in the rat knee joint.

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83P DIBUTYRYL cAMP INCREASES RESPONSIVENESS TO A B_1 BRADYKININ RECEPTOR AGONIST IN PRIMARY CULTURES OF RAT URINARY BLADDER SMOOTH MUSCLE CELLS

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Bradykinin (Bk) and its carboxypeptidase metabolite desArg^{9}Bk mediate their actions through two classes of receptor. DesArg^{9}Bk is selective for the B_1 receptor and Bk is selective for the B_2 receptor. A feature of the B_1 receptor is its ability to be upregulated under certain conditions (Deblois et al., 1988). In this study we have isolated smooth muscle cells from the rat urinary bladder and investigated expression of B_1 and B_2 receptors. Bladders were removed from Sprague-Dawley rats and subjected to enzymic digestion (Levesque et al., 1993). The isolated cells were cultured for up to 9 days in Medium 199 plus 10% foetal calf serum. The cells were confirmed as smooth muscle (90%) using an antibody to smooth muscle actin. Agonist-stimulated ^{45}Ca efflux (Smith et al., 1995) was used as a measure of receptor activation. Results have been expressed as the rate of ^{45}Ca efflux during a 1min application of agonist minus the mean rate in the 2min preceding agonist application. Bk stimulated ^{45}Ca efflux from bladder smooth muscle cells with an EC_{50} value of 2.9 ± 0.7nM (n=3). The ^{45}Ca efflux induced by 3nM Bk was inhibited by HOE140 (a B_2 antagonist) with an IC_{50} value of 1.8 ± 0.5nM (n=3) but was unaffected by 10μM desArg^{9}Leu^{8}Bk (a B_1 antagonist). The net increase in ^{45}Ca efflux evoked by a maximal concentration of desArg^{9}Bk (1μM) was unaffected by HOE140 (up to 100nM) but completely blocked by 10μM desArg^{9}Leu^{8}Bk. The EC_{50} value for desArg^{9}Bk-evoked ^{45}Ca efflux (in the presence of 30nM HOE140) was 43.4 ± 7.6nM (n=5). DesArg^{9}Bk-evoked ^{45}Ca efflux increased with time in culture. The net increase in the rate of efflux in response to desArg^{9}Bk (1μM) at 8 days (0.093 ± 0.012 min^{-1}, n=7) was significantly greater ($p<0.01$) than at 1 day (0.043 ± 0.008 min^{-1}, n=9). The response to Bk also increased with time in culture; at 8 days the increase in ^{45}Ca efflux evoked by 3nM Bk (0.129 ± 0.016min^{-1}, n=5) was significantly ($p<0.05$) greater than the response at 1 day (0.064 ± 0.012min^{-1}, n=5). The basal efflux was the same at 8 days (0.040 ± 0.006min^{-1}, n=5) and 1 day (0.049 ± 0.011min^{-1}, n=5). Treatment of 7-9 day old cells with 1mM dibutyryl cAMP (dbcAMP) for 4h increased ($p<0.05$) the response to a maximal concentration of desArg^{9}Bk (1μM) from 0.114 ± 0.023min^{-1} (n=8) in naive cells to 0.195 ± 0.035min^{-1} (n=8) in treated cells, with no effect ($p>0.2$) on the EC_{50} value (29.7 ± 6.6nM, n=3). Cycloheximide prevented the increase in responsiveness to desArg^{9}Bk. The response to desArg^{9}Bk (1μM), in cells treated with a combination of 70μM cycloheximide and 1mM dbcAMP for 4h (0.067 ± 0.022min^{-1}, n=3), was not significantly different ($p>0.2$) from the response in naive cells. This suggests that protein synthesis was required for the effect of dbcAMP. The ^{45}Ca efflux induced by Bk (3nM) following treatment with 1mM dbcAMP for 4h (0.214 ± 0.042min^{-1}, n=3) was no greater than in untreated cells (0.173 ± 0.048min^{-1}, n=3). DbcAMP was also without effect on the response to a maximal concentration of Bk (100nM) (data not shown). Basal ^{45}Ca efflux was the same in dbcAMP-treated cells (0.036 ± 0.007min^{-1}, n=5) and naive cells (0.038 ± 0.004min^{-1}, n=5). In summary, cultured bladder smooth muscle cells express a mixture of B_1 and B_2 receptors. Responsiveness to both B_1 and B_2 agonists increased with time in culture. Treatment with dbcAMP increased responsiveness to the B_1 agonist desArg^{9}Bk but not to the B_2 agonist Bk.

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84P THE EFFECTS OF SILDENAFIL, AN INHIBITOR OF TYPE 5 cGMP PHOSPHODIESTERASE, ON cGMP AND cAMP LEVELS IN RABBIT CORPUS CAVERNOSUM, *IN VITRO*

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Nitric oxide (NO) plays a key role in mediating penile erection and impairment of its release has been implicated in the pathophysiology of erectile dysfunction (Finberg *et al.*, 1993). Sildenafil (VIAGRA™), a potent selective inhibitor of type 5 cGMP phosphodiesterase (PDE5), enhances neuronal NO-dependent relaxation of human corpus cavernosal smooth muscle (Ballard *et al*, 1996). Sildenafil has also proved effective in the treatment of erectile dysfunction following oral administration in man (Gingell *et al*, 1996). Since the action of NO is mediated by activation of the synthesis of cGMP, the clinical efficacy of sildenafil is likely to result from inhibition of cGMP breakdown. In order to investigate further the mode of action of sildenafil, its effects on cGMP and cAMP levels in the corpus cavernosum of the rabbit were investigated. Responses were compared to those obtained with papaverine, which is widely used therapeutically as an erectogen via intracavernosal injection (Virag, 1982).

Male new Zealand White rabbits (2.5kg) were killed with an overdose of pentobarbitone and their penes rapidly excised and cut into discs. Following preincubation in Dulbecco's Minimum Essential Medium (DMEM) to allow the effects of preparative handling to subside, penile discs were then incubated with varying concentrations of sildenafil or papaverine (from 1x10^{-8}M to 1 x 10^{-5}M) and cGMP synthesis stimulated with increasing concentrations of sodium nitroprusside (NaNP). Following incubation at 37°C for 30 min, cGMP and cAMP were then extracted with perchloric acid, the supernatants neutralised, acetylated and nucleotides measured by radioimmunoassay as previously described (Miller *et al*, 1994).

In the presence of 1μM NaNP, sildenafil enhanced the rate of accumulation of cGMP in rabbit penile tissue in a dose-dependent manner (Table 1), the EC_{50} for sildenafil at this concentration of NaNP being 440 nM. cAMP concentrations were unaltered by sildenafil (up to 1 x 10^{-4}M). In the absence of NaNP, sildenafil increased the rate of accumulation of cGMP from 0.8 ± 0.05 fmoles/mg tissue/min to 3.9 ± 0.1 fmoles/mg tissue/min; the EC_{50} being 1.3μM. Papaverine also enhanced cGMP accumulation in response to 1μM NaNP, but at much higher concentrations than sildenafil (EC_{50} > 10 μM).

The finding that sildenafil increases cGMP, but not cAMP, accumulation in corpus cavernosal tissue is consistent with its known profile as a potent inhibitor of cGMP hydrolysis catalysed by PDE5 (IC_{50} 4nM, Ballard *et al*, 1996). It is concluded that the enhancement of NO-dependent relaxation of corpus cavernosal smooth muscle elicited by sildenafil *in vitro* is mediated by a specific elevation of cGMP. *In vivo*, sildenafil may therefore enhance penile erection via augmentation of NO-stimulated cGMP-dependent pathways leading to cavernosal smooth muscle relaxation.

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Table 1. Effect of sildenafil on NaNP (1μM)-stimulated accumulation of cGMP in corpus cavernosum (fmoles/mg tissue/min; mean ± SEM; n=6).

Sildenafil (M):	0	1 x 10^{-8}	1 x 10^{-7}	3 x 10^{-7}	1 x 10^{-6}	3 x 10^{-6}	1 x 10^{-5}
cGMP	0.8 ± 0.1	1.2 ±0.1	5 ± 0.05	7.7 ± 2	10.8 ± 1	22.1± 1.3	25.6 ± 3.5