71P OXIDATIVE STRESS INDUCES INTRACELLULAR RELOCATION OF THE THROMBOXANE RECEPTOR IN COS-7 CELLS

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8-Iso-prostaglandin $F2_\alpha$ (8-iso-$PGF2_\alpha$), a member of the recently discovered family of isoprostanes, is produced *in vivo* by cyclooxygenase-independent, free-radical-catalysed lipid peroxidation (Patrono *et al.*, 1997). It has been shown that 8-iso-$PGF2_\alpha$ acts as a potent vasoconstrictor in the oxidatively-stressed isolated perfused rat heart by an action on the thromboxane A_2 receptor (TXA_2R) (Kromer *et al.*, 1999). The aim of our study is to establish the effect of oxidative stress on TXA_2R.

TXA_2R β isoform GFP-tagged at the C-terminus was transiently transfected into COS-7 cells using a pcDNA 3.1/CT-GFP vector. Quantification of the TXA_2R and determination of its half-life were determined by radioligand binding assays and Western blot respectively. Using immunofluorescence microscopy, we also determined the effect of oxidative stress on the intracellular localisation of the TXA_2R reserve, by quantification of the fluorescence of the GFP.

Immunofluorescence suggested that the presence of H_2O_2 (10 μM, 40 min) increased the trafficking of the TXA_2R from the endoplasmic reticulum to the Golgi complex (oxidative stress increased the presence of the TXA_2R in the Golgi complex by 1.8 ± 0.2 fold, n=25, $P < 0.05$ by Student's t test). H_2O_2 treatment increased the TXA_2R antagonist ($[^3H]SQ29548$, 10^{-8} M) binding on plasma membrane (2235± 260 *vs* 1353 ± 230 cpm/mg protein, n=3, $P < 0.05$ by Student's t test). The degradation of TXA_2R following cycloheximide treatment (200 μg, 3 h), a protein synthesis inhibitor, was reversed in the presence of H_2O_2.

These results support the possibility that oxidative stress induces the maturation and the stabilisation of the TXA_2R protein probably by intracellular translocation. This mechanism could increase the quantity and/or ligand affinity of the TXA_2R on the plasma membrane and explain the potentiation of the 8-iso-PGF2-induced vasoconstriction during oxidant injury.

Supported by the British Heart Foundation

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72P DOES ION TRAFFICKING CONTRIBUTE TO SURVIVAL UNDER SEVERE STRESS IN THE EUKARYOTIC CELL?

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The universality of basic cellular and molecular mechanisms made yeast a model system for the pharmacological investigation of the cellular stress response (Tiligada *et al.*, 2002). We sought to establish whether microenvironmental conditions originating from protected cells enable non-pretreated cells to withstand severe stress. Since membrane components may play a critical role (van der Rest *et al.*, 1995) a preliminary insight into the ionic contribution in triggering and responding to this protection was also attempted.

Late-logarithmic phase growing cells of *Saccharomyces cerevisiae* ATCC 2366 acquired tolerance to heat shock (HS, 53°C, 30min), following heat pretreatment at 37°C for 2h (Tiligada *et al.*, 1999). After washing, the cell-free pretreated-culture supernatant (S) was transferred to the non-pretreated cell precipitate (P) and exposed to HS. Survival under acute HS, mediated by ion-coupled cellular processes was investigated by incubating non-pretreated cells in the presence of amiodarone (AMI), omeprazole (OME), tetraethyl-ammonium ions (TEA) (Sigma, USA) and mepivacaine (MEP, Orgamol, Switzerland), either for 2h before or during HS (Figure 1). Cell viability was subsequently determined under the light microscope, using the vital exclusion dye methylene blue. Culture pH was recorded at all times. Statistical analysis was performed by ANOVA followed by Dunnett's test.

S conferred thermotolerance to P upon exposure to severe HS (Figure 1). Significant induction of thermotolerance was observed when the proton pump inhibitor OME was added in the cultures either for 2h before or during HS. AMI

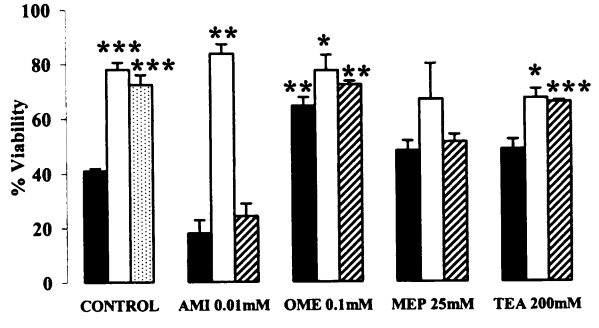

Figure 1. Effect of drugs on non-pretreated (closed bars) and heat pretreated (open bars) cells and on heat shock (stripped bars). Dotted bar denotes P receiving S. Data are expressed as mean±SEM of % viable cells in each culture. $*P<0.05$, $**P<0.01$ $***P<0.001$ *vs* control non-pretreated cells (n=3-20)

marginally reduced viability of P, but this effect was not significant. OME had no effect on pretreated cells. Blockade of Na^+ channels with MEP induced no significant alterations in viability of P, but blockade of K^+ channels with TEA led to thermotolerance induction (Figure 1).

In conclusion, eukaryotic cells are able to survive a severe stress under favourable microenvironmental conditions, partly arising from the intra- and extra-cellular ionic homeostasis.

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Tiligada, E. *et al.* (1999). *Lett. Appl. Microbiol.* **29**, 77-80.
van der Rest, M.E. *et al.* (1995). *Microbiol. Rev.* **59**, 304-322.

73P THE USE OF NEBULISED CADMIUM CHLORIDE TO DEVELOP ANIMAL MODELS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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We have previously shown that nebulised cadmium chloride ($CdCl_2$) induces inflammatory cell recruitment and elevated Penh (enhanced pause, an index of bronchoconstriction, Hamelmann E. *et al.* 1997) in Sprague-Dawley rats (Vincke *et al.*, 2002). The aim of the present study was to continue this work with a view to the development of a novel, accurate model of chronic obstructive pulmonary disease (COPD) in rats and mice. The effects on inflammatory cell proliferation, cytokine levels and emphysema were investigated.

Male Sprague-Dawley rats (200-400g) were exposed to a 1 h nebulisation of 0.9% (w/v) NaCl (control) or 0.1% (w/v) $CdCl_2$ (treated) on days 1 and 3. After anaesthetising the animals with sodium pentobarbital (60 $mgkg^{-1}$, i.p.) bronchoalveolar lavage (BAL) was performed at 0, 1, 2 and 4 h timepoints. Differential cell counts and enzyme-linked immunosorbent assays (ELISAs) for inflammatory cytokines were carried out on the BALf.

Male BL/Jlco mice (20-25g) were exposed to two nebulisations as described for rats. BAL was taken immediately following the second nebulisation and analysed for macrophage inflammatory protein 2 (MIP-2) content by ELISA. The experiment was repeated with a third nebulisation on day 6, immediately after which BAL was performed for KC (murine IL-8 homologue) ELISA and differential cell counts. In a further experiment mice were exposed to 3 nebulisations per week for 3 weeks, straight after which the lungs were excised and fixed in formol for histological analysis. Results were given as a subjective report by an experimenter unaware of the treatments given.

Data was analysed by Student's *t* test, with a *P* value of less than 0.05 being considered significant.

$CdCl_2$ induced a significant increase in lymphocyte, neutrophil and eosinophil numbers detected in rat BAL fluid at all timepoints tested ($P<0.05$, $n=5$). However it did not affect levels of interleukin-6 (IL-6) or tumour necrosis factor α (TNFα). A significant increase was observed in cytokine-induced neutrophil chemoattractant 1 (CINC-1) at all timepoints ($P<0.05$, $n=5$), peaking at 1h, although levels remained approximately 15% of those induced by nebulised lipopolysaccharides (unpublished data).

No significant change in KC levels were observed despite an increase in lymphocyte and macrophage numbers in the treated mice ($P>0.05$, $n=4$). MIP-2 levels also remained similar in the treated and control groups although there were elevated neutrophil numbers in BAL from the $CdCl_2$-treated animals.

Histological analysis revealed evidence of emphysema in the lungs of the treated mice but not in the control animals.

The present study has confirmed that nebulised $CdCl_2$ can cause inflammatory cell influx in the lungs of the species tested, although this was unlikely to be induced by IL-6, TNFα, KC or MIP-2. CINC-1 levels were increased by $CdCl_2$ and may play a role in the mechanism. $CdCl_2$ also induced some emphysematous changes in mice.

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74P THE EFFECT OF A CANNABINOID (CB_2) AGONIST ON ACTIVATION OF AIRWAY SENSORY NERVES IN VITRO AND THE COUGH REFLEX

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Cough is initiated by activation of vagal sensory nerve endings in the airways via a central reflex pathway. Sensory nerve activity may be enhanced in inflammatory lung diseases such that the cough reflex becomes exacerbated. *In vivo* data suggests that sensory nerve activity is modulated by cannabinoid (CB) receptor activation (Harris *et al.*, 2000). CB_1 receptors are distributed on central and peripheral nerves, whereas CB_2 receptors are expressed in the periphery.

We studied the effects of the CB_2 receptor agonist, JWH 133 (Huffman et al., 1999) on (i) capsaicin-induced sensory nerve depolaristaion in guinea-pig and human vagus nerve *in vitro* and (ii) on citric acid-induced cough in concious guinea pigs. Male Dunkin Hartley guinea pigs (350-450g) were used in animal experiments. Depolarisation of guinea-pig or human vagus nerve was measured using a 'grease-gap' technique as previously described (Birrell *et al.*, 2002). After control responses to capsaicin (1μM, 4 min) tissues were treated with JWH 133 (0.3 – 10μM) or vehicle for 20 min. Capsaicin was reapplied in the continued presence of JWH 133. In some tissues, the CB_1 antagonist SR141716A, the CB_2 antagonist SR144528 (both at 0.01μM) or vehicle (0.1% DMSO) were perfused for 10 min prior to JWH 133 (3μM). For cough experiments, vehicle (0.5% methylcellulose with 0.2% Tween 80 in saline, i.p.) or JWH 133(10 mg/kg i.p.) was dosed for 20 min prior to exposure to aerosolised citric acid (0.3M) for 10 min during which time the number of coughs were counted.

Data from *in vitro* studies ($n=4$) and cough studies ($n=8$) were analysed using a paired and unpaired t-test respectively (** $p<0.01$). Capsaicin-induced depolarisations of 0.44 ± 0.07 mV and 0.19 ± 0.05 mV in guinea pig and human vagus nerve respectively. JWH 133 inhibited this response in guinea-pig vagus nerve (Fig. 1A). The inhibitory action of JWH 133 (3μM) was abolished in the presence of SR144528 (8.5 ± 2.6 % ↑) and unaffected by SR141716A (55.2 ± 1.7 % ↓). JWH 133 (10μM) inhibited capsaicin-induced depolarisations in human vagus nerve (40%, $n=1$). This was completely blocked by SR144528 (n=1) and was unaffected by SR141716A (46.6% ↓, $n=1$). JWH 133 also inhibited citric acid-induced cough in guinea pigs by 53.7%; Fig. 1B).

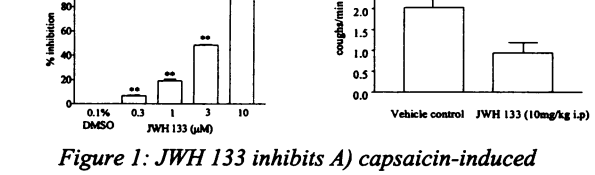

Figure 1: JWH 133 inhibits A) capsaicin-induced depolarisation in guinea-pig vagus nerve and B) citric acid-induced cough in guinea pigs.

Hence, CB_2 receptor agonists, devoid of central activity, may have therapeutic potential for the treatment of cough.

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75P PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ) AGONISTS: A POSSIBLE THERAPY FOR CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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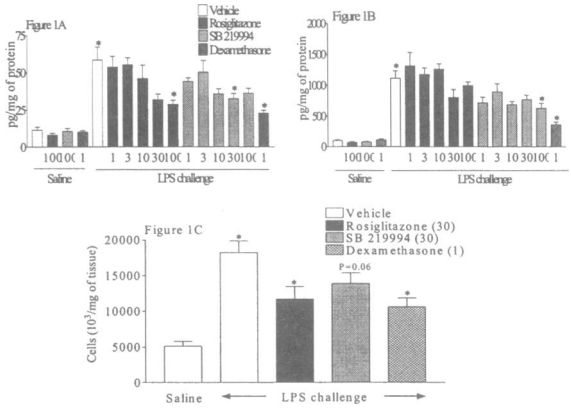
COPD remains one of the leading causes of morbidity and mortality in many parts of the world. Currently no drugs are available to control or prevent the progression of COPD and so there is an urgent need to develop disease-modifying agents. COPD is characterised by a chronic inflammatory process in the lung that includes increased numbers of inflammatory cells (including neutrophils) in the airways and parenchyma (Barnes, 1999). It is believed that these inflammatory cells are involved in the development and progression of the disease (Barnes, 1999). We have previously shown PPARγ agonists to inhibit the release of inflammatory cell survival factors (including granulocyte-colony stimulating factor, G-CSF) and induce apoptosis *in vitro* (Patel *et al*, 2001). The aim of this study was to determine the effect of two structurally different PPARγ agonists in an *in vivo* model of COPD-like inflammation.

Male Balb/c mice (18-20 gm, n=8) were orally dosed with vehicle, the PPARγ agonists rosiglitazone (1, 3, 10, 30 or 100 mg kg^{-1}), SB 219994 (1, 3, 10, 30 or 100 mg kg^{-1}) (Murphy & Holder, 2000) or dexamethasone (1 mg kg^{-1}) one-hour prior to challenge with aerosolised saline or LPS (0.01 mg ml^{-1} for 30 minutes). The mice were sacrificed with an overdose of pentobarbitone (200 mg kg^{-1} i.p.) 3 hours after challenge and the lungs removed and flash frozen. Supernatant from the lung tissue homogenate was assessed for G-CSF and KC (murine IL-8) content by ELISA in accordance with manufactures instructions. The protocol for the second experiment was as before except the dose of PPARγ agonists was 30 mg kg^{-1} and the end point was neutrophil recruitment into the lung tissue using a method by Underwood *et al* (1997). The data was analysed using one-way ANOVA with the appropriate post-test. A p value of less than 0.05 was considered to be significant (* = $p < 0.05$ compared to relevant control).

Figure 1: Effect of PPARγ agonist (mg/kg, p.o.) on G-CSF (a) and KC (b) and neutrophil (c) levels in the lung tissue.



We found that treatment with Rosiglitazone or SB 219994 inhibited LPS-induced neutrophilia and the associated increase in chemoattractants/survival factors (KC and G-CSF) in the lung (Figure 1). We postulate that if a PPARγ agonist has the same effect in man and inflammation is important in the progression of COPD then a compound with this profile could be a potential therapy.

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76P EFFECTS OF A NOVEL NO-RELEASING GLUCOCORTICOID (NCX-1015) IN A RAT MODEL OF GOUT

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Glucocorticoids (GC) are highly effective in controlling chronic inflammatory diseases such as arthritis. However, their long-term administration results in a considerable array of side effects, most notably secondary osteoporosis. We have recently described a novel GC that is the nitro-releasing derivative of prednisolone, termed NCX-1015 (Paul-Clark *et al.*, 2000). Here we examined the anti-arthritic properties of this compound in relation to the derivative deprived of nitric oxide (NO), termed NCX-1016, and prednisolone.

Male Sprague Dawley rats (220-250 g) were pre-treated intraperitoneally with peanut oil (vehicle), prednisolone, NCX-1015 (prednisolone 21-[4'-(nitrooxymethyl)benzoate) or with NCX-1016 (prednisolone 21-[4'-(oxymethyl)benzoate) at doses of 1 and 4 μmolkg^{-1}. One hour later, animals were anaesthetized and the right knee injected intra-articular (i.a.) with 50 μl of MSU crystals (20 mg/ml) with the left knee receiving 50 μl PBS. After 16 h, the knee joints were exposed, joint size measured callipers, and the arthritic score (from 0= normal to 4= severe damage) calculated. Sixteen hours later, knee joints were lavaged with 1 ml PBS containing 3 mM EDTA. Cell-free supernatants were analysed for interleukin (IL)-1β and IL-6 contents (R&D Systems, Abingdon, UK). The cell pellet was re-suspended in 100 μl Turk's solution for differential cell counts (Neubauer haemocytometer): the majority (>90%) of cells recovered were neutrophils (NØ). Data were analysed (mean ± s.e.mean of n rats per group) by ANOVA and Bonferroni test with $P<0.05$ taken as significant.

Injection of MSU crystals induced an intense inflammatory response as measured at the 16 h time-point, with values of cell migration of 1.73 ± 0.18 x10^5 NØ vs. a PBS-injected knee value of 0.2 ± 0.01 x10^5 NØ (n=10, $P<0.05$). Cell influx was paralleled by macroscopic evaluation of the joints, with an increase in joint size of 0.32 ± 0.05 mm and a clinical score of 1.51 ± 0.29 arbitrary units (n=10). Regarding soluble mediators, high contents of IL-1β and IL-6 were measured in knee joints injected with MSU crystals (16.64 ± 2.52 and 61.8 ± 2.40 $pgml^{-1}$ respectively) compared to PBS-treated joints, whose levels were below detection. Treatment of rats with 1 μmol kg^{-1} NCX-1015 brought all inflammatory parameters back to control levels (n=11). NCX-1015 (1 μmol kg^{-1}) reduced IL-1β levels to 1.2 ± 0.2 $pgml^{-1}$ (95% inhibition) and IL-6 below detection. In contrast, 1 μmol kg^{-1} prednisolone and NCX-1016 produced significant reduction of NØ influx (45% and 53% inhibition, respectively, n=6, $P<0.05$), with no significant effect on clinical score and joint size. IL-1ß levels in cell-free exudates were reduced by 54% and 72% following prednisolone and NCX-1016 treatment (n=6, $P<0.05$), whereas IL-6 levels were only affected by NCX-1016. At the higher dose of 4 μmolkg^{-1} all GC abrogated MSU crystal induced inflammation, diluting in this manner potential differences amongst the treatments.

In conclusion, NCX-1015 has higher anti-inflammatory properties than prednisolone and NCX-1016 in a model of gouty arthritis. Coupled to recent data obtained in a model of rheumatoid arthritis (Paul-Clark *et al.*, 2002), these findings indicate that this nitro-releasing GC could be developed as a novel treatment for several forms of arthritis.

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77P EFFECTS OF PROTEIN KINASE C INHIBITORS ON EQUINE EOSINOPHIL ADHERENCE AND SUPEROXIDE PRODUCTION

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Equine eosinophils produce superoxide anions when stimulated with serum treated zymosan (STZ) or histamine (Foster & Cunningham, 1997). Histamine, as well as human recombinant (hr)IL-5, also causes adherence of these cells to serum-coated plastic and protein kinase C (PKC) has been implicated in histamine-induced superoxide production but not adherence (Foster et al, 1997; Greenaway et al, 2002). In the present study the effects of PKC inhibitors on STZ-induced superoxide production and hrIL-5-induced adherence by eosinophils from normal ponies have been examined.

Eosinophil superoxide production and adherence were measured colorimetrically as described previously (Foster & Cunningham, 1997; Foster et al., 1997). The effects of the non-selective PKC inhibitor, Ro31-8220 (Ro; n=3), the conventional PKC inhibitor Gö6976 (Gö; n=6) and rottlerin, which inhibits novel PKCδ (Rott; n=5-7), on STZ (333.3μg/ml)-induced superoxide generation were studied. The effects of Ro (n=5), Gö (n=5) and Rott (n=5-7) on hrIL-5 (10^{-8}M)-induced adherence were then examined. Results are expressed as means±SEM and the effects of PKC inhibitors examined by 2-way ANOVA and Bonferroni's post hoc test.

STZ-induced superoxide production was significantly reduced by Ro and Gö. In contrast, Rott (10μM) significantly increased this response (Table 1). HrIL-5-induced adherence was not affected by Ro, as found previously using histamine (Greenaway et al., 2002). However Gö caused a significant reduction in adherence and Rott had no effect (Table 2).

Table 1: Effect of PKC inhibitors on STZ-induced superoxide production (nmol reduced cytochrome C/10^6 cells)

control	STZ	Concentration (μM)				
1.4±1.4	44.8±3.7	Ro	0.1	1	3	10
			54.9 ±1.1	54.8 ±5.1	33.6 ±5.4	#3.0 ±1.9
-0.1±1.5	55.2±6.6	Gö	0.02	0.05	0.2	0.5	2
			51.9 ±5.6	42.9 ±4.9	*24.1 ±6.5	*6.8 ±4.4	*-2 ±2.6
-0.9±1.9	42.7±5.0	Rott	1		10	
			54.1±6.4		*108.8±10.3	

Table 2: Effect of PKC inhibitors on hrIL-5 induced adherence (% cells added initially to each well)

control	hrIL-5	Concentration (μM)			
2.0±0.8	8.9±2.2	Ro	1	3	10
			10.3±2.4	9.2±1.8	7.1±2.0
2.0±0.8	8.9±2.2	Gö	0.02	0.05	0.2	0.5
			6.4±2.1	#4.1±1.9	*1.6±0.6	*1.7±0.7
1.2±0.2	11.1±0.9	Rott	1	5	10
			11.7±0.8	12.7±1.2	7.8±1.7

= p<0.05; * = p <0.001 versus STZ or hrIL-5

This study suggests that PKC is involved in both STZ-induced superoxide production and hrIL-5-induced adherence by equine eosinophils. Furthermore, individual PKC isotypes may be differentially involved in the regulation of both responses, with a stimulatory function suggested for conventional PKCs.

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78P THE EFFECT OF A NUMBER OF INFLAMMATORY MEDIATORS ON THE HUMAN EOSINOPHIL SHAPE CHANGE RESPONSE

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Eosinophils are thought to play a role in allergic inflammatory diseases. However, pharmacological studies of the effects of inflammatory mediators on eosinophils are hampered by their low abundance in peripheral blood. In humans, studies tend to be performed on hypereosinophilic donors and require lengthy isolation procedures which can compromise cell responsiveness (Casale *et al.*, 1999). Sabroe *et al.* (1999) have measured the changes in leukocyte morphology which precede chemotaxis using a flow cytometric technique to study the effects of a variety of chemokines on immune effector cells. This technique allows the response of eosinophils to be measured in a mixed cell population removing the need for extensive cell purification. The aim of this study was to investigate the applicability of this technique by studying the effects of a number of inflammatory mediators on human eosinophils.

Shape change assays were performed essentially as described in Sabroe *et al.* (1999). Concentration-response curves were constructed to C5a, formyl-methionyl-leucyl-phenylalanine (fMLP), eotaxin, histamine and leukotrienes B_4 and D_4 (data are summarised in table 1). C5a was the most potent agent tested in this study. With eotaxin, it also caused the largest proportion of cells to change shape. Concentration-response curves to eotaxin and histamine were markedly bell-shaped (data in table 1 are for the stimulatory phase of the response to these agonists). The highest concentration used of each agonist (100 nM and 10 μM, respectively) produced a significantly lower response than the peak value. The peak eotaxin response was 52.0±2.2% of cells responding (sem, n=18) whilst at 100 nM it was 38.8% (p<0.001, t-test). The peak histamine response was 43.2±3.1% responding (sem, n = 9), at 10 μM it was 30.5±2.9% (p<0.01, t-test). Responses to the leukotrienes, particularly LTD_4, were very variable. Some donors responded well to this agonist whilst others were essentially unresponsive.

A number of inflammatory mediators with known effects on eosinophils caused these cells to change shape. This assay should, therefore, be suitable for quantitative pharmacological studies on a range of eosinophil receptors. fMLP and C5a gave simple, monophasic concentration-response curves. The variability in the leukotriene response may indicate variable expression of the receptors for these ligands within the population. The responses to eotaxin and histamine were bell-shaped. This may suggest the presence of inhibitory as well as stimulatory receptors for these agonists or that the receptors can interact with an inhibitory signal transduction system at high agonist concentrations. Preliminary studies show that the effects of histamine were unaffected by mepyramine (1 μM) but were non-competitively inhibited by thioperamide (1 μM).

Table 1. Potency, maximal response (as % cells changing shape) and Hill coefficients for induction of shape change. Values are the mean of n separate determinations ± sd.

Agonist	pEC_{50}	Maximal Response	Hill Coefficient	n
Eotaxin	9.06 ± 0.30	54.2 ± 7.3	2.77 ± 1.04	18
C5a	9.90 ± 0.31	55.2 ± 7.3	2.35 ± 0.75	8
Histamine	7.40 ± 0.24	41.8 ±10.1	1.89 ± 0.61	9
fMLP	8.48 ± 0.26	48.8 ± 6.4	1.43 ± 0.20	4
Leukotriene B_4	8.30 ± 0.58	45.3 ± 15.0	1.16 ± 0.38	8
Leukotriene D_4	6.98 ± 1.00	44.9 ± 21.1	0.64 ± 0.11	10

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79P IL-17, PRODUCED BY LYMPHOCYTES AND NEUTROPHILS, IS NECESSARY FOR LIPOPOLYSACCHARIDE-INDUCED AIRWAY NEUTROPHILIA

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IL-17 is a pro-inflammatory cytokine secreted by $CD4^+$ cells (Infante-Duarte *et al.* 2000). When given locally into the lungs of mice or rats, IL-17 selectively recruits neutrophils. In this report we investigate the relative importance of this cytokine in a murine model of neutrophilic lung inflammation.

Five weeks old female BALB/c and T and B cell-deficient BALB/c C.B.-17 *scid/scid* mice (SCID) were intranasally anaesthised with 2% isoflurane in oxygen and challenged with lipopolysaccharide (LPS, 0.3 mg kg^{-1}) or 50 µl phosphate buffered saline (PBS) sacrificed at different time points for bronchoalveolar lavage (BAL) as described previously (Corteling *et al.* 2002). Total BAL cells, obtained 48 h after the LPS challenge, were sorted into $CD4^+$ (99% pure), $CD8^+$ (99% pure) and CD4/CD8 double negative cells. From the latter population, neutrophils (98.5% pure) were isolated from macrophages (99% pure) by adherence to plastic (2 h at 37°C). Total RNA was isolated using the Rneasy mini RNA isolation kit (Qiagen) and RT-PCR for IL-17 mRNA performed (primer forward: 5' GGT CAA CCT CAA AGT CTT TAA CTC 3', and reverse: 5' TTA AAA ATG CAA GTA AGT TTG CTG 3'). Statistical significance ($P < 0.05$) was determined using a Mann-Whitney test with Bonferroni correction for multiple comparison. Data, mean ± s.e.mean, are expressed as 10^5 cells ml^{-1} or pg ml^{-1} for the cell counts and cytokine levels, respectively.

LPS induce a biphasic influx of neutrophils into the BAL peaking at 24 and 48 h. (PBS: 0.08 ± 0.03, LPS 24 h: 5.8 ± 0.8, LPS 30 h: 3.1 ± 0.3, LPS 48 h: 6.3 ± 0.8). IL-17 production was increased only during the late phase neutrophilia (48 h, PBS: 5 ± 2, LPS: 210 ± 50). Intranasal treatment with a neutralising anti-IL-17 antibody (200 µg, R&D Systems), 24 h after the LPS challenge, significantly inhibits the late phase neutrophilia (control: 3.8 ± 0.6, treated: 1.7 ± 0.2). Among the BAL cell population recovered at 48 h post challenge and in agreement with the published literature mRNA for IL-17 was detected in $CD4^+$ and $CD8^+$ cells but not in macrophages. Unexpectedly IL-17 mRNA was also associated with neutrophils (Figure 1).

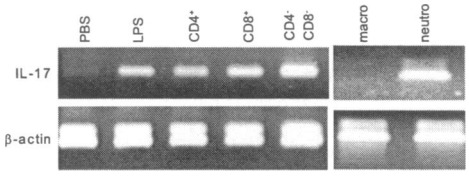

Figure 1. IL-17 mRNA expression 48 h after the challenge

To further demonstrate that neutrophils could be a source of IL-17, LPS-induced lung inflammation was studied in SCID mice. These mice also developed a neutrophilic response at 24 h (PBS: 0.02 ± 0.01, LPS: 8.3 ± 0.7) which decline by 48 h (PBS: 0.01 ± 0.01, LPS: 3.5 ± 1.6). Despite the absence of lymphocytes, a significant increase in IL-17 levels was observed at both 24 h (PBS: 3.1 ± 0.1, LPS: 6.2 ± 0.1) and 48 h (PBS: 3.9 ± 0.1, LPS: 14.2 ± 0.2). However, when compared with BALB/c, BAL IL-17 levels were significantly lower.

Our results show that IL-17, produced by mainly by T cells but also neutrophils, is responsible for the late phase neutrophilic infiltration following LPS challenge.

Corteling *et al.*, 2002, *BMC Pharmacol.*, 2:1
Infante-Duarte *et al.*, 2000, *J. Immunol.*, 165:6107

80P EFFECTS OF PHOSPHODIESTERASE INHIBITORS ON IL-4 AND IL-13 GENERATION FROM HUMAN BASOPHILS

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Human basophils have been shown to generate Interleukin-4 (IL-4) and IL-13 as well as histamine. Previous studies have shown that the cAMP-specific phosphodiesterase (PDE), PDE4, is the major isoform regulating the release of histamine from basophils (Weston *et al.*, 1997). The aim of the present study was to determine, using a range of PDE inhibitors, which isoform(s), if any, of PDE regulates the generation of IL-4 and IL-13 from human basophils.

Basophils were isolated from venous blood by Percoll density sedimentation purity, 7±3%, n=11). Cells were incubated with a range of PDE inhibitors for 15 min and were then challenged with anti-IgE (1/10000) for 4 h (for IL-4 and histamine release) or 24 h (for IL-13 and histamine release) for optimal generation of the cytokines. The release of histamine in the supernatants was measured by an automated fluorometric technique. To measure the levels of IL-4 and IL-13 in supernatants, commercially-available ELISA kits were used. Values are means±s.e.mean. Results were analysed statistically using ANOVA (GraphPad InStat, version 3.0a).

The non-selective PDE inhibitor, theophylline (1mM), and the selective inhibitors (all at 10µM), rolipram (PDE4-selective) and Org30029 (mixed PDE3/4 inhibitor) significantly suppressed the IgE-mediated generation of IL-4 (n=7, $P<0.05$ at least), IL13 (n=4, $P<0.01$) and histamine (n=4 or 7, $P<0.05$ at least) from basophils (see Table 1). The isozyme-selective PDE inhibitors (all at 10µM), siguazodan (PDE3-selective), zaprinast (PDE5-selective) and 8-methoxymethylisobutyl-methylxanthine (8-Me-IBMX, PDE1-selective) did not inhibit the generation of IL-4, IL-13 or histamine ($P>0.05$). These data indicate that inhibition of PDE4 attenuates IL-4, IL-13 and histamine generation from basophils.

	% Inhibition			
Inhibitor (class)	*Histamine**	*IL-4**	*Histamine***	*IL-13***
Theophylline (NS)	81±6	76±10	73±9	78±4
8–Me-IBMX (1)	2±3	-1±7	19±11	35±9
Siguazodan (3)	3±2	9±7	7±71	14±6
Org 30029 (3/4)	33±7	47±11	36±5	89±6
Rolipram (4)	46±7	61±12	54±14	83±7
Zaprinast (5)	2±4	10±4	-4±7	8±8

Table 1 Values are expressed as the percent inhibition of the control IL-13 (20±1 pg per 10^6 basophils), IL-4 (53±17 pg per 10^6 basophils) and histamine (22±5% in IL-4 and 18±7% in IL-13 experiments) release. Incubations were for 4 h* or 24 h**. NS, non-selective.

These results indicate that modulation of PDE4 regulates not only the release of histamine but also the generation of IL-4 and IL-13 from human basophils.

Weston, M.C. *et al.*, (1997). *Br. J. Pharmacol.*, **121**, 287–295.

81P EFFECT OF NITROPARACETAMOL ON IL-1β and TNF-α PRODUCTION IN LIPOPOLYSACCHARIDE CHALLENGED HUMAN BLOOD

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Nitroparacetamol (a nitric oxide [NO] releasing derivative of paracetamol) is a potent inhibitor of carrageenan-induced hindpaw oedema (Al-Swayeh *et al.*, 2000) and lipopolysaccharide (LPS)-induced hypotension (Marshall & Keeble, 2002) in the rat. In each case, nitroparacetamol is more potent (mol for mol) than paracetamol. The mechanism of action of nitroparacetamol is not clear but inhibition of caspase-1 (Fiorucci, 2001) and/or disruption of the intracellular NF-κB pathway (Cui *et al.*, 2001) may be involved. The aim of these experiments was to examine the ability of nitroparacetamol and paracetamol to affect LPS-induced IL-1β and TNF-α production in human blood.

Heparinised blood (12.5 U/ml) was obtained from human volunteers (n=6) and diluted 1:1 v/v in RPMI-1640 medium. Aliquots (1.5 ml) were incubated (37°C, 1-5 h) with LPS (*E. Coli,* serotype 0127) and increasing concentrations (1-1000μM) of nitroparacetamol, nitroflurbiprofen, paracetamol, flurbiprofen or vehicle (DMSO, 15 μl). At the end of the incubation period, aliquots of plasma (10-100 μl) were assayed (ELISA, R&D Systems) for IL-1β and TNF-α.

Plasma concentrations of both cytokines in the absence of LPS were low (< 30 pg/ml). Incubation of blood with LPS resulted in the time-dependent formation of both IL-1β (5h; 3.81 ± 0.33 ng/ml, n=12) and TNF-α (5h; 3.16 ± 0.88 ng/ml, n=12). DMSO did not affect cytokine formation. Nitroparacetamol caused dose-related inhibition of LPS-induced IL-1β (e.g. IC_{50}, 812.0 ± 101.2 μM, n=12) and TNF-α (IC_{50}, 9.0 ± 2.1 μM, n=12,) formation. In contrast, paracetamol (up to 1 mM) did not affect the production of either cytokine. For comparison, nitroflurbiprofen also inhibited LPS-induced IL-1β (IC_{50}, 362.0 ± 36.8 μM, n=12) whilst flurbiprofen was without effect. In contrast, both nitroflurbiprofen and flurbiprofen inhibited TNF-α formation in blood (e.g. 100 μM; 83.0 ± 4.2% and 87.3 ± 9.1% inhibition respectively, both n=12).

Both nitroparacetamol and nitroflurbiprofen inhibit IL-1β and TNF-α formation in LPS-challenged human blood. Nitroparacetamol was 90 times more potent as an inhibitor of TNF-α (c.f. IL-1β) production. That paracetamol did not affect IL-1β/TNF-α formation whilst flurbiprofen inhibited TNF-α (but not IL-1β) production suggests that NO, released from nitroparacetamol (and possibly nitroflurbiprofen), during incubation contributes to the inhibition of cytokine production observed. The molecular target for NO in this respect is not known and warrants further investigation.

We wish to thank NicOX Ltd for financial support.

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82P HYPERTHYROIDISM INDUCES CONJUNCTIVAL MAST CELL DISRUPTION WITHOUT SIMULTANEOUS MODIFICATION OF THE EARLY PHASE RESPONSE TO THE HISTAMINE-RELEASER C48/80

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An interaction between thyroid hormones and mast cell activity has been reported in various conditions, including the inflammatory and allergic responses (Manzolli *et al.*, 1999; Davani *et al.*, 2002). The aim of this study was to evaluate the anaphylactoid response in the hyperthyroid rat conjunctiva.

One drop of 100mg/ml C48/80 (a mast cell degranulator) was instilled under light ether anaesthesia into the lower conjunctival fornix of normal (NORM) and hyperthyroid (THYR, receiving a daily s.c. dose of 0.025mg/100g L-thyroxine -T_4- for 14 days) male Wistar rats of 250-300g and a drop of vehicle into the contralateral control eye (license K/4358/01). Forty-five min after challenge, the animals were sacrificed and the conjunctival histamine was quantified fluorometrically (Tiligada et al., 2000), while mast cells were obtained by routine conjunctival scraping, stained for 3min with 0.25% (w/v) toluidine blue, pH 1.6 and examined under the light microscope. Histamine content was expressed as mean±SEM % of the control eye. Statistical analyses were performed by t-test and ANOVA. Following challenge, histamine levels in NORM were significantly reduced to 44±4% (n=13, $P<0.001$), compared to the control. A similar reduction to 52±10% (n=6, $P<0.01$) was detected in the THYR group, the response to C48/80 not being significantly different between the two groups ($P>0.2$). The microscopic appearance of mast cells in the THYR conjunctivae was consistently disrupted as shown in Figure 1.

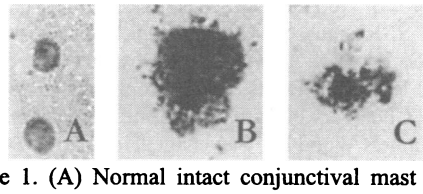

Figure 1. (A) Normal intact conjunctival mast cells, (B) C48/80-induced degranulation, (C) disrupted mast cells in unchallenged hyperthyroid conjunctiva. x 400

These preliminary results showed, for the first time, that long-term T_4 administration induced extensive conjunctival mast cell structural disorganization, yet without parallel disruption of functionality. Without disregarding mast cell heterogeneity, the underlying mechanisms and the functional significance remain elusive, although a relation to T_4-induced alterations in PKCdelta (Pantos *et al.*, 2002; Leitges *et al.*, 2002) or TR orphan receptors (Siebler *et al.*, 2002) may be hypothesized. These novel observations provide the lead for the investigation of the hormone-regulated mast cell integrity and activity, which may have implications in allergic responses.

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The work was supported by the Grant 70/4/5900 of the UOA.

83P AGONIST FINGERPRINT OF THE RECOMBINANT HUMAN CRTH2 PROSTANOID RECEPTOR EXPRESSED IN HEK293(T) CELLS

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The chemoattractant receptor-like molecule of Th2 lymphocytes (CRTh2; DP2) is a family A 7TM receptor activated by prostaglandin D_2 (PGD_2; Hirai *et al.*, 2001). PGD_2 also activates DP prostanoid receptors however CRTh2 is structurally distinct from the prostanoid receptor family. Thus far, published pharmacological profiling of the receptor has been limited to PGD_2, close metabolites of PGD_2, and DP-selective compounds (eg. Monneret *et al.*, 2001). Here, we describe an extensive agonist profile of recombinant hCRTh2 receptors expressed in HEK293 (T) cells obtained by measuring [^{35}S]-GTPγS accumulation.

Briefly, membranes were prepared from HEK293 (T) cells transiently expressing human CRTH2 receptors and $G\alpha_{i5}$ G-proteins. Assays were performed in 100µl (total) of assay buffer (20mM HEPES, 100mM NaCl, 10mM $MgCl_2$, pH 7.4) containing: membranes (15µg / well), [^{35}S]-GTPγS (0.2nM), GDP (0.4µM), saponin (10µg/ml), and agonists (30µM-0.7pM). Reactions were started by the addition of membranes. SPA beads were added 30mins later (0.5mg / well in 25µl). Plates were centrifuged after a further 30 mins at 100G and counted on a Wallac Microbeta scintillation counter. Concentration / effect curve parameters were estimated by fitting a four-parameter logistic function to quench-corrected cpm. data; n=3-6. The data in Table I show that prostanoid receptor agonists possessing the PGD and PGF ring systems are also agonists at CRTh2 receptors, differentiating CRTH2 from DP receptors. Thus CRTh2 & prostanoid receptor agonist binding domains may be similar. Indeed, the rank order of 15(R) >15(S)-15methyl-PGD_2 suggests that in common with other prostanoid receptors, stereoselectivity around the 15C position exists. It is also interesting to note that 15(R),15 methyl PGD_2 is more potent than PGD_2 itself. Further work in a well-coupled assay system is required to establish agonist relative potencies.

Table I. Agonist potencies at CRTH2 receptors. Values quoted are mean ± sd. Hill slope values are geometric mean (95% C.I.)

	pEC50	nH	max cf PGD2
PGD_2	8.2±0.2	0.7 (0.6-0.8)	100
13,14 dihydro 15 keto PGD_2	7.8±0.2	0.7 (0.6-0.9)	97±15
15(S) 15 methyl PGD_2	7.2±0.1	0.9 (0.7-1.2)	95±13
15(R) 15 methyl PGD_2	9.1±0.4	0.7 (0.2-1.9)	93±5
PGJ_2	6.9±0.2	1.3 (1.2-1.4)	87±1
Latanoprost	6.0±0.1	2.1 (0.9-5.1)	80±5
Cloprostonol	5.9±0.1	2.3 (1.1-4.9)	79±7
$PGF_{2\alpha}$	6.1±0.2	1.6 (0.4-6.7)	62±6
15(S) 15 methyl $PGF_{2\alpha}$	5.7 (n=2)	8.4	52±16
U46619	6.0±0.1	3.2 (0.7-14.6)	52±6
Fluprostenol	5.6 (n=2)	5	32±6
Butaprost (methyl ester)			15±7
19(R) hydroxy PGE_2	5.8 (n=1)	12.5	14±10
PGE_2	5.8 (n=1)	9.3	13±16
Cicaprost	6.3 (n=1)	11.4	12±10
BW245C90			9±7
PGI_2	6.0 (n=1)	1	8±8

Butaprost (free acid), PGB_2, 17 phenyl PGE_2, Iloprost, PGE_1, Misoprostol, 11 deoxy PGE_1, 16,16 dimethyl PGE_2 were without effect.

Further work in a well-coupled assay system is required to establish agonist relative potencies.

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84P GW627368X: A NOVEL, POTENT AND SELECTIVE EP4 PROSTANOID RECEPTOR ANTAGONIST

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AH23848, a prostaglandin analogue, has been the antagonist of choice for characterising the role of the EP_4 prostanoid receptor. AH23848 has low potency at EP_4 receptors in piglet saphenous vein (pA_2 = 5.4; Coleman *et al.*, 1994), low binding affinity vs. recombinant human EP_4 receptors (pKi = 4.9; Abramowitz *et al.*, 2000) and is relatively non-selective over other prostanoid receptors. We report here the discovery of GW627368X, a potent and selective EP_4 receptor antagonist

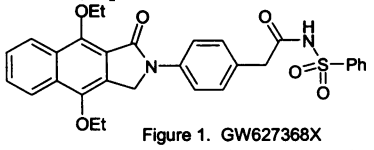

Figure 1. GW627368X

Radioligand competition binding: Scintillation Proximity Assay (SPA) was used except for EP1 (filtration binding). Membranes were prepared from CHO, BHK or HEK293(T) cells transiently expressing recombinant human prostanoid receptors. Assays were performed in 100µl (total) of assay buffer (50mM HEPES, 10mM $MgCl_2$, pH7.4) containing membranes, SPA beads (0.75 or 1 mg/well), radioligand (≈ 1 x Kd of radioligand), GW627368X (1nM - 10µM) or cold competitor to determine non-specific binding (1-100µM). Data are mean pKi ± sd, or % inhibition ± sd at 10µM compound.

cAMP accumulation assay: HEK293(T) cells stably expressing hEP4 were incubated with GW627368X (30nM - 10µM) for 30mins prior to the addition of PGE_2 (3nM - 10µM) in 100µl assay buffer (DMEM-HAMF12 [Gibco-BRL, UK], 100µM IBMX, 3µM indomethacin). Reactions were stopped by the addition of 150µl ice-cold ethanol and cAMP accumulation determined using a proprietary [^{125}-I] cAMP SPA kit [Amersham, UK]. *Piglet Saphenous Vein bioassay:* 5mm rings of piglet saphenous vein were mounted in tissue baths containing Krebs solution supplemented with 3µM indomethacin and 10µM GR32191B for isometric force recording. PGE_2 response curves (0.3nM - 10µM) were produced in the presence of 1µM phenylephrine and GW627368X (1nM - 0.3µM).

Radioligand competition binding assay data.

Receptor	DP	EP1	EP2	EP3	EP4	FP	IP	TP
pKi ± sd	<5.0	<5.1	<5.0	<5.1	**7.0±0.3**	<5.1	<5.3	**6.9**
% I ± sd	18±13	0	0	2±6	100	0	0	100
n	7	2	5	5	10	5	5	2

Functional assay data.

hEP_4 pK_b = 7.6±0.3, Schild slope = 1.1±0.1; n=4; no agonism.
pEP_4 pK_b = 9.2±0.2, Schild slope = 0.9±0.2; n=3; no agonism.
pTP pA_2 = 5.6±0.5; n=3; no agonism to 10µM.

GW627368X is a marked advance over previously available EP_4 receptor antagonists and is therefore a valuable research tool for the investigation of EP_4 receptor pathophysiology.

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85P EFFECT OF NEUROPEPTIDE Y, Y_1 AND Y_2 AGONISTS ON CUTANEOUS MICROVASCULAR RESPONSES

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Neuropeptide Y (NPY) has been suggested to mediate inflammatory effects via the Y_1 receptor in the mouse (Naveilhan *et al.*, 2001). This has led us to investigate the effects of NPY and related ligands, in the mouse cutaneous microvasculature. Responses were examined in C57BL/6 mice, $Y_2^{+/+}$ and $Y_2^{-/-}$ mice which were anaesthetised with urethane (25% w/v; 100 µl $10g^{-1}$ i.p.). Oedema formation was assessed by extravascular accumulation of ^{125}I-albumin (1.25 µCi i.v.), neutrophil accumulation by myeloperoxidase assay (Chu *et al.*, 2000), and blood flow by local clearance of ^{99m}Tc (Chu *et al.*, 2001).

Intradermal (i.d.) injection of Y agonists either alone, or in the presence of the vasodilator CGRP (30 pmol/site), had non-significant effects on oedema formation and NPY (300 pmol $site^{-1}$) had no significant effect on neutrophil accumulation. However, NPY, as expected, induced a dose-related vasoconstrictor effect. The ED_{50} values for NPY (a Y_1 and Y_2 agonist), Pro^{34}-NPY (Y_1>Y_2) and PYY_{3-36} (Y_2>Y_1 agonist) are 62, 5.6 and 31 pmol $site^{-1}$, respectively. The Y_1 antagonist (BIBO3304; 0.5 µmol kg^{-1} i.v. -5 min) caused a rightward shift of the Pro^{34}-NPY dose-response curve, abolishing responses to ≤10 nmol (Table 1). By comparison, the decreased blood flow induced by PYY_{3-36} (30 pmol $site^{-1}$) was attenuated in $Y_2^{-/-}$ mice and significantly inhibited when both Y_1 and Y_2 (BIIE0246TF 3 µmol kg^{-1} i.v., -5 min) antagonists were given together, rather than separately (Table 1).

Our results suggest only a weak inflammatory activity of NPY. By comparison, NPY agonists possess potent vasoconstrictor effects, acting predominantly via the Y_1 receptor. A role for the Y_2 receptor cannot however be ruled out as PYY_{3-36} responses (not blocked by a Y_1 receptor antagonist at the lower 30 pmol dose), were significantly inhibited by the further addition of a Y_2 receptor antagonist, and were significantly reduced in $Y_2^{-/-}$ mice (when compared with $Y2^{+/+}$).

Chu *et al.*, (2000). *Br.J.Pharmacol.*, 130:1589-96.
Chu *et al.*, (2001). *Neurosci. Letts.* 310:169-72.
Naveilhan *et al.*, (2001). *Nature* 409:513-7.

This project was supported by a grant from GKT Trustees. NPY antagonists were gifts from Boehringer-Ingelheim, Germany.

Ligand	Dose (pmol)	$Y_2^{+/+}$	BIBO3304 (Y_1) 0.5µmol kg^{-1}	BIIE0246 (Y_2) 3µmol kg^{-1}	Y_1 + Y_2 antagonist	$Y_2^{-/-}$
Pro^{34}-NPY (Y_1)	1	9.7 ± 3.5 (7)	*1.3 ± 2.8 (7)	ND	ND	6.5 ± 2.0 (8)
	10	14.2 ± 2.6 (11)	*2.9 ± 3.1 (11)	ND	ND	9.7 ± 2.0 (12)
PYY_{3-3} (Y_2)	30	6.6 ± 1.0 (35)	5.0 ± 0.9 (5)	3.8 ± 2.3 (12)	*1.0 ± 2.4 (9)	*1.8 ± 1.9 (10)
	100	8.7 ± 1.1 (35)	4.5 ± 0.9 (5)	6.1 ± 1.6 (12)	**1.2 ± 2.6 (9)	6.3 ± 2.6 (10)

Table 1. Effect of NPY antagonists BIBO3304 (Y_1) and BIIE0246 (Y_2) on Pro^{34}-NPY and PYY_{3-36} induced decreased blood flow responses over 30 min. Data expressed as change in % decreased clearance compared to vehicle, mean ± s.e.m., n numbers in brackets. Statistics by unpaired Student's t-test, *P<0.05, **P<0.01 compared to $Y_2^{+/+}$ vehicle control.

86P THE COMPARISON OF THE MICROVASCULAR ACTIVITY OF CALCITONIN GENE-RELATED PEPTIDE (CGRP) AND OTHER VASODILATORS IN MOUSE SKIN

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Calcitonin gene-related peptide (CGRP) is one of the most potent microvascular vasodilators (Brain *et al.*, 1985). CGRP has been implicated in the onset of migraine, stimulating the search for a non-peptide CGRP antagonist. We have not, to date, found a direct method to measure the effect of microvascular vasodilators in mouse skin. However, these vasodilators potentiate the oedema formation caused by mediators of increased microvascular permeability in species including the mouse (Cao *et al.*, 1999). The aim of this study was to learn about the ability of CGRP and the related peptide adrenomedullin (AM) to potentiate oedema formation when compared with other microvascular vasodilators. Thus, we have investigated the ability of CGRP, AM, vasoactive intestinal peptide (VIP) and prostaglandin (PG) E_1 to potentiate plasma extravasation induced by substance P (SP) in mouse skin.

Female CD1 mice (20-25g) were anaesthetised with urethane (2.5mg/g, i.p.) and 3.6kBq ^{125}I-albumin (BSA) injected i.v. Plasma extravasation was assessed as the extravascular accumulation of ^{125}I-albumin. Agents were administered intradermally in the presence or absence of SP (300pmol/site). After thirty minutes, blood samples were taken by cardiac puncture, and plasma and skin sites assessed for radioactivity. Plasma extravasation was expressed as µl of plasma per gram of tissue. Results are shown as mean ± s.e.mean and statistical analysis was by ANOVA with Bonferroni's post test.

When administered alone, the vasodilators induced no plasma extravasation, as expected. However, all agents significantly potentiated the plasma extravasation to SP (see Table 1). The results indicate that CGRP is a highly potent vasodilator in the mouse skin in comparison with the other vasodilator agents tested. A potency difference of approximately 300 times is demonstrated between CGRP and AM.

Table 1. Effect of vasodilator agents on the potentiation of SP-induced plasma extravasation. *=p<0.05, **=p<0.01 compared to SP alone (n=5-9).

	Agent alone	Agent + SP
Tyrode	0.5 ± 1.7	33.3 ± 3.6
CGRP		
0.3pmol	1.3 ± 0.7	118.0 ± 22.8*
1pmol	4.3 ± 3.0	128.6 ± 20.4*
AM		
100pmol	0.1 ± 1.7	29.6 ± 12.9$^{n.s.}$
300pmol	7.8 ± 2.1	139.7 ± 39.8*
VIP		
3pmol	-6.8 ± 3.8	84.8 ± 11.8*
10pmol	-4.7 ± 2.2	120.1 ± 40.0
PGE_1		
10pmol	1.4 ± 2.4	78.1 ± 19.9**
100pmol	7.6 ± 5.4	91.7 ± 20.4**

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C. T. and A.G. are funded by the British Heart Foundation.

87P MECHANISMS INVOLVED IN *Phoneutria nigriventer* SPIDER VENOM-INDUCED INFLAMMATORY RESPONSES IN MOUSE SKIN: EFFECT OF A 5-HT_4 RECEPTOR ANTAGONIST

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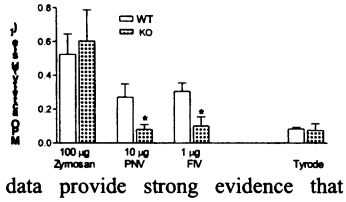
Pro-inflammatory effects (*in vivo*) and vagus nerve depolarization (*in vitro*) evoked by *Phoneutria nigriventer* venom (PNV) are markedly reduced by capsaicin pretreatment, suggesting involvement of sensory C-fibres (Costa *et al.*, 1997; 2001). PNV-induced depolarization is inhibited by a 5-HT_4 receptor antagonist, implying a link between PNV-evoked neurogenic inflammation and prejunctional 5-HT_4 receptors (Costa *et al.*, 2001). We have now investigated the inflammatory effect of PNV and a semi-purified fraction IV (MW 10-14 kDa) in the microvasculature of wild type (WT) and NK_1 receptor knockout (KO) mice. The involvement of 5-HT_4 receptors was also examined.

Male and female mice (Sv129 + C57BL/6; 25-30 g), either WT or KO were anaesthetized with urethane (25% w/v; 100 µl 10^{-1} g, i.p) for measurement of plasma extravasation and (isoflurane:O_2 flow, 2.5%:2.5%) for myeloperoxidase (MPO) assay. Responses to intradermal injection (i.d.) of PNV and test agents were assessed in the shaved dorsal skin. Tyrodes was used as a vehicle throughout. Plasma extravasation was measured by the extravascular accumulation of ^{125}I-albumin after 30 min and neutrophil accumulation by MPO assay after 4 h. Mice were killed by cervical dislocation and the skin sites were removed. Either the radioactivity was measured in skin and plasma or MPO activity evaluated. Results are mean ± s.e.mean. Statistical analysis was by ANOVA plus Bonferroni's modified t-test. $P<0.05$ was taken as significant.

The i.d. injection of either PNV (10 µg), fraction IV (1 µg) and zymosan (100 µg, a known complement-dependent mediator of neutrophil accumulation) caused significant neutrophil stimulation in WT mice. The accumulation of neutrophils induced by PNV and fraction IV, but not zymosan-treated sites, was attenuated in NK_1 KO mice (Figure 1). The plasma extravasation evoked by PNV was also significantly greater in WT mice than NK_1 KO mice (25 ± 6.5 and 1.3 ± 2.3 µl g^{-1} plasma extravasation $site^{-1}$, respectively, $P<0.01$, n = 7). PNV results were similar when the fraction IV was used, except that fraction IV was more potent than PNV. The selective 5-HT_4 receptor antagonist RS39604 (30 pmol $site^{-1}$; n = 7) markedly reduced PNV (30 µg; 43 ± 9.9 to 1 ± 7.7 µl g^{-1})- and fraction IV-induced plasma extravasation (40 ± 10 to 12 ± 5 µl g^{-1}, $P<0.01$). Neither 5-HT-induced plasma extravasation nor neutrophil accumulation was changed by RS39604.

Figure 1. Effect of test agents on neutrophil accumulation. Mean ± s.e.mean, n = 6. $P<0.05$ compared to WT group.



The present data provide strong evidence that PNV-induced neutrophil accumulation, in addition to oedema, is mediated by NK_1 receptors and supports the concept that PNV-induced responses are due to the release of an NK_1 agonist component from sensory fibres. Furthermore, we now provide evidence that the activation of prejunctional 5-HT_4 receptors plays a pivotal role in PNV-induced plasma extravasation. The inability of RS39604 to block 5-HT-induced responses is unclear and further studies with selective 5-HT_4 receptor agonists are required.

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This study is supported by the British Heart Foundation.

88P ANTIBODIES TO THE LEUKOCYTE INTEGRIN MOLECULES α4 AND LFA-1 INHIBIT DSS-INDUCED COLITIS IN RATS

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The integrin molecules alpha4beta1 (α4β1, VLA-4), alpha4beta7 (α4β7, LPAM-1) and alphaLbeta2 (αLβ2, LFA-1) play a critical role in the recruitment of leukocytes to sites of inflammation. Oral administration of dextran sulfate sodium (DSS) induces colitis in rodents (Okayasu *et al.*, 1990) bearing some resemblance to ulcerative colitis, with crypt destruction and infiltration of inflammatory cells, including lymphocytes and neutrophils, into the colon. We investigated whether blocking antibodies to α4 or LFA-1 would inhibit DSS-induced mucosal colonic inflammation in rats.

DSS (3%) was added to the drinking water of male Wistar rats (200g - 230g, n = 8/group) continuously for 11 days. The rats were treated twice weekly with either MAX68P (mouse anti-human α4 antibody which cross-reacts with rat α4), WT-1 (mouse anti-rat LFA-1) or 101.4 (isotype matched negative control antibody) at a dose of 10 mg/kg i.p. The first dose of antibody was administered 1 hour before the addition of DSS to the drinking water. A second control group of age-matched rats (n = 6) received normal tap drinking water only. Body weight was monitored daily and rats killed with an overdose of halothane on day 12 and the colon removed. The length of the colon from the rectum to the caecum was measured and the most distal 2 cm snap frozen in isopentane for later analysis of myeloperoxidase (MPO) levels as a marker of neutrophilia (Krawisz *et al.*, 1984). The remaining colon was placed in formalin for later analysis of CD3+ T cell infiltration by immunocytochemistry. Data are presented as mean ± sem and analysed by one-way ANOVA with Bonferroni's multiple comparison post test.

Addition of DSS (3%) to drinking water resulted in a significant reduction in body weight. A weight loss to 93±3.9 % of starting weight was noted by day 11 in 101.4 treated rats whereas normal control animals receiving normal drinking water gained weight to 118±1.0 % starting weight ($p<0.001$). Treatment with either MAX68P or WT-1 reduced this DSS-induced weight loss significantly, with rats showing a gain in weight to 107±1.4 % starting weight ($p<0.05$) and 109±1.4 % ($p<0.01$) starting weight respectively at day 11. Colon length was significantly reduced in rats receiving DSS in their drinking water and treated with 101.4 (8.0±0.4 cm compared with 15.5±0.6 cm in normal control rats). Treatment with either MAX68P or WT-1 protected against the colon shortening induced by DSS with colon lengths of 12.6±0.7 cm ($p<0.001$) and 13.5±0.5 cm ($p<0.001$) respectively. MAX68P also reduced the level of MPO noted in 101.4 control antibody treated animals from 0.14±0.04 U/mg colonic tissue to 0.02±0.01 U/mg ($p<0.01$). A reduction in the number of CD3+ cells in the colonic lamina propria of DSS colitis mice was also noted after treatment with MAX68P or WT-1 (from 57±12 to 33±4 and 35±2 cells per 500µm x 500µm, respectively ($p<0.05$)).

These results indicate that antibodies to α4 and LFA-1 reduce DSS-induced colitis in rats and support α4 and LFA-1 as potential therapeutic targets for inhibiting intestinal inflammation.

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89P CONTRACTILE EFFECTS OF THE TACHYKININS RANAKININ AND RANATACHYKININ A ON THE RAT ISOLATED ILEUM PREPARATION

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Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) contract the rat ileum by acting at NK_1, NK_2 and NK_3 receptors. In this tissue, NK_2 receptors are believed to predominate and NKA has been shown to be the most potent tachykinin (Regoli *et al.*, 1994). Ranakinin (O'Harte *et al.*, 1991; RK: KPNPERFYGLMamide) and ranatachykinin A (Kanagawa *et al.*, 1993; RTKA: KPSPDRFYGLMamide) are tachykinins isolated from frogs of the genus *Rana* and are reported to be NK_1 agonists (Lippe *et al.*, 1998). The aim of this study was to evaluate contractile effects of the 5 peptides in the absence and presence of the NK_1 antagonist N-acetyl-L-tryptophan-3,5-bis(trifluoromethyl) benzyl ester (NATBE) (MacLeod, *et al.*,1993) which has been shown (Coltman *et al.*, 2002) to block SP-induced contraction of rat ileal tissue.

Ileal segments (4 sections taken 15-30 cm from the ileo-caecal junction) were obtained from male Wistar rats (220 – 250g) and mounted in 10ml organ baths containing Tyrode's solution at 35 °C (plus 1 μM atropine, gassed with 95% O_2 : 5% CO_2) under a tension of 1g. Tissues were equilibrated for 20 min before non-cumulative dose response curves to SP, NKA, NKB, RK and RTKA were obtained using a 4 min cycle with 1 min contact time using an isotonic transducer. NATBE (2 – 5 μM) was added to the buffer and tissues equilibrated for 20 min before retesting the effects of the 5 tachykinins. Data are mean ± s.e. and statistical significance was determined using Student's *t*-test for pair differences.

SP (10 nM–1 μM) caused dose-dependent tonic contraction with an EC_{50} value of 95±9 nM (n=8). EC_{50} values for RKTA, RK, NKA and NKB were 47±5 nM (n=8; P<0.001), 55±4 nM (n=8; P<0.05), 71±4 nM (n=8; P<0.05) and 253±167 nM (n=8; P<0.0001) respectively. The maximum response to SP occurred at 0.8 μM while those to RTKA, RK and NKA occurred at 0.2 μM and were 80±30% (n=8; P<0.01), 30±5% (n=8; P<0.005) and 38±7% (n=8; P<0.005) greater than SP at the same concentration. Addition of NATBE (2-5 μM) to the saline caused non-competitive inhibition of RTKA-, RK-, NKA-, SP- and NKB-induced contraction. At 5 μM, NATBE reduced the maximum responses caused by RTKA, RK, NKA, SP and NKB by 95±3% (n=7; P<0.0001), 76±8% (n=7; P<0.001), 39±10% (n=5; P<0.05), 80±15% (n=8; P<0.001) and 81±10% (n=6; P<0.001) respectively. NATBE (2 μM) had no significant effect on contraction induced by NKA or NKB.

These data confirm earlier observations that the order of potency in ileal tissue is NKA>SP>NKB and therefore NK_2 receptors predominate. This is supported by the fact that NATBE had greater antagonist potency against SP than NKA. RTKA and RK were the most potent contractants of the tissue and were antagonised by NATBE to the same extent as SP providing evidence that they are selective for NK_1 receptors.

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90P DIFFERENTIAL ROLE OF Y_1 AND Y_2 RECEPTORS MEDIATING NEUROPEPTIDE Y'S CONTRIBUTION TO VERATRIDINE-INDUCED ION TRANSPORT ACROSS MOUSE COLON

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Neuropeptide Y (NPY) is a prominent enteric neuropeptide (Sang & Young, 1996) with prolonged antisecretory effects (measured as decreased short circuit current (I_{sc})) in rodent intestine (Cox *et al.*, 2001; Tough & Cox, 1996). Y_1 and Y_2 receptors mediate Y agonist effects in rat colon mucosa and are located both pre- and post-junctionally (Tough & Cox, 1996), however the cellular location of Y receptors in mouse colon is unknown. Veratridine stimulates intrinsic enteric nerves, resulting in increased epithelial ion secretion (Sheldon *et al.*, 1990) which is a combination of inhibitory and excitatory neurotransmission. Previously we described NPY as an inhibitory enteric neuropeptide (Hyland & Cox, 2002) and the aim of this study was to determine the specific mediation of veratridine responses by Y_1 and Y_2 receptors, using either $Y_1^{-/-}$ and $Y_2^{-/-}$ tissue or selective Y_1 and Y_2 antagonists, BIBO3304 and BIIE0246 respectively.

Mucosal preparations from adult wild-type ($^{+/+}$), $NPY^{-/-}$, $Y_1^{-/-}$ and $Y_2^{-/-}$ colon were placed between two halves of an Ussing chamber and voltage-clamped at 0 mV as described previously (Cox *et al.*, 2001). Basolateral veratridine (30 μM, in 95% ethanol) was added either in the presence or absence of BIBO3304 (300 nM, 15 mins; in 10% DMSO), BIIE0246 (1 μM, 15 mins; in 10 % DMSO) or atropine (1 μM) and hexamethonium (10 μM, 15 mins). Any remaining veratridine I_{sc} component was abolished by tetrodotoxin (100 nM). Data was pooled and the means ± 1 s.e. mean were compared using Student's unpaired *t*-test a $P<0.05$ was considered significant.

Veratridine increased I_{sc} in $^{+/+}$ tissue reaching a maximum at 7 min (45.9 ± 4.0 μA.cm^{-2}; n=30), and was blunted in $NPY^{-/-}$ colon (30.1 ± 6.4 μA.cm^{-2}; n=12) significantly so between 1-5 mins ($P<0.05$). BIBO3304 slightly increased veratridine-stimulated secretion (55.9 ± 8.8 μA.cm^{-2}; n=16, $P>0.05$), and this was absent in $Y_1^{-/-}$ tissue (46.7 ± 9.8 μA.cm^{-2}; n=7). Veratridine responses were however significantly increased by BIIE0246 (69.8 ± 12.1 μA.cm^{-2}; n=12, $P<0.05$; 2-15 min) and to a lesser extent in $Y_2^{-/-}$ (54.7 ± 10.9 μA.cm^{-2} ; n=5, $P>0.05$) compared with $^{+/+}$ controls. Cholinergic blockade did not alter veratridine-stimulated I_{sc} in either $^{+/+}$ (49.8 ± 9.2 μA.cm^{-2}; n= 19) or $NPY^{-/-}$ colon (27.7 ± 4.8 μA.cm^{-2}; n=7).

Our data shows that veratridine-induced responses in mouse colon are non-cholinergic (NC) and that NPY can act pre-junctionally upon submucosal neurons which innervate epithelial cells. The elevation in veratridine-induced I_{sc} in $Y_2^{-/-}$ tissue and in $^{+/+}$ tissue in the presence of BIIE0246 (and BIBO3304) suggests that Y_2 (and to a lesser extent Y_1) receptors are located pre-junctionally and that activation of these will inhibit enteric NC neurotransmission.

We thank Dr. H. Herzog (Garvan Institute of Medical Research, Sydney) and Prof. D. Wynick (University of Bristol) for wild-type and mutant mice. Supported by the BBSRC.

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91P MUCOSAL CHOLERA TOXIN RESPONSES ARE PARTIALLY AND DIFFERENTIALLY MEDIATED BY 5-HT_4 AND Y_4 RECEPTORS IN THE MOUSE DESCENDING COLON

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Cholera toxin (CTX)-induced intestinal secretion is partly mediated by the release of 5-hydroxytryptamine (5-HT) from mucosal endocrine cells, which then activates several neuronal reflexes one of which involves intrinsic primary afferent neurones (Lundgren, 2002). Additionally, in rat colon the monoamine stimulates a tetrodotoxin (TTX)-insensitive secretory response (Budhoo *et al.*, 1996) involving 5-HT_4 receptor-mediated increases in epithelial cAMP-dependent chloride secretion in isolated crypts (Albuquerque *et al.*, 1998). This led us to investigate 5-HT mediation of CTX responses in mouse colon mucosa and to assess whether neuropeptide Y Y_4 receptors play a role by comparing toxin responses in Y_4 knockout ($Y_4^{-/-}$) and wild-type ($Y_4^{+/+}$) tissue.

Muscle stripped colonic mucosal sheets from male $Y_4^{+/+}$ and age-matched $Y_4^{-/-}$ mice (Sainsbury *et al.*, 2002) were voltage-clamped at 0 mV as described previously (Cox *et al.*, 2001). Vectorial ion transport was continuously measured (as short-circuit current, I_{sc}; $\mu A.cm^{-2}$) and additions made basolaterally, with the exception of CTX (10 $\mu g.ml^{-1}$) which was applied apically. A non-cumulative 5-HT response curve (100 nM - 30 μM) yielded a pEC_{50} of 6.1 ± 0.2 in $Y_4^{+/+}$ colon. 5-HT antagonists (ketanserin, 5-HT_2; tropisetron, 5-HT_3; or RS39604, RS, 5-HT_4, all at 1 μM in 0.01% DMSO vehicle) were added 20 min prior to either 5-HT (1 μM), HTF-919 (a 5-HT_4 agonist, 1 μM also in 0.01% DMSO) or CTX. The TTX (100 nM) sensitivity of 5-HT responses was also assessed. Values are the mean ± 1 s.e. mean and unpaired Student's *t*-test was used to compare data groups.

In $Y_4^{+/+}$ mucosa, RS abolished 5-HT ($P < 0.001$) and HTF-919 responses ($P < 0.01$). Tropisetron reduced 5-HT responses by 48.6 ± 10.0% ($P < 0.05$) while ketanserin and TTX had no effect. $Y_4^{+/+}$ CTX responses were biphasic with an initial decrease in I_{sc} of -2.7 ± 1.9 $\mu A.cm^{-2}$ (n=18) at 60 min, followed by a prolonged I_{sc} increase, which was maximal at 200 min (64.2 ± 9.4 $\mu A.cm^{-2}$, n=15). RS significantly attenuated the latter (30.0 ± 5.6 $\mu A.cm^{-2}$, n=5; $P < 0.01$) compared with controls. In contrast, $Y_4^{-/-}$ CTX responses were monophasic; no initial inhibition was observed ($P < 0.05$) and the elevated I_{sc} levels were blunted (42.8 ± 12.1 $\mu A.cm^{-2}$, n=8, 200 min) and partially RS-sensitive (22.5 ± 11.5 $\mu A.cm^{-2}$, n=3) though not significantly in either case.

These results indicate that in $Y_4^{+/+}$ colon mucosa devoid of myenteric ganglia, CTX stimulates a 5-HT_4 post-junctional secretory response which is also present, but to a lesser extent in $Y_4^{-/-}$ tissue. The absence of initial inhibitory CTX effects in $Y_4^{-/-}$ mucosa implicates a Y_4 receptor mechanism that is most likely stimulated by endogenous pancreatic polypeptide released from colonic enteroendocrine cells. The identity of secretagogue(s) mediating residual RS-insensitive CTX effects has yet to be elucidated.

We thank Dr H. Herzog (Garvan Institute of Medical Research, Sydney) for the mice and Dr D. Hoyer (Novartis Pharma AG, Basel) for HTF-919.

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92P TO INVESTIGATE THE EFFECT OF 8-OHDPAT (5-$HT_{1A/7}$ RECEPTOR AGONIST) ON THE PROXIMAL REGION OF THE *SUNCUS MURINUS* INTESTINE

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In previous studies we have shown that different 5-HT receptor agonists mediate a contractile response in the *Suncus murinus* intestine (Javid & Naylor, 1999). The aim of the present study was to investigate the effect of the 5-$HT_{1A/7}$ receptor agonist 8-OHDPAT in the proximal region of the *Suncus murinus* intestine.

Segments (1.5 cm length) taken from the intestine (1-2 cm distal to the pyloric sphincter (proximal region) of adult female Japanese House Musk shrew, *Suncus murinus* (30-35 g) were mounted in 10 ml organ baths containing Krebs' solution (37 °C, 95% O_2, 5% CO_2), and left to equilibrate for 60 min and washed every 20 min. The resting tension was maintained at 0.5 g and contractions were recorded isometrically. Using a paired experimental design, the non-cumulative concentration-response to 8-OHDPAT (1 nM - 30.0 μM) was established in non-contracted and in tissues pre-contracted with KCl (120 mM), in the absence and presence of WAY-100635 (5-HT_{1A} receptor antagonist, 1.0 μM, 60 min pre-treatment) (Fletcher et al., 1990) and SB269970A (5-HT_7 receptor antagonist, 1.0 μM, 60 min pre-treatment) (Hagan et al., 2000), using a 20 min drug cycle. Tension changes were expressed as the mean ± s.e. mean of the absolute values or percentage of KCl-induced contraction of n=5 and analysed using paired student's t-test.

8-OHDPAT did not induce a contractile response at any concentration examined. Indeed, 8-OHDPAT at 3.0 to 30 μM produced a concentration-dependent relaxation in pre-contracted tissues with KCl. The relaxation response induced by 8-OHDPAT was not significantly modified in the presence of WAY-100635 (1.0 μM) or SB269970 A (1.0 μM) (figure 1).

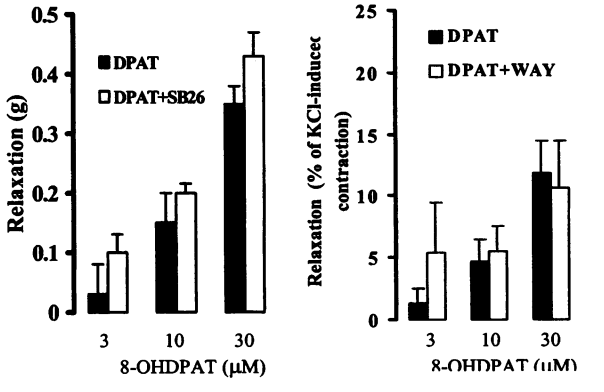

Figure 1. The effect of WAY-100635 (WAY, 1.0 μM) and SB269970A (SB26, 1.0 μM) on the relaxation response to 8-OHDPAT in the proximal region of *Suncus murinus* intestine.

The data suggest that the relaxation-induced by 5-$HT_{1A/7}$ receptor agonist, 8-OHDPAT, is mediated by 5-HT_{1A} or 5-HT_7 receptors as antagonism at these receptors did not modify the relaxation response.

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93P RENAL DOPAMINERGIC SYSTEM ACTIVITY IN CHRONIC RENAL FAILURE

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Dopamine exerts natriuretic and diuretic effects by activating D_1-like receptors located at various regions in the nephron (Jose *et al.*, 1992). Uninephrectomy results in increased renal dopaminergic activity and dopamine-sensitive enhanced natriuresis with no changes in blood pressure values (Vieira-Coelho *et al.*, 2000). The present study evaluated renal adaptations in sodium handling and the role of dopamine in ¾ nephrectomized (¾nx) rats, a model of chronic renal insufficiency.

Adult male Wistar Han rats (Harlan Ibèrica) weighing 201±2 g were anaesthetized with pentobarbital sodium (60 mg.kg bw^{-1}, ip) and submitted to nephrectomy of the right kidney and excision of both poles of the left kidney (¾nx rats, n=6) as previously described by Isaac *et al.* (1993). Control rats were Sham-operated animals (Sham rats, n=6). The animals were kept in metabolic cages and sacrificed with sodium pentobarbital two weeks after surgery. During this period the gain in body weight in ¾nx rats was not different from Sham controls. The assay of urinary levels of dopamine and precursor L-3,4-dihydroxyphenylalanine (L-DOPA) was performed by HPLC with electrochemical detection. The enzymatic assay of renal aromatic L-amino acid decarboxylase, the enzyme responsible for the synthesis of renal dopamine, was performed as previously described by Soares-da-Silva *et al.* (1998). All values are mean±sem. Statistical analysis was performed by one-way ANOVA, followed by Student's t-test for unpaired comparisons.

Two weeks after the surgery, remnant renal mass in ¾nx rats increased 97±7% in weight. In comparison with Sham rats, the creatinine clearance was reduced by 61±5% in ¾nx rats whereas the fractional excretion of sodium was markedly increased by 243±15%. This was accompanied in ¾nx rats by increases in both systolic (148±4 vs 122±1 mmHg, $p<0.0001$) and diastolic (116±4 vs 80±4mmHg, $p<0.0001$) blood pressure. The ¾nx rats presented lower urinary levels of dopamine (22.7±2.4 vs 32.7±2.3 nmol.g kd^{-1}.kg bw^{-1}.day^{-1}, $p<0.001$) as well as lower urinary dopamine/L-DOPA ratios (35.8±11.5 vs 84.2±18.5, $p<0.05$). This was accompanied in ¾nx rats by decreases in both V_{max} and K_m values for renal aromatic L-amino acid decarboxylase (V_{max}, 203±5 vs 265±3 nmol.mg $prot^{-1}$.$15min^{-1}$, $p<0.001$; *Km*, 2.7±0.2 vs 3.3±0.1 mM, $p<0.01$). Blockade of dopamine D_1 receptors (Sch-23390, 2×30µg.kg^{-1}.$3h^{-1}$, ip) in ¾ nx rats did not change the urinary excretion of sodium (4.1±0.4 vs 3.9±0.2 mmol.kg bw^{-1}.$6h^{-1}$).

We conclude that in contrast to uninephrectomized rats, the enhanced fractional excretion of sodium in ¾nx rats is accompanied with a decrease in renal dopaminergic activity, which may contribute to the increase in blood pressure.

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94P AGONISTS OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-α (CLOFIBRATE AND WY14643) REDUCE ISCHAEMIA/REPERFUSION INJURY OF THE RAT KIDNEY

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The peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (Evans, 1988). PPAR are expressed in the kidney (Guan & Breyer, 2001) and there is limited evidence that the PPAR-α agonist etoxomir can reduce renal dysfunction caused by ischaemia/reperfusion (I/R) of the kidney (Portilla *et al.*, 2000). The aim of this study was to investigate the effects of the PPAR-α agonists clofibrate (CLO) and WY14643 (WY) on the renal dysfunction and injury caused by I/R of the kidney of the anaesthetised rat.

Forty male Wistar rats (220-330 g) were anaesthetised using sodium thiopentone (120 mg kg^{-1} i.p.) and subjected to either bilateral renal ischaemia (45 min) followed by reperfusion (6 h) (20 rats) or sham-operation (20 rats) as described previously (Chatterjee *et al.*, 2000). Rats were administered either CLO (3 mg kg^{-1}), WY (1 mg kg^{-1}) or vehicle of PPAR-α agonists [10 % (v v^{-1}) dimethylsulphoxide, (DMSO)] twice as an i.v. bolus (2 ml kg^{-1}) 5 min before reperfusion and again after 3 h reperfusion. At the end of the experimental period, renal dysfunction and injury were assessed by measurement of serum creatinine (sCr), fractional excretion of Na^+ (FE_{Na}) and urinary *N*-acetyl-β-D-glucosaminidase (uNAG) activity. A histological score (HS) of renal injury (out of a total of 300) was determined as described previously (Chatterjee *et al.*, 2000) (Table 1).

Table 1	*N*	sCr (µM)	FE_{Na} (%)	uNAG (iu L^{-1})	HS
Sham+DMSO	12	44±2★	1±0★	3±2★	0±0★
Sham+CLO	4	44±2★	1±0★	9±4★	0±0★
Sham+WY	4	33±1★+	1±0★	6±2★	0±0★
I/R+DMSO	7	176±7+	17±1+	32±9+	240±6+
I/R+CLO	7	152±6★+	7±0★+	10±2★+	198±8★+
I/R+WY	6	144±8★+	7±0★+	4±1★	156±8★+

Table 1: Effect of PPAR-α agonists on biochemical and histological indicators of renal dysfunction and injury. ★$P<0.05$ *vs.* I/R+DMSO, +$P<0.05$ *vs.* Sham+DMSO. Data are expressed as mean±s.e.mean, analysed using one-way ANOVA followed by Dunnett's *post-hoc* test for multiple comparisons.

Renal I/R produced significant increases in sCr, FE_{Na}, uNAG and HS which were reduced significantly by the PPAR-α agonists clofibrate and WY14643 (Table 1). These results demonstrate that administration of PPAR-α agonists during reperfusion can significantly reduce the renal dysfunction and injury caused by I/R of the kidney. The mechanisms underlying this beneficial effect and the role of the PPAR-α in the development of renal I/R injury warrant further investigation.

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P.K.C. is supported by the NKRF (Project Grant R41/2/2000)

95P THE EFFECT OF ASCORBIC ACID ON ARSENIC INDUCED RELEASE OF N-ACETYL-BETA-D-GLUCOSAMINIDASE FROM RAT KIDNEY LYSOSOMES IN VITRO

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There is evidence that arsenic-induced cell toxicity is mediated through the generation of reactive oxygen species (ROS) (Wang et al, 1996). Ascorbic acid (AA) is a free radical scavenger but recent studies indicate that it can also act as pro-oxidant, particularly in the presence of compounds that increase the production of ROS (Sakagami & Satoh, 1997; Bijur et al., 1997). We have therefore investigated if arsenic induced renal toxicity is affected by the presence of AA. Lysosome-rich fractions from the renal cortex of freshly killed male Wistar rats (300-325 g) were prepared (Win-Aung et al., 1998) and incubated at 37^{0}C with varying amounts of sodium arsenate (SA) for 30 min to 2 h. The release of N-acetyl-β-D-glucosaminidase (NAG) isozymes to indicate lysosomal damage was estimated using spectrophotometric methods. The protein content of renal cortex was also determined (Hartree,1972). The statistical significance of differences between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's all pair-wise comparison.

Arsenic caused concentration and time dependent release of NAG isozymes from the renal lysosomes, which was either increased or decreased in the presence of AA. The results show that NAG isozyme release with 32 mM SA was increased with AA concentrations below 6mM but was inhibited with AA concentration above 11mM table1). Arsenic compounds can increase urinary NAG activity in animals whether given chronically in low doses (Lin et al.2000) or acutely as a single high doses (Price 1979). AA is interchangeably converted into dehydroascorbic acid (DHA) which can cause lysosomal labilisation (Chari et al., 1983). Since arsenic compounds also have oxidant property it seems that the biphasic effect AA on NAG isozyme release by SA was the result of changed AA: DHA ratio with a higher formation of DHA in the presence of lower AA concentrations in the incubating medium.

Table 1. The lysosomal release of NAG isozymes after incubation at 37^{0}C for 60 min with 32 mM of SA.

AA (mM)	SA	NAG isozymes (EU*	p value
-	-	0.641 ± 0.185	-
-	+	2.801 + 0.049	<0.01[a]
0.71	+	3.928 ± 0.091	<0.01[b]
2.84	+	3.560 ± 0.455	<0.01[b]
5.86	+	2.917 ± 0.083	>0.05[b]
11.36	+	1.859 ± 0.084	<0.01[b]

[a] Compared with control without SA; [b] compared with SA containing control. (Mean ± SD; n =6)
* 0ne EU will hydrolyze 1nM of substrate/min/mg protein

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96P FLUORIDE CAUSES RELEASE OF RENAL LYSOSOMAL N-ACETYL-BETA-D-GLUCOSAMINIDASE ISOZYMES WITH BIPHASIC EFFECT ON MDA LEVELS IN VITRO

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N-acetyl-beta-D-glucosaminidase (NAG) isozymes of lysosomal origin in urine are widely used for the diagnosis of kidney disease (Xu et al., 1999) and free radicals play an essential role in the nephrotoxicity induced by various xenobiotics (Cuzzocrea et al., 2002). While the mechanism of renal cell damage is not clearly understood some studies have indicated the cellular injury induced by free radicals may be mediated through lysosomal damage (Ollinger & Brunk, 1995). Since fluoride (F) can cause lipid peroxidation (Guan et al., 2000) and can release NAG from renal cells (Cittanova et al., 2002), we have investigated if a temporal relationship exists between the two events when renal lysosomes are exposed to F under in vitro conditions.

Lysosome-rich fractions (Win-Aung et al. 1998) obtained from the renal cortex of freshly killed male Wistar rats (300-325 g) were incubated for 2 hr at 37^{0}C with varying amounts of F ranging from 0.37 to 192 mM. The release of NAG isozymes and malondialdehyde (MDA) in the supernatant was estimated by the spectrophotometric and HPLC techniques respectively. The protein content of renal cortex was also determined (Hartree,1972). F causes a concentration dependent effect on MDA formation and the release of NAG isozyme from renal lysosomes (Fig.1). In low concentrations it causes a stimulation of MDA formation without releasing NAG isozymes. With F concentrations higher than 24 mM there is a concentration dependent release of NAG isozymes from the renal lysosomes and a marked fall in MDA values in the supernatant. Since F induced NAG release occurs at higher concentrations than those producing increased free radical formation /lipid peroxidation the fall in free radical activity may be related to the antioxidant potential of some of the released lysosomal enzymes and may form part of a defence mechanism for these organelles.

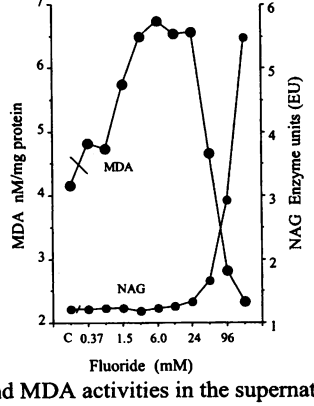

Fig 1.NAG and MDA activities in the supernatant of renal lysosomes incubated with sodium fluoride at 37^{0}C for 2 hr. 0ne EU will hydrolyze 1nM of substrate/min/mg protein (n=6). For clarity, mean values without SD are shown.

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97P EVIDENCE FOR A HETEROGENEOUS RECEPTOR POPULATION MEDIATING CONTRACTILE RESPONSES TO OXYTOCIN IN RAT UTERUS

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Radioligand binding assays have suggested that rat myometrium may contain both oxytocin (OT) and vasopressin (VP) receptors (Chan *et al.*, 1990). However, the functional relevance of this heterogeneous receptor population has not been assessed in detail. The aim of the present experiments was to characterise the receptors mediating OT-induced contractions in rat uterus using an analytical pharmacological approach.

Female, Sprague-Dawley rats (150-250g) were pre-dosed with oestradiol (0.5mg/kg subcutaneously) 24 hours prior to euthanasia. Uterine strips were mounted longitudinally in organ baths and perfused with Krebs buffer at 32°C. Cumulative concentration-effect (E/[A]) curves were constructed to the agonists OT or [Thr^4,Gly^7]OT (TGOT) in the absence or presence (30 min pre-incubation) of the selective OT receptor antagonist, L-368,899 (1-(((7,7-dimethyl-2(S)-(2(S)-amino-4-(methylsulfonyl)-butyramido)-bicyclo[2.2.1]-heptan-1(S)-yl)methyl)sulfonyl)-4-(2-ethylphenyl)piperazine). Individual E/[A] curves were fitted to the Hill equation and EC_{50} values in the absence and presence of antagonist were used to calculate pK_B values according to the method of Lew & Angus (1995). Data are presented as mean ± s.e.mean or 95% confidence intervals and statistical comparisons were made using two-sided Student's t-test or one-way analysis of variance (ANOVA).

OT and TGOT caused concentration-dependent contractions (EC_{50} 5.99±0.53 and 2.62±0.31nM respectively; n=20) that were inhibited by L-368899 (10-300nM) in a concentration dependent manner with similar potency (pK_B 7.75±0.06 and 8.00±0.10 respectively; n=8-11). Schild slope parameters were not significantly different from unity (0.71-1.16 and 0.98-1.18) for OT and TGOT, respectively), however the Hill slope (n_H) of the OT (but not TGOT) E/[A] curve significantly increased with increasing doses of L-368,899 [n_H = 0.81±0.03, 0.91±0.04, 0.93±0.03, 0.91±0.04 and 0.99±0.07 in the absence and presence of 10, 30, 100, and 300nM L-368,899, respectively; n=6-22], inconsistent with expectations for simple competitive antagonism.

Our thesis is that the complex antagonism of OT by L-368,899 provides further evidence for the involvement of a heterogeneous receptor population of OT and VP or 'VP-like' (Anouar *et al.*, 1996) receptors, the exact identity of which is currently under investigation. This study also supports previous findings (Van der Graaf *et al.*, 1996) that analysis of Hill slope parameters might be a more sensitive analytical tool to detect receptor heterogeneity than the more commonly used Schild regression or agonist-dependency of antagonist potency.

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98P THE ROLE OF ATP AND ADENOSINE IN THE INHIBITORY EFFECTS OF THE UROTHELIUM ON DETRUSOR MUSCLE OF THE PIG

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In recent years it has become clear that the urothelium has many roles in both the sensory and motor functions of the bladder. The pig urothelium has been shown to release an unidentified factor (not nitric oxide or a prostaglandin), which inhibits the underlying smooth muscle (Hawthorn *et al* 2000). Since it has been shown that ATP can induce relaxation responses in the marmoset bladder (McMurry *et al* 1998) and stretch of the rabbit urothelium induces ATP release (Ferguson *et al* 1997), the aim of the present study was to examine whether the inhibitory factor could be ATP or its metabolite adenosine.

The bladders from female pigs were obtained from a local abattoir. Paired longitudinal strips of detrusor smooth muscle were isolated from the pig bladder dome (approx 10 x 5 mm) and the urothelium removed from one strip of each pair. The tissues were suspended in 30 ml organ baths, bathed with Krebs-bicarbonate solution (gassed with 95% O_2 / 5% CO_2), placed under 1g of tension and maintained at 37°C. Cumulative concentration response curves to carbachol were constructed and the responses of urothelium-denuded tissues were compared with control intact bladder strips. Experiments were then repeated in the absence and presence of various antagonists and inhibitors of ATP and its metabolite adenosine. All results are expressed as mean ± standard error and comparisons made using paired Student T-test.

In the presence of an intact urothelium, responses to carbachol were reduced to 42.9 ± 4.3% (n=10, P<0.001) compared with the control urothelium-denuded muscle strips whilst detrusor sensitivity to carbachol was similar in the intact (pEC_{50} = 5.6 ± 0.12) and urothelium-denuded tissue (pEC_{50} = 5.39 ± 0.11).
In the presence of the P1-receptor antagonist 8-phenyltheophylline (10μM, n=5) or adenosine deaminase (0.1 units ml^{-1}, n=4) maximum responses to carbachol were still depressed in the presence of an intact urothelium (47.2 ± 8.4%, 51.1 ± 8.8% respectively). Similarly the P2-receptor antagonist suramin (100μM, n=6) and the P2X-receptor antagonist PPADS (10μM, n=5) failed to alter maximum responses obtained in the presence of the urothelium (48.4 ± 15.2 % and 50.5 ± 5.1% respectively). In the presence of the P2Y antagonist cibacron blue (100μM, n=6) the urothelium failed to inhibit responses to carbachol, maximum responses in the intact tissues being similar to denuded tissues (88.3 ± 20.0 %).
In a separate series of experiments neither ATP or adenosine produced relaxation in carbachol or AVP- precontracted tissues.
In conclusion, neither ATP nor adenosine appears to be involved in the inhibitory influence of the urothelium on the underlying detrusor muscle. The actions of cibacron blue may be non-specific effects unrelated to P2Y receptor antagonism.

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99P INVESTIGATION OF THE EFFECTS OF PHENOL RED $P2X_1$ AND $P2X_3$ RECEPTOR ANTAGONIST ON THE MICTURITION REFLEX IN ANAESTHETIZED FEMALE RATS

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Using a selection of purinoceptor ligands α, β-methylene ATP, PPADS (Knowles *et al.*, 2000), IP_5I, and MRS 2179 (Knowles *et al.*, 2001) a role for $P2X_1$ and $P2X_3$ receptors has been identified in the control of the micturition reflex in anaesthetized female rats. The present experiments were carried to determine the effects of Phenol Red (phenolsulfonphthalein sodium salt), which has recently been demonstrated to block both $P2X_1$ and $P2X_3$ receptors (King *et al,* 2002), on the micturition reflex.

Experiments were performed on spontaneously breathing female Sprague-Dawley rats (230-250g), anaesthetized with urethane (1.2 g kg^{-1}, i.v.). Simultaneous recordings were made of urinary bladder and urethral perfusion pressures and external urethral sphincter (EUS) EMG as well as BP and HR, as previously described (Wibberley *et al.*, 2002). Micturition reflexes were evoked by distension of the urinary bladder with saline infusion (0.05 ml min^{-1}). Reflex- and drug-evoked responses (in mmHg) were expressed as percentage changes before and after the administration of drug, saline or DMSO. Changes caused by drug were compared with that of vehicle controls using an unpaired Student's *t*-test. All values are mean ± s.e. mean.

Administration (i.v.) of Phenol Red (0.1 and 1.0 μg kg^{-1}, n=5) caused a significant ($P< 0.05$) increase in the pressure (84 ± 9% and 60 ± 3% respectively) and volume (72 ± 8% and 58 ± 5%) thresholds required to evoke micturition. At the low dose, there was no effect of the size of the evoked bladder contractions; however, at the higher dose the evoked bladder contraction was significantly reduced in amplitude by 20 ± 6%. Phenol Red at both doses failed to affect the associated changes in urethral relaxation and increases in EUS–EMG activity. This is somewhat surprising as a reduced bladder contraction is usually associated with a reduced urethral relaxation. Baseline values were unaffected.

The effects of Phenol Red were similar to IP_5I, in that both cause a reduction the reflex-evoked isovolumetric contractions. For IP_5I, this was associated with a reduction in urethral relaxation. All the other compounds tested caused a maintained contractions during the filling phase, whereas Phenol Red caused an increase pressure and volume thresholds, although not dose related. The combined data favours a role for $P2X_1$ receptor in the evoked bladder contraction. If $P2X_3$ is associated with sensory signalling (Chen *et al.*, 1995) the ability of these compounds to interfere with threshold and to cause maintained contraction during filling could be attributed to interference with $P2X_3$ receptors, although Phenol Red has a qualitative difference to the other ligands in this respect.

Supported by Roche Bioscience, Palo Alto, U.S.A.

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100P COMPARISON OF DIFFUSION OF AN 18-MER ANTISENSE MOLECULE ACROSS THREE HOLLOW FIBRE DIALYSIS MEMBRANE TYPES *IN VITRO*

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Selective receptor 'knockdown' *in vivo* by antisense oligodeoxynucleotides (ODN) is an increasingly used tool in the neurosciences. Due to limited access to the CNS following systemic administration, studies to date have employed intracerebroventricular administration or focal injection in target structures. Reverse microdialysis potentially offers advantages over both these procedures, allowing discrete administration within the CNS without introducing volume. However, the high molecular weight of antisense molecules (typically 4-6 kDa) may limit the utility of this approach due to low diffusion across the dialysis membrane. In order to assess this we have determined the delivery of an 18-mer ODN (phosphorothioate (PTO) 'end capped' and unmodified forms) across three membrane types *in vitro*.

Microdialysis probes equipped with a 2 mm length of either a polycarbonate (PC) or polyethersylfone (PES) membrane (nominal molecular weight (MW) cut-off 20 and 100 kDa respectively; CMA/Microdialysis, Sweden) or a polyamide (POLY) membrane (Polyflux®, MW cut-off 30 kDa; Gambro-Hospal, UK) were compared. Membrane fibre outer diameter dimensions were 0.5, 0.5 and 0.27 mm respectively. The antisense ODN was designed to hybridise with a homologous sequence of mRNA encoding for the rat $GABA_B$ R1a and $GABA_B$ R1b subunits (Morris *et al.*, 1998). Molecular weights were 5451 and 5483 Da for unmodified and PTO-modified ODN respectively (MWG-Biotech (UK) Ltd.). Probes were perfused at 0.5 μl.hr^{-1} with an artificial cerebrospinal fluid solution (aCSF) containing 1 mM ODN and the membrane immersed in 1.5 ml aCSF at ambient temperature (20-22°C). After a period of 20-22 hr, the concentration of ODN in the bathing solution was determined by absorbance spectroscopy (260 nm). Results are displayed in Table 1.

	% *in vitro* ODN delivery	
	unmodified	PTO-modified
PES	82.2 ± 2.3	70.2 ± 1.6*
POLY	69.1 ± 1.3	65.6 ± 0.7
PC	5.6 ± 1.3	5.0 ± 1.0

Table 1: Data is presented as mean ± s.e. mean, n=3. *$p<0.05$ compared to 'unmodified', Student's t-test (two-sided).

The rank order of delivery was in line with, but not well correlated to, the nominal MW cut-off of the membranes employed. Thus, diffusion across the PC membrane was markedly lower than either the PES or POLY membranes. The reason for lower delivery of the PTO-modified ODN across the PES membrane is not known, but is unlikely to be due to the small difference (<1%) in molecular weight between these molecules. Rather, this finding may reflect a higher degree of binding of the PTO-modified molecule to the PES membrane. Regardless, the high rate of diffusion of ODN across both the PES and POLY membranes indicates that are suitable for delivery of antisense molecules *in vivo* (see Bowery *et al.*, this meeting).

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101P THE FUNCTIONAL –759C/T POLYMORPHISM OF THE 5-HT2C RECEPTOR GENE IS UNRELATED TO [3H]MESULERGINE BINDING IN HUMAN BRAIN

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Polymorphisms of the promoter region of the 5-HT2C receptor gene show association with obesity and diabetes (Yuan *et al*, 2000), with drug-induced weight gain (Reynolds *et al*, 2002) and with the incidence of tardive dyskinesia (Zhang *et al*, 2002). It has been suggested that these functional polymorphisms have effects on promoter activity (Yuan *et al*, 2000). We have investigated whether a polymorphism that can influence drug-induced weight gain has effects on the density of 5-HT2C receptors in human brain.

Post-mortem tissues from a series of 53 brains were successfully genotyped for the C-759T polymorphism of the 5-HT2C receptor gene (Reynolds *et al*, 2002). 6 of 28 males (hemizygotes) and 9 of 25 females (heterozygotes) were identified as having the variant –759T allele of this X-linked gene. Binding of 4nM [3H]mesulergine to 5-HT2C receptors was determined in tissue from the putamen of 44 of these cases following a modification of the method of Marazziti *et al* (1999), in which the tissue was homogenized and centrifuged at 50,000g; the pellet was then used following reconstitution in buffer. Binding to hypothalamic 5-HT2C receptors was also undertaken in genotypically-differing males matched for age.

A significant negative correlation (p=0.045) between age and 5-HT2C binding in the putamen was observed. Age was thus included as a covariate in ANOVA. Sex was shown to have a significant effect on 5-HT2C receptor binding but genotype did not (table 1a), nor was there a significant interaction between genotype and sex. Comparison of radioligand binding to the 5-HT2C receptor in both putamen and hypothalamus for the matched male subjects also showed no significant differences between genotype (table 1b).

Table 1a Effect of –759 genotype and sex on binding to the 5HT2C receptor in the putamen

-759 genotype:	C or C/C (n=33)	T or C/T (n=11)
	3.57 ± 1.50	4.43 ± 2.19
Sex:	Male (n=24)	Female (n=20)
	3.37 ± 1.36	4.30 ± 2.00*

Table 1b Effect of –759 genotype on binding to the 5HT2C receptor

-759 genotype:	C (n=6-7)	T (n=5)
putamen	3.67 ± 1.54	3.33 ± 1.57
hypothalamus	4.55 ± 3.51	3.19 ± 1.89

Data expressed as mean ± SD in pmol/g tissue. *p=0.021

These results suggest that, within the substantial limitations of the radioligand binding technique, the –759C/T polymorphism does not substantially influence the levels of 5-HT2C receptors in the human striatum and hypothalamus. Both age and sex of subjects appear to influence receptor binding. It remains unclear what might be the molecular mechanisms underlying the association of the polymorphism with, for example, drug-induced weight gain.

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102P THE 5-HT_{1A} RECEPTOR ANTAGONIST ROBALZOTAN ENHANCES SSRI-INDUCED EJACULATION DELAY IN THE RAT

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Selective serotonin reuptake inhibitors (SSRIs), which are primarily indicated for the treatment of depression (Feighner & Boyer, 1991) have also been used, off-label, to treat premature ejaculation (PE) by prolonging intravaginal ejaculatory latency time (Waldinger *et al.*, 1998). A rat behavioural model of copulation was used to explore the role of 5-HT_{1A} receptors in the delay in ejaculation with the SSRIs fluoxetine (FLX) and citaolopram (CIT). The selective 5-HT_{1A} receptor antagonist robalzotan (ROB, aka NAD-299) (Johansson et al., 1997) was administered with and without SSRIs. The effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT on copulatory behaviour were also measured.

Sexually experienced male rats (Sprague Dawley, 350-450g, n=7-11 per dose group), were dosed with either vehicle (VEH 1, 0.9% saline) or FLX or CIT (10 $mg.kg^{-1}$ sc) once daily for 11 days. ROB (15 $ug.kg^{-1}$ sc) was administered 15 minutes prior to each test. Sexual behaviour was tested 4h after dosing with SSRIs on days 1 and 11 of the study (males were placed into an open-field arena with red light, in the dark portion of a 12h light:dark cycle with a sexually experienced female rat (Sprague Dawley, 200-300g) in behavioural oestrous). Ejaculation latency (EL, time in seconds from first intromission to first ejaculation) was measured. The acute effects of 8-OH-DPAT (0.25 $ug.kg^{-1}$ sc, n=4, 15 minutes pre-test) were similarly tested against a separate control group of rats (VEH 2). Statistical analysis was made using ANOVA and subsequent pair-wise comparison of means.
The EL in each treatment group is shown in the table below. Data are shown as mean ± s.e. mean with statistical significance indicated at the 5% level (* = p<0.05).

	Day 1	Day 11
VEH 1	146 ± 19	118 ± 15
FLX	282 ± 107	457 ± 188*
CIT	208 ± 31	304 ± 59
VEH + ROB	202 ± 31	211 ± 49
FLX + ROB	954 ± 355*	854 ± 234*
CIT + ROB	523 ± 177*	487 ± 273*
VEH 2	436 ± 99	Not tested
8-OH-DPAT	54 ± 22*	Not tested

There is a synergy between SSRIs and 5-HT_{1A} receptor antagonists in the delay of ejaculation. If this result translates to man, therapeutic approaches that inhibit serotonin re-uptake and 5HT_{1A} receptors may be useful as an effective on-demand treatment for PE.

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103P IMETIT INDUCES SCRATCHING WHICH IS ANTAGONISED BY THIOPERAMIDE IN BALBC MICE

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Pruritus (itch) is clinically important; histamine plays a role in mediating itching in some disorders (e.g. urticaria), but, in disorders such as atopic eczema, H_1 and H_2 histamine receptor antagonists are largely ineffective at relieving itching (Hagermark, 1992).
We are studying experimentally induced scratching in mice to investigate the mechanism(s) of action of putative mediators of itch. We recently reported that the H_3 receptor antagonist thioperamide reduced histamine-induced scratching in BalbC mice (Bell et al, 2002). We have now investigated the itch inducing properties of the histamine H_3 agonist, imetit.

Scratching was induced in female BalbC mice by intra-dermal (i.d.) injections of imetit into the back of the neck; phosphate buffered saline (PBS, pH=7.4; 100µl; 26G needle) was used as a control. Itch was measured by recording scratching of the neck by the hind limbs during the 20 min period directly following an injection. Six mice (18-21g) were given three doses of imetit ($3x10^{-8}$ – $3x10^{-6}$mol) at hourly intervals and the number of bouts of scratching (3 or more individual scratch movements = 1 bout) evoked was recorded.
Thioperamide (20mgkg^{-1}, i.p.) was given 30 mins prior to the highest dose of imetit ($3x10^{-6}$mol) in three mice (21-22g). Experiments lasted for up to 4 hours and all mice were later killed with an overdose of sodium pentobarbitone (24mg i.p.).

Imetit induced scratching at all three doses administered, dose dependently. Scratching increased from 9±6 at $3x10^{-8}$mol to 23±9 at $3x10^{-7}$mol and 56±10 at $3x10^{-6}$mol (n=6). Imetit induced scratching was significantly greater than that induced by saline vehicle (5±1, n=12) at the highest dose ($P<0.05$) but not at the two lower doses ($P>0.05$, Kruskal-Wallis test with Dunn's post hoc test).

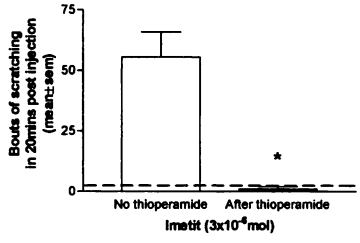

Figure 1. Scratching induced by imetit was significantly reduced by thioperamide (*$P<0.05$, Mann Whitney test) and did not differ significantly from PBS ($P>0.05$, Mann Whitney test) Dashed line illustrates level of PBS-induced scratching.

In summary, imetit induces scratching in BalbC mice and this effect can be reduced by thioperamide. This suggests a role of H_3 receptors in the perception of itch. Human studies are needed to establish whether this finding has a clinical application.

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Bell, J.K. *et al* (2002). *British Journal of Pharmacology* (Glasgow meeting, in press)

Acknowledgment: We wish to thank the British Skin Foundation for a PhD studentship to J. Bell.

104P SUB-CHRONIC ADMINISTRATION OF THE CB_1 RECEPTOR ANTAGONIST, SR 141716, PREFERENTIALLY DECREASES BODY WEIGHT IN OBESE (*fa/fa*) COMPARED TO LEAN ZUCKER RATS

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It is widely reported that CB_1 receptor agonists increase food intake, and that CB_1 receptor antagonists reduce food intake, in a range of animal species (Kirkham and Williams, 2001). Furthermore, levels of endocannabinoids are increased in obese mice and rats (Di Marzo et al., 2001). The objective of the present study was to investigate whether treatment with the selective CB_1 receptor antagonist, SR 141716, had a preferential effect on the body weight and daily food intake of genetically obese (*fa/fa*) Zucker rats compared to lean (*FA/FA or FA/fa*) controls. In addition, groups of pair-fed obese (*fa/fa*) Zucker rats were included to assess whether the effects of SR 141716 on body weight in obese animals were attributable to decreased food intake.

Male obese (*fa/fa*) Zucker rats (449-602g at start) held on a 12h light/dark cycle (lights on 0800h) were administered vehicle, SR 141716 (3, 10 or 30 mg/kg; p.o.) or sibutramine (SIB; 5 mg/kg; p.o) once daily for 7 days (n=5) between 09.00 and 10.00h. Lean male age-matched (*FA/FA or FA/fa*) Zucker rats (306-415g at start) were treated in an identical manner (n=5).

SR 141716 (3-30 mg/kg) decreased mean daily food intake and body weight in both obese (*fa/fa*) and lean Zucker rats (Table 1). The effects on body weight were significantly greater in obese animals (Table 1), while at the top dose, 50% and 16% reductions in mean daily food intake were observed in obese and lean animals respectively. Since pair-fed controls exhibited a similar reduction in body weight gain compared to drug-treated animals, the data suggest that the more pronounced effects of SR 141716 on weight gain in obese animals is likely to be attributable to the greater reduction in food intake over 7-days. In contrast, the monoamine reuptake inhibitor sibutramine did not have a more pronounced effect in obese animals..

Table 1. Effect of SR 141716 or Sibutramine treatment on mean body weight and mean daily food intake of obese (*fa/fa*) and lean Zucker rats.

DOSE mg/kg	BODY WEIGHT CHANGE (g)			DAILY FOOD INTAKE (g)	
	Obese	Pair-fed obese	Lean	Obese	Lean
0	12.6 (3.5)	-	6.2 (1.5)	30.5	20.5
3	-8.2 (3.7)**	-7.8 (3.5)	-1 (2.8)	21.7**	19.3
10	-13 (1.8)**	-14.2 (2.2)	-10 (2.3)**	19.5**	18.2**
30	-34 (3.7)** $	-31.8 (2.6)	-10.6 (2.2)**	14.3**	17.2**
SIB	-13 (6.0)**	-13 (7.2)	-19.4 (2.5)**	20.0**	14.9**

Data are mean ± (s.e.m). *$p<0.05$, **$p<0.01$ significantly different to vehicle using Dunnett's test. $$p<0.01$ significant Newman-Keuls comparison of obese and lean body weight changes after significant dose x phenotype interaction $F(4,40)=7.2$, $p<0.001$.

These data demonstrate that sub-chronic, once daily, treatment with SR 141716 preferentially reduces the body weight of obese (*fa/fa*) Zucker rats compared to lean controls. The results obtained with pair-fed animals indicate that the effects of SR 141716 on body weight are likely to be attributable to the effects of the drug on daily food intake.

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105P EFFECT OF THE CB_1 RECEPTOR ANTAGONIST SR 141716 ON FOOD INTAKE, BODY WEIGHT AND THE BEHAVIOURAL SATIETY SEQUENCE IN OBESE (*fa/fa*) ZUCKER RATS

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Peripheral administration of the selective CB_1 receptor antagonist SR 141716 (Rinaldi-Carmona *et al.*, 1994) has been shown to reduce ingestive behaviour in animals (Simiand *et al.*, 1998; Colombo *et al.*, 1998), however sub-chronic efficacy has not as yet been demonstrated in an animal model of obesity. The present study investigated the behavioural specificity of the reduction in food intake and body weight gain induced by SR 141716 in a genetic model of obesity.

Male obese (*fa/fa*) Zucker rats (509-689 g, n=7 for all treatment groups) held on a 12-hour light/dark cycle with free access to food and water were used. Animals were habituated to a daily one-hour presentation of a weighed amount of palatable mash diet. SR 141716 (1, 3 or 10 mg $kg^{-1}day^{-1}$), sibutramine (sib; 3 mg $kg^{-1}day^{-1}$; positive control) or vehicle was orally administered 1 hour before mash presentation for 5 days. On days 1, 3 and 5 the behaviour of the rats was observed during the 60-minute mash access period. Terminal blood samples were taken on day 5 and plasma triglyceride levels were determined. Data were analysed using ANOVA and Dunnett's test.

SR 141716 (1-10 mg kg^{-1}) significantly reduced palatable mash and daily chow intake compared to vehicle treated animals for the duration of the study (Table 1). There was also a dose-related decrease in body weight gain with a significant fall in plasma triglyceride levels after sub-chronic SR 141716 administration (Table 1). Behavioural analysis revealed that SR 141716 preserved the satiety sequence of the animals. Hence, on day 1 eating was succeeded by a period of active behaviour before initiation of an extended grooming sequence followed by resting. SR 141716 (1-10 mg kg^{-1}) led to an overall decrease in total feeding behaviour which was maintained from days 1 to 5 (number of feeding observations on day 1, mean ± s.e.m.: (0) 41.1 ± 4.2, (1) 28.0 ± 3.8, (3) 18.7 ± 1.8 $p<0.01$, (10) 19.9 ± 2.7 $p<0.01$, (sib) 38.1 ± 2.4). This was accompanied by an increase in total grooming behaviour (number of grooming observations on day 1, mean ± s.e.m.: (0) 16.1 ± 2.6, (1) 30.7 ± 6.1, (3) 57.9 ± 6.7 $p<0.01$, (10) 62.7 ± 6.4 $p<0.01$, (sib) 8.0 ± 1.9). The incidence of total active and resting behaviours was not affected. Interestingly on days 3 and 5 the behavioural satiety sequence appeared temporally advanced with an earlier offset of feeding and earlier onset of active and grooming behaviours. This is similar to the profile typically observed after treatment with compounds that enhance satiety e.g. *d*-fenfluramine (Hewitt *et al.*, 2002) or sibutramine (Halford *et al.*, 1998).

Table 1: Effect of SR 141716 (1, 3, 10 mg kg^{-1}) and sibutramine (3 mg kg^{-1}) on palatable mash consumption, daily chow intake, daily body weight gain and plasma triglyceride levels in obese (*fa/fa*) Zucker rats. Day 5 data.

Treatment mg kg^{-1} (n=7)	Mash intake (g)	Chow intake (g)	Body weight change (g)	Triglycerides (mmol/l)
Vehicle	30.9±2.2	22.3±1.7	+4.3±1.5	5.1±0.5
1	18.7±0.9**	19.5±0.9	-4.1±1.3	5.0±0.4
3	13.1±2.2**	14.8±2.4**	-15.7±3.3**	3.5±0.3*
10	13.3±1.4**	10.9±1.3**	-20.3±4.4**	2.6±0.3**
Sib	19.4±2.0**	14.5±1.2**	-10.6±0.8**	3.2±0.5**

Mean ± s.e.m., *$p<0.05$ **$p<0.01$ compared to vehicle using Dunnett's test after significant ANOVA.

Thus, once-daily oral administration of SR 141716 significantly reduced food intake and body weight gain in obese (*fa/fa*) Zucker rats, an effect maintained for 5 days that also resulted in a significant fall in plasma triglycerides. Behavioural observation of the animals revealed a classical satiety sequence, suggesting that the hypophagic action of SR 141716 was not attributable to non-specific disruption of behaviour.

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Simiand J., *et al.*, (1998) Behav. Pharmacol., 9, 179-181

106P THE ANTICONVULSANT LAMOTRIGINE IMPROVES THE SELECTIVE IMPAIRMENT IN REVERSAL LEARNING INDUCED BY PCP (PHENCYCLIDINE) IN THE RAT

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Recent clinical findings suggest that lamotrigine may usefully augment clozapine in the treatment of patients with chronic refractory schizophrenia (Dursun et al, 1999). Human volunteer studies have shown that lamotrigine, unlike most antipsychotic drugs, can reverse psychotomimetic symptoms induced by ketamine (Anand et al, 2000). The aim of the present study was to determine whether lamotrigine could improve a PCP-induced deficit in performance of a reversal learning task in the rat. Subjects were 40 adult female hooded-Lister rats (Harlan, UK) housed under standard laboratory conditions on a 12h light:dark cycle (lights on 0700h). Rats were maintained at 85% free-feeding weight (approx 225g) and trained to perform an operant reversal learning task to 90% criterion by a lever-pressing selection method previously described in detail (Abdul-Monim et al. 2003). Data are expressed as mean ± SEM (n=8-10 per group) of percentage correct responding in the initial and reversal phases of the task (5 min per phase). Statistical comparisons were made using a 2 way ANOVA with *post-hoc* Bonferroni corrected t-test. PCP was injected ip 30 min prior to testing, lamotrigine isethionate was injected ip 90 min prior to testing, and clozapine was injected ip 60 min prior to testing. All doses are base equivalent weight. Doses of 1.5 and 2.0 mg/kg of PCP induced a selective and significant reduction in performance of the reversal task only ($p<0.01$ and $p<0.05$ respectively).

Percent correct responding was reduced from 77.9±5.0 to 48.3±7.0 in the reversal task following 1.5mg/kg PCP; the initial task was unaffected by PCP. Lamotrigine alone (5-30mg/kg) had no effect on cognitive performance. However, doses of 20 and 30mg/kg significantly improved the selective impairment in reversal task performance induced by 1.5mg/kg PCP ($p<0.01$). Percent correct responding in the reversal task increased from 26.4±6.2 (PCP alone) to 70.2±3.7 (PCP plus 20mg/kg lamotrigine) and 61±8.1 (PCP plus 30mg/kg lamotrigine). Clozapine (5mg/kg) also significantly improved the selective PCP-induced deficit in reversal task performance ($p<0.05$). Percent correct responding in the reversal task increased from 40.7±6.6 (PCP alone) to 66.3±6.5 (PCP plus 5mg/kg clozapine). These data provide strong evidence that lamotrigine, at anticonvulsant doses, and clozapine, can attenuate behavioural symptoms induced by PCP in rodents. The novel antipsychotic, ziprasidone, but not haloperidol, has recently been shown to improve PCP-induced deficits in this task which may represent cognitive impairment of particular relevance to the pathology of schizophrenia (Abdul-Monim et al. 2003). Results suggest that lamotrigine may improve cognitive dysfunction associated with schizophrenia.

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107P EFFECT OF LEVETIRACETAM ON THE DECREASED [^{14}C] 2-DEOXYGLUCOSE UPTAKE THAT IS SEEN IN PENTYLENETETRAZOLE-KINDLED MICE

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Levetiracetam (LEV) is an antiepileptic drug with a unique profile in animal models of epilepsy. It has little efficacy against seizures in naïve animals, but high efficacy against kindled seizures (Klitgaard et al., 1998). Currently the mechanism of action of LEV is unknown, although a unique stereoselective binding site has been identified in the CNS (Noyer et al., 1995). In order to determine if LEV acted in specific brain areas, we investigated the effect of LEV on [^{14}C] 2-deoxyglucose (2-DG) uptake in pentylenetetrazole (PTZ) kindled mice.

Male NMRI mice (20-24 g) were injected (IP) with 37 mg/kg PTZ or saline every other weekday for 3 weeks. Following a five-day drug-free period, the PTZ-kindled animals were divided into three groups (n=5-6), which received an injection (SC) of saline, 40 mg/kg or 80 mg/kg LEV (relevant anticonvulsant doses). The chronic saline-treated mice (n=6) were injected with saline. Twenty minutes after drug or saline injection, the mice received an IV injection of 0.1 μCi/g 2-DG and were decapitated 45 minutes later. Brains were removed, frozen on dry ice and cut into 20 μm sections on a cryostat. These sections and Amersham standards were covered with Kodak BioMax film for 6 days.

Optical density measures for numerous brain regions were then made for each mouse, and are shown in Table 1 as mean ± SEM. Comparisons within the PTZ treatment groups were performed by one-way ANOVA and Dunnett's test. The acute saline treated groups were compared using Student's t-test.

Table 1 shows that in the 6 brain regions investigated, kindled mice showed a decreased 2-DG uptake compared to control mice. This reduction in 2-DG uptake was reversed by LEV in the amygdala, and partially reversed in the caudate putamen.

These results clearly demonstrate that 5 days after their last injection of PTZ, the kindled mice have a decreased uptake of 2-DG. This has also been demonstrated in amygdala-kindled rats (Namba et al., 1989). This decrease in 2-DG uptake seems to be reversed by LEV in some, but not all, brain areas. Further investigation of LEV's activity in these specific brain areas may give additional insight into its mechanism of action.

Chronic treatment	saline	PTZ	PTZ	PTZ
Acute treatment	saline	saline	40 mg/kg LEV	80mg/kg LEV
Caudate Putamen	262 ± 16	157 ± 6*	177 ± 5	195 ± 13#
Amygdala, basolat.	169 ± 16	104 ± 8*	146 ± 9#	142 ± 9#
Thalamus, ventral	245 ± 20	153 ± 9*	181 ± 20	176 ± 13
Piriform Cortex	169 ± 16	132 ± 3*	176 ± 15	148 ± 8
Entorhinal Cortex	162 ± 8	105 ± 11*	134 ± 8	131 ± 6
S. Nigra, reticulata	157 ± 10	110 ± 5*	121 ± 7	124 ± 4

Table 1: [^{14}C] 2-deoxyglucose uptake (nCi/g ± SEM) in chronic saline or PTZ treated mice. * P<0.05 cf chronic saline group; # P<0.05 cf chronic PTZ saline group.

Klitgaard, H. et al., (1998) *Eur. J. Pharmacology*, **353**, 191-206
Namba, H. et al. (1989) *Brain Res.*, **486**(2), 221-7
Noyer, M. et al. (1995) *Eur. J. Pharmacology*, **286**, 137-146

108P BLOCKADE OF N-TYPE CALCIUM CHANNELS IN THE CENTRAL NUCLEUS OF THE AMYGDALA ATTENUATES CONDITIONED BUT NOT UNCONDITIONED AVERSIVE BEHAVIOUR IN THE RAT

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It is well established that the amygdalaloid complex plays an integral role in the modulation of aversive behaviours (Davis et al. 1994) and that the processing of conditioned and unconditioned aversion involves one or more amygdaloid nuclei (LeDoux 2000). However, precisely which regions are recruited in the processing and expression of different forms of aversion is less clear.
The present study explores the role of the central nucleus of the amygdala (CEN) using local administration of the N-type calcium channel blocker, ω-conotoxin GVIA in two distinct established models of conditioned and unconditioned aversive behaviour.

Male Sprague Dawley rats (200-250g Charles River UK) were anaesthetised (isoflurane, N_20 in O2) and stereotaxically implanted with unilateral guide cannulae aimed at the CEN (AP -2.5mm, ML –4.6mm, DV –7.3mm relative to bregma,). One week post-surgery animals received an intra-CEN injection of vehicle (250nl artificial CSF) or ω-conotoxin GVIA (0.2μg in 250nl vehicle) 10 minutes prior to aversive conditioning (10x 1s foot shocks, 0.4mA at 1min intervals). After 24 hours, animals were returned to the testing apparatus and the amount of contextually-induced freezing was determined by computer tracking. Seven days later, animals received an opposite injection of vehicle or ω-conotoxin GVIA and after 10 minutes were exposed to a 1 min unconditioned aversive ultrasonic 20kHz, 98dB tone in an open field arena (Beckett et al 1996). Locomotor behaviour was determined by computer tracking.

Vehicle treated animals in the conditioned aversion group displayed typical freezing in response to the contextual cue 24 hours post conditioning (Fig1). This was significantly attenuated by ω-conotoxin GVIA ($p<0.01$) (Fig 1). Exposure to the unconditioned ultrasound cue evoked typical hypolocomotor behaviour throughout the duration of the stimuli in vehicle treated animals. This was unaltered by pretreatment with ω-conotoxin GVIA (Fig 2). Data from animals with cannulae located outside the CEN were excluded from the study

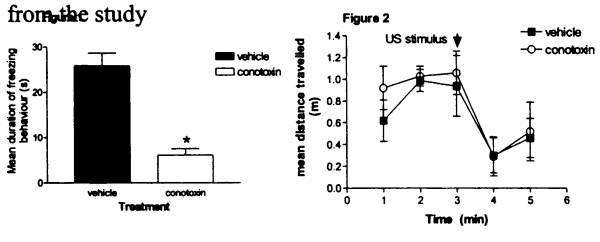

Figure 1 Effect of intra-CEN ω-conotoxin GVIA (0.2μg in 250nl) on freezing behaviour on exposure to a conditioned aversive contextual cue. *p<.001 vs vehicle (mean ± sem, n=7 unpaired t test).
Figure 2 Lack of effect of intra-CEN or ω-conotoxin GVIA (0.2μg in 250nl) on hypolocomotion induced by 20kHz 98dB artificial tone (mean ± sem, n=7 one way ANOVA)

These data demonstrate that the functional presence of some calcium channels within the CEN are important for the expression of conditioned aversion but not unconditioned aversion. This implicates the CEN in the acquisition phase of conditioning aversion but not in innate aversive responses.

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Davis M *et al* (1994) *Trends Neurosci.* 4, 208-214
LeDoux J (2000) *Ann Rev Neurosci.* 23, 155-184

109P THE ADENOSINE RECEPTOR ANTAGONIST CGS15943 ELICITS PLACE PREFERENCE IN THE LISTER HOODED RAT

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A_{2A} adenosine receptors are found in the brain to high density in dopamine-rich areas, such as the caudate nucleus, putamen and nucleus accumbens (Ferre, 1997). We have previously reported that antagonism of the A_{2A} receptor leads to enhancement of dopamine (DA) release in the nucleus accumbens of Lister Hooded Rats *in vitro* (Harper et al., 2002). This prompted an investigation of the effect of the non-selective adenosine receptor antagonist, CGS15943 *in vivo* using a conditioned place preference (CPP) paradigm (Cheer *et al.*, 2000)

Male Lister Hooded rats (n=24) weighing 250-350 g were used in the study. The CPP apparatus was similar to that described by Cheer et al. (2000). It consisted of two compartments each with a different visual cue in the form of either thick (2.5 cm) or thin (1 cm) black and white stripes, separated by a removable partition. Animals were randomly assigned to groups (n=6 per group) following a habituation period in which the amount of time spent in each compartment was determined. The assay was validated with 10 mg kg^{-1} cocaine in separate animals. Three groups received three pairings of either 1 mg kg^{-1}, 2mg kg^{-1} or 4mg kg^{-1} CGS15943 i.p. with one compartment, alternated with three pairings of the other compartment with saline. A fourth (control) group received vehicle for all pairings. Forty-eight hours after the last pairing (to allow for drug clearance), the amount of time spent in each compartment was determined (test day) using Ethovision and Videotrack behavioural software packages. All data were analysed using Student's t-test.

No significant preference was observed for either compartment during habituation. The control group also displayed no preference for either compartment on the test day. CGS15943 was found to produce a preference for the drug-paired compartment at 1 mg kg^{-1} (thick stripes, $P<0.001$; thin stripes, $P<0.01$), 2mg kg^{-1} (thick stripes, $P<0.05$; thin stripes, $P<0.05$) and 4 mg kg^{-1} (thick stripes, $P<0.001$; thin stripes, $P<0.05$).

These results show that adenosine receptor inhibition produces conditioned place preference at a range of doses, indicating a role for adenosine in reward mechanisms. These results, however, partly contrast with an earlier study which showed a significant place preference in Wistar rats using CGS15943 at only 1 mg kg^{-1}, but not at 0.5 or 2 mg kg^{-1} (Brockwell & Beninger, 1996). This divergence may reflect a strain difference in response to behavioural tests and drugs administered. This should be considered when carrying out further studies on adenosine receptor ligand effects both *in vivo* and *in vitro*.

Given that there is an association between the nucleus accumbens and reward, further investigation into the effects of adenosine receptor ligands in behavioural paradigms to measure reward may give more insight into the role of adenosine and the particular receptors involved in reward mechanisms *in vivo*.

LKH is a BBSRC Research Committee Student.

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110P CHRONIC PAROXETINE TREATMENT TO YOUNG RATS INCREASES A CONDITIONED EMOTIONAL RESPONSE

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Acutely, selective serotonin reuptake inhibitors (SSRIs) increase the synaptic availability of serotonin, but in the long-term the mechanism underlying their therapeutic effect remains unknown, though desensitization of pre-synaptic serotonin autoreceptors resulting in increased serotonin release, may be a factor (Kreiss and Lucki, 1995). Chronic administration of SSRIs drugs to neonatal rats produces behavioural and physiological changes that resemble a depressive state in the adult animal (Vogel et al., 1990). The present study investigated the effect of chronic paroxetine, an SSRI given to young rats on contextual fear conditioning in young adults.

Male Lister hooded rats were obtained at weaning (22-24 postnatal day) and divided into groups of 8. The rats were treated with paroxetine (200 mg/l ≅ 20 mg/kg) in the drinking water for 25 days either with or without one-week drug withdrawal and controls were given normal water. For behavioural testing, rats on day 1 were subjected to inescapable foot shock (0.4mA, 10ms x 10 with an intershock interval of 1min) in a conditioning chamber. The day after the footshock, the rats were again placed into the same chamber for 10min, but given no footshock and their behaviour observed. Freezing was recorded in seconds. After behavioural testing on day 2 rats were killed and brain regions (cortex, striatum and hippocampus) collected for the determination of 5-HT turnover using HPLC with electrochemical detection. Levels of paroxetine in blood plasma during treatment were measured on day 21 in a separate group of animals (n=6) using HPLC with UV detection. Data were analysed using Student's unpaired t-test.

The levels of paroxetine in plasma were 262 ± 18.2 ng/ml when the drug was given in the drinking water. Chronic paroxetine increased freezing time ($P<0.05$) on day 2 both after 25 days treatment and after 7 days withdrawal compared to controls (Figure 1). 5-HT turnover was significantly higher ($P<0.05$) in the three brain regions after 7 days withdrawal from paroxetine treatment compared to controls. These results indicate that chronic paroxetine treatment to rats immediately post-weaning enhances a conditioned fear response and that this effect is maintained on withdrawal. It remains to be determined whether the altered behaviour is a direct effect of enhanced serotonergic function or a change in development produced by early SSRI treatment (Norrholm & Quimet, 2000).

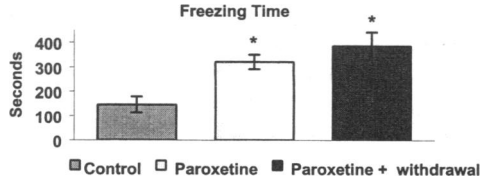

Figure 1. Effect of chronic paroxetine treatment for 25 days with or without withdrawal on CER induced freezing Results are mean ± SEM (n=8, each group). *$P<0.05$ vs controls.

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111P EVIDENCE SUGGESTING A ROLE FOR $P2Y_{13}$ RECEPTORS IN THE REGULATION OF RAT HEPATOCYTE CYCLIC AMP LEVELS

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Extracellular ATP and other purine nucleotides regulate liver glycogenolysis by: 1. Acting on $P2Y_1$ and $P2Y_2$ receptors to raise cytosolic Ca^{2+}; 2. Acting on unidentified P2Y receptors to inhibit glucagon or forskolin-elevated cyclic AMP levels (Keppens & De Wulf, 1991; Dixon et al., 2000). These 2 sets of regulatory events would be expected to have opposite effects on glycogenolysis, stimulating and inhibiting respectively. Here we further characterise the P2Y-mediated response coupled to inhibition of adenylyl cyclase in the light of recently cloned P2Y receptors, and provide the first evidence for a functional $P2Y_{13}$ receptor.

Hepatocytes were isolated from rat liver by collagen perfusion and used within 60 min as a suspension of $2x10^6$ cells ml^{-1} in a final volume of 300 µl. Cells were shaken at 37°C in the presence of 300 µM IBMX, and antagonists were added either 10 min, or in some cases 20 sec prior to incubation with the agonist for 3 min. The nucleotide agonists were added simultaneously with glucagon (10 nM). Cyclic AMP was extracted and estimated using a [^{3}H]cyclic AMP protein binding assay.

As expected glucagon stimulated cyclic AMP production. Various purine nucleotides attenuate this response (Keppens & De Wulf, 1991), and here 2-methylthioADP (2MeSADP) inhibited glucagon-stimulated cAMP accumulation, e.g. basal, 6.30 ± 0.06; 10 nM glucagon, 34.2 ± 0.7; glucagon + 300 µM 2MeSADP, 14.7 ± 0.5 (pmol cyclic AMP, mean ± sem, n = 3, from a representative experiment, $P<0.001$ Students t-test).

2MeSADP is an agonist at human $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors. $P2Y_{11}$ is reported to stimulate adenylyl cyclase and so is an unlikely mediator. This was confirmed by observation that ARC67085 (an agonist of $P2Y_{11}$ receptors at µM concentrations) had no effect on cyclic AMP levels up to 300 µM. We tested the effects of $P2Y_1$-selective antagonists and saw no effect of MRS 2179 (Boyer et al., 1998) or A3P5P on 2MeSADP-mediated inhibition of glucagon-stimulated cAMP production, when 2MeSADP was used at either maximal, or submaximal concentrations (response to 300 µM 2MeSADP plus 300 µM antagonist as % of response with no antagonist was: MRS 2179, 114.1 ± 11.2%; A3P5P, 113.9 ± 10.5%). $P2Y_{12}$ has recently been characterised as the receptor responsible for the inhibitory adenylyl cyclase response to purine nucleotides in platelets, C6-2B glioma cells and PC12 cells. ARC67085, a potent and selective antagonist at $P2Y_{12}$ receptors, had no effect on the 2MeSADP-evoked inhibition of cAMP, both when the agonist was used at maximal, and submaximal concentrations (300 µM 2MeSADP plus 100 nM ARC67085, 113.9 ± 12.3 %). In each case data were from 3 separate experiments each in triplicate.

These experiments demonstrate that 2MeSADP inhibits glucagon-stimulated cyclic AMP accumulation in rat hepatocytes in a manner consistent with agonist action at $P2Y_{13}$ receptors. We predict therefore, that a selective $P2Y_{13}$ agonist would inhibit glycogenolysis in the rat liver.

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112P COMPARISON OF LANTHANIDE SENSITIVITY OF RECEPTOR-ACTIVATED CALCIUM INFLUX IN THE HUMAN PROMYELOCYTIC LEUKAEMIC CELL LINE HL60 AND HUMAN NEUTROPHILS

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Lanthanides are amongst the most widely used Ca^{2+} channel blockers available to characterise Ca^{2+} entry pathways, and have been used in a variety of cell types including voltage non-excitable cells such as leukocytes. A previous study (Bahra *et al.*, 2001) suggested that UTP-stimulated Ca^{2+} influx in HL60 cells was relatively insensitive to blockade by the lanthanide cations La^{3+} and Gd^{3+}. However, subsequent studies in the human neutrophil found that Gd^{3+} and La^{3+} were more potent blockers of formyl-methionyl leucyl phenylalanine (fMLP)-stimulated Ca^{2+} influx when used in a phosphate-free (PO_4^--free) buffer. We have now extended our early study in HL60 cells with La^{3+} and Gd^{3+} to evaluate their potency in a PO_4^--free buffer.

Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured at room temperature (RT) in Fluo-4 (Molecular Probes)-loaded HL60 cells (ATCC) and human neutrophils in 96 well plates ($2x10^5$ per well) using FLIPR (FLuorescence Imaging Plate Reader, Molecular Devices). HL60 cells were stimulated with UTP (10µM) in Hanks Buffered Salt Solution (HBSS) containing 0.07mM $CaCl_2$ and 1mM $MgCl_2$ (Bahra *et al.*, 2001) or PO_4^--free buffer containing in mM concentrations, 140NaCl, 5.4KCl, 1.0$MgCl_2$, 0.07$CaCl_2$, 15HEPES. Neutrophils were stimulated with fMLP (0.1µM) in HBSS containing 0.3mM $CaCl_2$ and 1mM $MgCl_2$ or PO_4^--free buffer with 0.3mM $CaCl_2$. When the $[Ca^{2+}]_i$ had returned to basal levels post-stimulation, 1mM extracellular $CaCl_2$ was added to enable quantitation of Ca^{2+} influx. Cells were pre-treated with the blockers $LaCl_3$ and $GdCl_3$ (Sigma Aldrich) for 2 min respectively (RT) prior to stimulation. Results are expressed as mean values ± s.e.m.

	HL60 cells; IC_{50} (µM)		Neutrophils; IC_{50} (µM)	
Blocker	HBSS	PO_4^--free	HBSS	PO_4^--free
La^{3+}	808 ± 62	0.19 ± 0.05	596 ± 198	0.02 ± 0.01
Gd^{3+}	316 ± 10	0.25 ± 0.03	475 ± 39	0.03 ± 0.01

Table 1. Comparison of IC_{50} data for La^{3+} and Gd^{3+} tested against UTP and fMLP-induced Ca^{2+} influx in HL60 cells and neutrophils respectively using HBSS and PO_4^--free buffer. Data represents mean ± s.e.m. (n=3).

This study shows that receptor-stimulated Ca^{2+} influx in HL60 cells like human neutrophils, is sensitive to blockade by sub-micromolar concentrations of La^{3+} and Gd^{3+} in a PO_4^--free buffer (Table 1). These data suggest that a PO_4^-- free buffer is required in all experiments with these lanthanide cations to determine their true efficacy and potency. However under the experimental conditions used in this study, the lanthanides were more potent than that reported by others also using PO_4^--free buffer both in HL60 cells (Demaurex *et al.*, 1992) and human neutrophils (Itagaki *et al.*, 2002).

The differences in lanthanide sensitivity of receptor-stimulated Ca^{2+} influx between HL60 cells and human neutrophils suggests that pharmacologically different channels may mediate Ca^{2+} influx in these cell types. However further comparisons with the La^{3+} and Gd^{3+} using UTP as a stimulus in human neutrophils along with the study of other Ca^{2+} channel blockers are required to determine whether this is the case.

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113P μ-OPIOID RECEPTOR MEDIATED Ca^{2+} SIGNALLING IN TRANSFECTED HEK293 CELLS REQUIRES CONCOMITANT M_3 MUSCARINIC RECEPTOR ACTIVATION

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Previously we have shown in SH-SY5Y neuroblastoma and CHO-δ cells that G_i/G_o-coupled receptor activation elevates intracellular free-Ca^{2+} ($[Ca^{2+}]_i$), but only during concomitant G_q-coupled receptor activation (Connor *et al.*, 1996; Yeo *et al.*, 2001). It has recently been reported that in transfected HEK293 cells, activation of the μ-opioid receptor alone can evoke a rise in $[Ca^{2+}]_i$ that does not require concomitant G_q-coupled receptor activation (Quillan *et al.*, 2002). Here, we investigate in HEK293 cells that we have stably transfected with the MOR1 form of the μ-receptor whether or not the μ-receptor mediated elevation of $[Ca^{2+}]_i$ requires concomitant G_q-coupled receptor activation.

HEK293 cells were stably transfected with the MOR1 subtype of the μ-receptor. Radioligand binding was performed using [^{3}H]-diprenorphine and showed the receptor density to be 175 ± 28 fmol mg^{-1}, which is comparable to endogenous expression levels previously reported (Tempel *et al.*, 1987). $[Ca^{2+}]_i$ was monitored in cell monolayers with fura-2 as described previously for SH-SY5Y cells (Yeo *et al.*, 2001). Cells were superfused with a modified Krebs buffer and drugs added to the superfusate in known concentrations. The Student's T-Test was used for statistical analysis.

First, we investigated if μ-receptor activation alone could evoke a rise in $[Ca^{2+}]_i$. However, exposure of cells to the μ-receptor agonist DAMGO (300nM) had no effect on resting $[Ca^{2+}]_i$ (n=7; Figure 1). Next we determined if μ-receptor activation could evoke a response during concomitant G_q-coupled receptor activation. Prolonged application of carbachol (1μM), which activates endogenous M_3 muscarinic receptors in these cells, evoked a small, sustained elevation of $[Ca^{2+}]_i$. In the continued presence of carbachol, exposure to DAMGO (300nM) evoked a large transient elevation of $[Ca^{2+}]_i$ (Figure 1) that decayed from its peak with a halftime ($t_{1/2}$) of 24 ± 3 s (n=3). The $[Ca^{2+}]_i$ response evoked by DAMGO was inhibited by the opioid receptor antagonist naloxone (1μM; n=3).

The response evoked by DAMGO (300nM) was maintained in the absence of extracellular Ca^{2+}, although the decay was significantly faster ($t_{1/2}$=14 ± 1 s; n=3) than in the presence of extracellular Ca^{2+}.

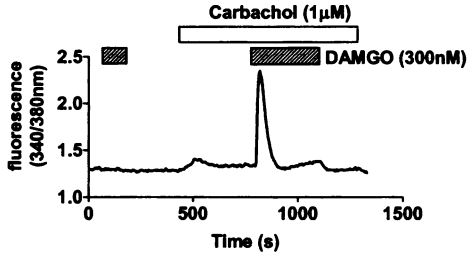

Figure 1. In HEK-MOR1 cells, DAMGO evoked a rise in $[Ca^{2+}]_i$ only during concomitant muscarinic receptor activation.

These data support our previous findings in SH-SY5Y and CHO-δ cells that μ-receptor activation can only evoke a rise in $[Ca^{2+}]_i$ if there is concomitant activation of G_q-coupled receptors.

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114P RATE OF DECAY OF THE μ-OPIOID RECEPTOR MEDIATED ELEVATION OF $[Ca^{2+}]_i$ IN SH-SY5Y NEUROBLASTOMA CELLS

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We have shown previously that in SH-SY5Y cells the activation of G_i/G_o-coupled receptors does not elevate intracellular free-Ca^{2+} ($[Ca^{2+}]_i$). However, activation of μ-opioid or sst_2 receptors during concomitant G_q-coupled m_3 muscarinic receptor activation evoked a transient, intracellular store-dependent elevation of $[Ca^{2+}]_i$ that decayed rapidly in the continued presence of the agonist (Connor *et al.*, 1996; 1997). Here, we investigate the decay kinetics of the μ-receptor mediated response.

$[Ca^{2+}]_i$ was monitored in cell monolayers as described previously (Yeo *et al.*, 2001). Cells were superfused with a HEPES buffered solution and drugs added to the superfusate in known concentrations. Data were analysed using the Student's T-test with a P value <0.05 considered significant.

Cells were exposed to the muscarinic receptor agonist carbachol (1μM) and a sustained elevation of $[Ca^{2+}]_i$ observed. In the continued presence of carbachol, prolonged application of the μ-receptor agonist DAMGO (1μM) evoked a transient rise in $[Ca^{2+}]_i$ that rapidly decayed back to baseline from its peak with a halftime ($t_{1/2}$) of 34 ± 5 s (n=4). When the experiment was repeated in Ca^{2+}-free buffer (100μM EGTA added) to block store operated Ca^{2+} influx the decay of the DAMGO-evoked response was significantly faster ($t_{1/2}$=16 ± 2 s; n=3).

The decay of the DAMGO-evoked response could be due to receptor desensitization, depletion of the available Ca^{2+} store or some unknown limiting factor. To examine the potential influence of μ-receptor desensitisation, cells were pre-exposed to DAMGO (1μM) for 30, 60, or 300 s before and then during the application of carbachol (1μM). It was observed that pre-exposure to DAMGO reduced the μ-receptor mediated rise in $[Ca^{2+}]_i$ in a time-dependent manner ($t_{1/2}$=19 s; n=4). This observation would be compatible with μ-receptor desensitization being responsible for the decay of the DAMGO response. However, responses evoked by morphine (10μM), a non-desensitising μ-receptor agonist (Whistler *et al.*, 1999; Bailey *et al.*, 2002), in the presence and absence of extracellular Ca^{2+} also decayed rapidly ($t_{1/2}$= 32 ± 2 s and 22 ± 4 s respectively).

To examine the role of Ca^{2+} store depletion cells were challenged with somatostatin in the presence of DAMGO. After a 5 min exposure to DAMGO (1μM) in the presence of carbachol (1μM), when the evoked rise in $[Ca^{2+}]_i$ had declined back to baseline levels, application of somatostatin (300nM) evoked a rise in $[Ca^{2+}]_i$ that was equal in amplitude to that evoked by somatostatin in the absence of DAMGO.

These data suggest that the decay of the μ-receptor response is not due to Ca^{2+}-store depletion as activation of a second G_i/G_o-coupled receptor, sst_2, after the μ-receptor response had fully decayed still evoked a further rise in $[Ca^{2+}]_i$. The decay of the response is also unlikely to be due to receptor desensitisation as morphine evoked a response that decayed at a rate equal to the DAMGO evoked response. Therefore, it is possible that the rapid decay is due to depletion or inactivation of some component of the second messenger pathway responsible for the Gi/Go-coupled receptor mediated elevation of $[Ca^{2+}]_i$.

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115P PRELIMINARY PHARMACOLOGICAL CHARACTERISATION OF UTP-INDUCED CA^{2+} INFLUX IN WELL-DIFFERENTIATED HUMAN BRONCHIAL EPITHELIAL CELLS

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Stimulation of human airway epithelial cells with nucleotide triphosphates causes an increase in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via Ca^{2+} release from intra-cellular stores and the influx of extra-cellular Ca^{2+} (Kerstan *et al.*, 1999; Paradiso *et al.*, 1996). Such an influx pathway has neither been described nor pharmacologically characterised in well-differentiated human bronchial epithelial cells (HBECs).

HBECs (Clonetics) were cultured on Snapwell permeable supports for 21 days, the final 14 days at an apical air interface. Epithelia were loaded with Fura2-AM (5μM) in culture media (60 min; 37°C). Cells were placed into a plastic chamber to enable simultaneous apical and basolateral fluid perfusion ($2.5mLmin^{-1}$) and were visualised using a CCD camera mounted on a microscope. Intracellular Fura2 was excited at 340nm and 380nm and consecutive emissions at 510nm were recorded enabling a fluorescence ratio (FR) to be calculated. The FR was continuously monitored (~2Hz) in response to UTP stimulation (30μM apical) in the presence of either normal (1mM) or low (0.1mM + 200μM EGTA) Ca^{2+} containing Hanks Balanced Salt Solution (HBSS) or in the presence of the Ca^{2+} channel blockers La^{3+} or SKF96365 (Merritt *et al.*, 1990). For the La^{3+} studies a phosphate-free solution was used. Data are expressed as absolute changes in FR (mean ± s.e.mean) at the times specified or as area under the curve between 300-600s ($AUC_{300\text{-}600s}$) after stimulation with UTP (mean ± s.e.mean). Groups were compared using Student t-test and significance assumed when $P<0.05$. Bonferonni correction was used for multiple comparisons.

In normal (1mM) and low Ca^{2+} containing HBSS, UTP (30μM) induced peak increases in FR of 1.482 ± 0.085 (n=5) and 1.389± 0.096 (n=5) respectively ($P=0.49$). By 300s after the initiation of the response the change in FR had returned to baseline in the low Ca^{2+} cells (–0.047 ± 0.010) but remained significantly elevated in the normal Ca^{2+} cells (0.452 ± 0.022) ($P<10^{-7}$).

La^{3+} at concentrations of 0.1μM and above significantly attenuated the sustained elevation of $Ca^{2+}{}_i$ measured as the $AUC_{300\text{-}600s}$ but was without effect on the peak increase in $Ca^{2+}{}_i$ (Table 1).

La^{3+} (μM)	UTP peak	$AUC_{300\text{-}600s}$	n	*P*
0	1.376±0.064	79.1±4.7	23	-
0.001	1.207±0.079	85.8±14.6	4	0.60
0.01	1.189±0.121	56.7±6.8	4	0.10
0.1	1.417±0.067	13.3±2.2	4	$<10^{-5}$
1.0	1.150±0.145	15.8±1.1	4	$<10^{-5}$
10.0	1.304±0.052	7.0±2.3	4	$<10^{-5}$
100.0	0.998±0.094	12.3±2.3	4	$<10^{-5}$

Table 1. The effects of La^{3+} on the UTP-stimulated peak increase in FR and $AUC_{300\text{-}600s}$. Vehicle control and La^{3+} $AUC_{300\text{-}600s}$ values were compared using a Student t-test with Bonferonni correction.

SKF96365 (30μM) also significantly reduced the $AUC_{300\text{-}600s}$ from 142.0±5.5 to 112.8±2.9 (n=6; $P<0.01$) in response to UTP (30μM) although was ineffective at 10μM (137.7±4.8) (n=6; $P=0.61$). There was no significant effect of SKF96365 on the UTP peak response.

HBECs stimulated with UTP respond with an increase in $[Ca^{2+}]_i$ that from 300s after activation is due exclusively to influx of extracellular Ca^{2+}. For the first time we have demonstrated that this pathway is sensitive to low concentrations of La^{3+} and although sensitive to SKF96365, requires relatively high concentrations to achieve a modest block when compared to its activity in other cell types (Merritt *et al.*, 1990). The functional consequences of this Ca^{2+} entry pathway are under investigation.

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116P CHARACTERISATION OF AN AGONIST SPECIFIC Ca^{2+} RESPONSE TO BOTH UTP AND ATP AT $P2Y_{11}$ RECEPTORS: AN EXAMPLE OF AGONIST DEPENDENT SELECTION OF SIGNALLING PATHWAYS

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Human $P2Y_{11}$ has been described as an ATP receptor, since this is reported to be the most potent of the native nucleotides (Communi et al, 1999; Zambon et al, 2001). The pyrimidine nucleotides (e.g. UTP) have been reported to be ineffectual at $hP2Y_{11}$. This has been based on the coupling of $hP2Y_{11}$ receptors to inositol phosphate production and the stimulation of adenylyl cyclase. Here we provide evidence that UTP is an agonist at $hP2Y_{11}$ receptors, and show that stimulation by ATP and UTP elicit Ca^{2+} responses by different signalling pathways.

For total [^{3}H]inositol (poly)phosphates ($InsP_x$) assay, $hP2Y_{11}$-transfected 1321N1 cells cultured in 24-well plates were [^{3}H]inositol-labelled. Agonists were added in the presence of 10mM lithium for 20 min. For cytosolic Ca^{2+} cells were loaded with fura 2-AM and continuously perfused with agonists for 30s, and responses monitored with a VisiTech imaging system. Data were pooled across 3 separate experiments (each 6-10 cells). Creatine phosphokinase regenerating system (CPK) was included in incubations where indicated. Agonist purity was monitored by HPLC analysis.

Stimulation of [^{3}H]inositol-labelled $hP2Y_{11}$-transfected cells with ATP led to a concentration-dependent increase in $InsP_x$ with a log EC_{50} of -4.91±0.13. No response was seen to UTP up to 300 μM. When fura 2 -loaded $P2Y_{11}$ transfected cells were perfused with different concentrations of ATP, a concentration-dependent response was seen with a log EC_{50} of -5.57±0.11. Unexpectedly, a similar concentration- response curve was also seen with perfusion with UTP (log EC_{50} 5.21±0.10) leading to similar maximal responses. This was true both in the presence and absence of CPK. Stimulation of untransfected 1321N1 cells gave no Ca^{2+} or $InsP_x$ response to either agonist. The response to ATP, but not to UTP, was significantly reduced in the absence of extracellular Ca^{2+}. Responses to maximally effective concentrations of ATP and UTP were not additive. On pretreatment with pertussis toxin (100 ng/ml, 24h) the maximum response to ATP was unaffected (97.8%) while the maximum response to UTP was significantly reduced (to 43.2%). The concentration-response curve to UTP was significantly attenuated by pertussis toxin ($P<0.0001$ by 2-way ANOVA). On repeated exposure to ATP, the Ca^{2+} response to ATP was reduced and the UTP response was lost. The presence of 2-aminoethyl diphenylborate, a tool for the attenuation of Ca^{2+} responses to the activation of G protein-coupled receptors, abolished the response to UTP but not the response to ATP.

We show that UTP acts as an agonist at $hP2Y_{11}$ receptors when the cytosolic Ca^{2+} response is measured, but not when the inositol phosphate response is measured. The 2 agonists signal to Ca^{2+} by different pathways. These results suggest agonist dependent selection of signalling pathways is a result of agonist trafficking and differential signal strength.

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117P THE MYOSIN PHOSPHATASE INHIBITOR CALYCULIN-A ABOLISHES NITRERGIC RELAXATION OF THE MOUSE ANOCOCCYGEUS

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It is now widely accepted that nitric oxide (NO) functions as a neurotransmitter in the peripheral nervous system, causing relaxation of a range of smooth muscle tissues, including the anococcygeus (Martin, 2000). In most cases, the second messenger system that is activated by neuronal NO in the smooth muscle is the guanylyl cyclase/cyclic GMP pathway. However, the mechanisms by which cyclic GMP then effects relaxation are still unclear, but may involve either reduction in cytoplasmic calcium concentration or modification of calcium sensitisation/desensitisation. In skinned preparations, NO donor drugs relax the mouse anococcygeus by promoting calcium desensitisation (Ayman *et al.*, 2001). Since myosin phosphatase is known to play a pivotal role in calcium sensitisation/desensitisation, we have now examined the effects of the myosin phosphatase inhibitor calyculin-A on intact mouse anococcygeus muscles to assess the importance of calcium desensitisation in nitrergic relaxation of this tissue.

Anococcygeus muscles from male mice (LACA; 25-35g) were set up for the recording of nitrergic relaxations as described previously (Ayman *et al.*, 2001). When tone was raised with thapsigargin, verapamil (10 μM) was included in the Krebs solution in order to inhibit voltage-operated calcium entry. Results are expressed as mean ± s.e. mean ($n \geq 5$). Statistical analysis was by Student's t test.

Carbachol (50 μM) produced sustained contractions (482 ± 44 mg tension) and field stimulation (10 Hz; 10 s trains) activated reproducible nitrergic relaxations of this carbachol-induced tone (47 ± 5% relaxation). When the nitrergic relaxations had stabilised, addition of 1 μM calyculin-A to the organ bath resulted in a further increase in induced tone (by 33 ± 6%) and, after 15 min, to complete inhibition of the nitrergic relaxations. The sarcoplasmic reticulum Ca-ATPase inhibitor thapsigargin also caused sustained contractions of the mouse anococcygeus (404 ± 26 mg tension) and field stimulation (10 Hz; 10 s trains) again produced reproducible nitrergic relaxations (42 ± 7%). As seen with carbachol, addition of 1 μM calyculin-A resulted in a further increase in the induced tension (by 16 ± 5%) and, after 15 min, to complete inhibition of the nitrergic relaxations. In muscles pre-contracted with carbachol, sodium nitroprusside (0.01 – 33 μM) produced concentration-related relaxations (maximum 96 ± 2%); when tone was raised with 1 μM calyculin-A (234 ± 20 mg tension) sodium nitroprusside was much less effective (maximum relaxation 27 ± 4%). Conversely, papaverine (1 – 333 μM) was equally effective as a relaxant of tone induced by either carbachol or calyculin-A.

Thus, calyculin-A abolished nitrergic relaxations whether muscle tone was raised by carbachol (receptor-dependent calcium release; store-operated calcium entry; voltage-operated calcium entry) or thapsigargin (receptor-independent calcium release; store-operated calcium entry). This implicates calcium desensitisation, via activation of myosin phosphatase, as a major mechanism underlying the nitrergic relaxations.

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118P DOPAMINE-MEDIATED INHIBITION OF RENAL NA^+/H^+ EXCHANGER ISOFORM 3: INVOLVEMENT OF PROTEIN KINASE A AND C PATHWAYS

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Dopamine D_1-like receptors are linked via G proteins to the multiple cellular signaling pathways namely adenylyl cyclase (AC) and phospholipase C (PLC) (Jose et al., 1996; Lokhandwala et al., 1998). We have previously shown that the D_1-mediated inhibition of Na^+-K^+ ATPase activity in OK cells involves the sequential activation of the AC-PKA and the PLC-PKC pathways (Gomes et al., 2002). The present study evaluated signaling cascades involved in dopamine-mediated inhibition of Na^+/H^+ exchanger isoform 3 in renal opossum kidney (OK) cells.

OK cells (ATCC 1840-CRL) were cultured in Minimum Essential Medium with 10% foetal bovine serum. After 6 days, the cells formed a monolayer and each cm^2 contained about 100 μg of cell protein. 24 h before the experiments the cell culture medium was changed to a serum free medium. Intracellular pH (pH_i) was monitored using the pH-sensitive dye BCECF-AM (Molecular Probes), as previously described (Thwaites et al., 1993). Na^+/H^+ exchanger activity was assayed as the initial rate of pH_i recovery after an acid load imposed by 10 mM NH_4Cl followed by removal of Na^+ from the Krebs' modified buffer solution. PLC activity was monitored in membranes and cytosol from OK cells, using the PC-PLC assay kit (Molecular Probes). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test with $P<0.05$ indicating a significant difference.

The transport kinetics displayed a simple Michaelis-Menten relationship for extracellular Na^+ with a K_m value of 25 ± 6 mM. Dopamine and the D_1-like receptor agonist SKF 38393 inhibited NHE3 activity with IC_{50} values of 72 (19, 278) and 176 (77, 397) nM, respectively. The D_2-like receptor agonist quinerolane was devoid of effect. The D_1-mediated inhibition of NHE3 (57.6 ± 4.7 % of control values) was completely prevented either by the D_1-like receptor antagonist SKF 83566 (1 μM), overnight treatment with cholera toxin (500 ng/ml), the PKA antagonist H-89 (10 μM), the PKC antagonist chelerythrine (1 μM), or the PLC inhibitor U-73,122 (3 μM), but not by the MAPK inhibitor PD 098059 (10 μM). In addition, cAMP (500 μM) was found to increase PLC activity, both in membranes and in cytosol (% of control values: membranes, 126 ± 3; cytosol, 147 ± 9) from OK cells; in contrast, PDBu (1 μM) did not have a significant effect on activity. Pre-treatment of OK cells with the anti-$G_s\alpha$ antibody, but not the anti-$G_{q/11}\alpha$ antibody, blunted the inhibitory effect of the D_1-like receptor agonist SKF 38393 on NHE3 activity.

It is concluded that D_1-mediated inhibition of NHE3 in renal OK cells involves both AC-PKA and PLC-PKC pathways, a mechanism similar to that described for Na^+-K^+ ATPase.

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Supported by grant POCTI/35747/FCB/2000

119P D_1-MEDIATED INHIBITION OF RENAL Na^+/H^+ EXCHANGER THROUGH A SIGNALLING CASCADE INVOLVING PROTEIN $G_S\alpha$, ADENYLYL CYCLASE (AC) AND PROTEIN KINASE A (PKA)

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Dopamine causes natriuresis via activation of D_1-like receptors located in renal proximal tubules (Jose et al., 1992). This effect occurs through the involvement of two key proteins: the Na^+/H^+ exchanger and Na^+/K^+ ATPase (Gomes et al., 2001). However, the molecular pathways involved from activation of D_1-like receptors to the inhibition of Na^+/H^+ exchanger are not yet completely understood. The present study evaluated the molecular events on D_1-mediated inhibition of Na^+/H^+ exchanger activity in immortalized renal proximal tubular cells (WKY).

WKY cells (Woost et al., 1996), were grown at 37° C in a humidified atmosphere (5% CO_2) in Dulbecco's Minimum Essential Medium supplemented with 5% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B, 100 μg ml^{-1} streptomycin, 4 μg ml^{-1} dexamethasone, 5 μg ml^{-1} transferrin, 5 μg ml^{-1} insulin, 5 ng ml^{-1}selenium and 10 ng ml^{-1}epidermal growth factor. After 4 days, the cells formed a monolayer and each 1 cm^2 culture well contained about 50 μg of cell protein. 24 h before the experiments the cell culture medium was changed to a serum free medium. Na^+/H^+ exchanger activity and cAMP measurement was determined as previously reported (Gomes et al., 2001). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test with $P<0.05$ indicating a significant difference.

The D_1-like receptor agonist SKF 38393 decreased the Na^+/H^+ exchanger activity with an IC_{50} value of 150 (13, 1760) nM. The effect of SKF 38393 (1 μM) (29 ± 3 % reduction) was completely prevented by the D_1-like receptor antagonist SKF 83566 (1 μM), the overnight treatment with cholera toxin (CTX, 500 ng ml^{-1}) and the PKA antagonist H-89 (10 μM). The PKA downregulation (overnight exposure to 100 μM cyclic AMP) also abolished the inhibitory effect of SKF 39393 (1 μM). Contrarily, neither the overnight treatment with pertussis toxin (PTX, 100 ng/ml), the PKC antagonist chelerythrine (1 μM) nor the PLC inhibitor U-73,122 (3 μM) affected the SKF 38393 (1 μM) induced decrease in Na^+/H^+ exchanger activity. The effect of SKF 38393 (1 μM) was accompanied by increases in cyclic AMP production (40 ± 12 %, increase). The inhibitory effect of the D_1-like receptor agonist SKF 38393 (1 μM) on Na^+/H^+ exchanger activity was abolished in cells treated with the anti-$G_S\alpha$ antibody, but not in cells treated with the anti-$G_{q/11}\alpha$ antibody.

It is concluded that D_1 agonists decrease Na^+/H^+ exchanger activity by stimulation of AC and PKA via $G_S\alpha$ proteins. Na^+/H^+ exchanger inhibition probably occurs as a result of phosphorylation by PKA.

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Supported by grant POCTI/35747/FCB/2000

120P DOPAMINE D_2-LIKE RECEPTOR-MEDIATED OPENING OF K^+ CHANNELS IN OPOSSUM KIDNEY CELLS

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It has been reported that dopamine D_2-like receptor activation leads to increases in potassium conductance (Vallar & Meldolesi, 1989). This study examined the effects of dopamine D_1- and D_2-like receptor activation upon basolateral K^+ currents (I_K) and changes in membrane potential in opossum kidney (OK) cells.

OK cells (ATCC 1840-CRL) were cultured in Minimum Essential Medium with 10% foetal bovine serum. After 6 days, the cells formed a monolayer and each cm^2 contained about 100 μg of cell protein. To evaluate the basolateral K^+ conductance of OK cells, cell monolayers were mounted in Ussing chambers in the presence of an apical-to-basolateral K^+ gradient (80:5 mM) (DuVall & O'Grady, 1993). After a 15-20 min stabilization period, and in the presence of ouabain, the apical membrane was permeabilized with amphotericin B. The resulting short circuit current (I_K) is due to the movement of K^+ through channels in the basolateral membrane. Cyclic AMP was determined with an enzyme immunoassay kit, as previously described (Gomes et al., 2001). Changes in membrane potential were evaluated using the bisoxonol fluorescent dye $DiBAC_4(3)$, as previously described (Gopalakrishnan et al., 1999). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test with $P<0.05$ indicating a significant difference.

The addition of amphotericin B (3 μg ml^{-1}) to the apical side resulted in a rapid increase in I_K, this effect being inhibited by the K^+ channel blockers barium chloride (1 mM; 78±2 % reduction) or glibenclamide (10 μM; 42 ± 2 % reduction), but not apamin (1 μM). The K^+ channel opener pinacidil increased I_K (50 μM; 37 ± 12 % increase). The selective D_2-like receptor agonist quinerolane increased (maximal effect at 1 μM: 147 ± 8 % of control), in a concentration dependent manner [EC_{50}=136 (108, 171) nM], I_K across the basolateral membrane. This effect was abolished by pre-treatment with pertussis toxin (PTX), S-sulpiride (selective D_2-like receptor antagonist) and glibenclamide. The selective D_1-like receptor agonist SKF 38393 did not change I_K. Both H-89 (PKA inhibitor) and chelerythrine (PKC inhibitor) failed to prevent the stimulatory effect of quinerolane upon I_K. Quinerolane did not change basal levels of cyclic AMP and also failed to affect the forskolin (3 μM)-induced increase in cyclic AMP levels (251±29 vs 205±21 % increase). D_2-like receptor stimulation was associated with an hyperpolarizing effect, whereas D_1-like receptor activation was accompanied by increases in cell membrane potential. The hyperpolarizing effect of quinerolane [EC_{50}=129 (75, 221) nM] was prevented by pre-treatment with PTX, S-sulpiride and glibenclamide.

It is concluded that stimulation of dopamine D_2-like, but not D_1-like, receptors coupled to PTX-sensitive G proteins of the $G_{i/o}$ class produce membrane hyperpolarization through opening of K_{ATP} channels.

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Supported by grant POCTI/35747/FCB/2000

121P CHARACTERISATION OF A NOVEL GLUCOCORTICOID RECEPTOR IN HUMAN PLATELETS

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Human platelets express receptors for oestrogen, progesterone and testosterone, members of the nuclear steroid receptor family (Khetawat *et al.*, 2000). The existence of such receptors in anuclear cells indicates potential non-genomic actions of steroid hormones on platelets. Is this the case for corticosteroids?
The aim of this study was to investigate glucocorticoid receptor (GR) expression in platelets and to examine the characteristics of corticosteroid binding to these cells.
Citrated venous blood was centrifuged at 180x g. for 10min and platelet-rich plasma removed. GR expression in platelets was determined by Western blot analysis, flow cytometry and confocal microscopy. A radioligand binding assay was used to quantify GR expression and ligand affinity.
Purified platelets were sonicated in presence of protease inhibitors and protein concentrations standardised. Samples were run under reducing conditions on 10% SDS-PAGE in conjunction with MW standards. Resolved proteins were transferred to nitrocellulose membranes and incubated with mouse anti-GR antibody (8E9, Serotech, Oxford) and thence detected using a goat anti-mouse horse-radish peroxidase conjugated antibody and ECL reagent. Purified whole platelets were also immunostained for GR. Following fixation with 2% paraformaldehyde, platelet membranes were permeabilised with 0.025% saponin and reacted with anti-GR antibodies at 4°C. Receptor staining was detected with goat anti-mouse IgG conjugated to fluorescein isothiocyanate.
For flow cytometry, platelets were discriminated by forward and side-scatter and CD31 expression. GR levels in platelets were compared with that of lymphocytes. GR distribution was also visualised by laser confocal microscopy. Ligand binding to GR was measured using a ^{3}H-dexamethasone (DEX) binding assay. Purified platelets at $1x10^7$ were reacted with 1 – 50pmol ^{3}H-DEX in the presence of absence of excess unlabelled DEX at 37°C for 1h. Following liquid scintillation counting, Scatchard analysis was performed.
Western blotting revealed a discrete GR band at 97kDa under reducing conditions. Flow cytometric analysis of $CD31^+$ platelets demonstrated positive GR staining of 248 mean fluorescence intensity (MFI) units compared to 10 MFI for pre-immune control IgG antibodies. $CD31^-$ lymphocyte GR levels were similar: 322 MFI compared to 18 MFI for IgG control antibodies. Confocal microscopy localised GR staining to the periphery of the platelet. The nature of the ^{3}H - DEX binding plots for 3 separate binding experiments suggested either negative cooperativity or the existence of multiple independent binding sites. The estimated hypothetical maximum Kd in the absence of ligand was 0.6 ± 0.2 nM with Bmax of 0.2 ± 0.02 nM, equating to 11500 ± 1300 binding sites per platelet.
In conclusion, we report the existence of GR in human platelets. The receptor is expressed in significant numbers and has a MW identical to that found in other blood cell types. The anuclear nature of platelets and atypical steroid binding profile of the receptor both indicate a non-classical function of GR in this cell type. Future work will relate these findings to both *in vitro* and *in vivo* effects of corticosteroids on platelet activation and function.

MP is a Senior Fellow of the Arthritis Research Campaign. RJF is Principal Research Fellow of the Wellcome Trust.

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122P GLUCOCORTICOID MODULATION OF RANKL AND OSTEOPROTEGERIN IN OSTEOBLAST CELL LINES

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The discovery of two novel cytokines osteoprotegerin (OPG) and RANK ligand (RANKL) has opened new avenues for therapeutic intervention on disabilitating disease such as osteoporosis and rheumatoid arthritis. RANKL induces osteoclastogenesis and activates mature osteoclasts, hence it is a potent inducer of bone resorption both *in vivo* and *in vitro* (Simonet *et al.*, 1997). OPG, a secreted glycoprotein, acts as a soluble decoy receptor for RANKL and is a potent inhibitor of bone resorption (Lacey *et al.*,1998). Here, we have tested the effect of glucocorticoids (GCs) on OPG and RANKL mRNA and protein expression in two human osteoblastic cell lines.
The osteoblastic cell lines Saos-2 and HTB-96 (ATCC, USA) were cultured in McCoy medium with 10% FCS and passaged two times a week. For determination of GC receptor (GR) expression, cells were incubated with 0.57-50 pmol [^{3}H]-dexamethasone (Amersham Biotechnology, Abingdon, Oxon) at 37°C for 1 h, in the presence or absence of 50 µmol cold GC. Scatchard plot analysis was run to measure the affinity constant K_D and the maximal binding B_{max}. HTB and Saos-2 cells ($5\text{-}20x10^5$) were incubated for 4 or 24 h with 1-100 nM prednisolone. Cell extracts were collected for mRNA detection using conventional RT-PCR: denaturation 92°C for 45 s, followed by annealing (60°-55°C for OPG and RANKL; 30 s) and extension (72°C, 30 s). Protein expression was measured by ELISA: OPG was measured with ELISA development reagents from R&D Systems (Abingdon, UK) while RANKL was measured with Peprotech antibodies (London, UK). OPG was readily detected in the cell incubation media, whereas RANKL protein was quantified in cell pellets. Data are mean ± s.e.mean, and were analysed by ANOVA followed Dunnet post-hoc analysis, with $P<0.05$ as significant.
As expected, HTB-96 cells expressed GR with K_D of 10.4 ± 1.3 nM, B_{max} of 53.6 ± 3.8 pM and 32,532 ± 2,993 sites per cell (n=3 experiments). HTB-96 cells released OPG in the medium, with values of 216 ± 3 and 1101 ± 40 $pgml^{-1}$, at 4 and 24 h, respectively (n=4). Cell incubation with 10 nM prednisolone decreased OPG release by 68±2%, as detected at the 24 h time-point (n=4; $P<0.05$), with no significant effect at the shorter time-point. Similar data were obtained in Saos-2 cells with 100 nM prednisolone at 24 h (data not shown). RANKL protein expression was not significantly modified by this GC in HTB-96 cells, but a significant increase was measured in Saos-2 cells: from 89.56 ± 2.66 $pgml^{-1}$ to 139.8 ± 26.25 $pgml^{-1}$, in basal and after 100 nM prednisolone (24 h). RT-PCR analysis confirmed these findings: cell incubation with 100 nM prednisolone decreased OPG mRNA by 76±16% in HTB-96 cells (n=3, $P<0.05$), whereas it induced an increase in RANKL mRNA by 155±24% in HTB-96 (n=3, $P<0.05$). Although in Saos-2 cells this GC increased OPG mRNA by 136 ± 16%, RANKL mRNA was increased by 214± 52% (n=3, $P<0.05$) giving a RANKL/OPG ratio of 1.57.

These results provide a molecular basis for GC-mediated osteoporosis, and are consistent with an overall increase in osteoclast activity *in vitro* as previously reported by our group (Paul-Clark *et al.*, 2002). In addition, if confirmed in *in vivo* settings, these data indicate that OPG could be exploited for the therapeutic control of GC side effects on bone.

This work was supported by a grant of the Joint Research Board of St Bartholomew's Hospital (grant XMNH)

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123P CHARACTERISATION OF MUSCARINIC RECEPTOR EXPRESSION IN DOG SUBMANDIBULAR GLAND

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Overactive bladder (OAB) is an under-reported syndrome (Abrams *et al.*, 2000; Ricci *et al.*, 2001) that affects more people worldwide than Alzheimer's disease (Milsom *et al.*, 2001). Antimuscarinic therapy, the mainstay of OAB treatment, is associated with dry mouth, which is largely attributed to blockade of M_3 receptors in the salivary gland. The present study describes a preliminary characterisation of muscarinic receptors in the dog submandibular gland.

Protein preparations obtained from tissue homogenates of dog submandibular gland were characterised using [^{3}H]-*N*-methyl scopolamine ([^{3}H]-NMS; Amersham) and non-specific binding was determined with atropine (1 μM). The affinities of a range of antimuscarinic agents were investigated using [^{3}H]-NMS (0.38nM) competition assays at room temperature with 2 h incubation time. The effects of the muscarinic M_1 subtype-selective toxin (MT-7; Peptides International; Karlsson *et al.*, 2000; 20 nM, 60 min pre-incubation at room temp.) on the binding parameters (K_D and B_{max}) of [^{3}H]-NMS (0.02-6.0 nM; 2 h incubation at room temp.) were determined from a 12-point saturation analysis (3-6 separate studies). Data from saturation studies were analysed using GraphPad PRISM curve fitting programs with K_D and B_{max} derived from the Langmuir equation. IC50 and hill slopes were derived from competition cures using non-linear curve fitting programs within GraphPad PRISM and either a 1- or 2-site model.

MT-7 toxin increased K_D values compared with no toxin (0.44 nM and 0.16 nM, respectively), while the B_{max} values of [^{3}H]-NMS were 464 fmole/mg in the absence and 216 fmole/mg in the presence of toxin. IC_{50} values are shown in Table 1.

Table 1. The IC_{50} values of a range of standard muscarinic ligands in the dog submandibular gland

	Muscarinic selectivity	IC_{50} (nM)	Hill slope	n
Atropine	All	4.4	-0.83	5
Pirenzepine	M_1	180	-0.69	7
MT-7	M_1	1.3[a]	-1.36	4
Methoctramine	M_2	4539	-1.16	4
Darifenacin	M_3	75.2	-0.91	4

[a] Calculated as the midpoint of the top and the bottom of the inhibition curve.

The saturation and competition analysis performed using the M_1 subtype-selective toxin MT-7 suggests that approximately 60% of the muscarinic receptors expressed in the dog submandibular gland are of the M_1-subtype. The remaining component is likely to be M_3 based on competition IC_{50} / Hill slope data for darifenacin and pirenzepine.

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124P DARIFENACIN HAS A LOW AFFINITY FOR MUSCARINIC M_1 RECEPTORS IN DOG SAPHENOUS VEIN CONFIRMING ITS M_3 SELECTIVITY

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Overactive bladder (OAB) is a common but under-reported syndrome in the general population (Abrams *et al.*, 2000), which may occur with or without incontinence (Stewart *et al.*, 2001). The estimated prevalence of OAB is 17% in patients aged 40 years or more (Milsom *et al.*, 2001), although many patients, especially those with incontinence, may not seek treatment due to embarrassment (Hampel *et al.*, 1997). The muscarinic M_3 receptor subtype is thought to be important in bladder control, and here, the potency and selectivity of darifenacin, tolterodine, oxybutynin and atropine for the M_1, M_2 and M_3 receptor subtypes have been compared.

Dog saphenous vein rings (M_1), guinea pig paired atria (M_2) or bladder strips (M_3) were attached to isometric transducers and suspended *in vitro* in organ baths containing Krebs solution gassed with 95% O_2/5% CO_2 at either 37°C or 32°C. Contractions were recorded using an in-house data acquisition system or a Grass 79D polygraph. Carbachol-evoked contractions of guinea pig bladder or atria and acetylcholine-evoked contractions of dog saphenous vein were recorded. Antagonist potencies were calculated by estimation of either a pK_B value using Schild analysis, following confirmation that Schild slopes did not differ from unity, or by use of the Gaddum equation to generate an apparent pA_2 value. The M_3 selectivity ratio was calculated by comparing M_3 vs M_1/M_2 affinities.

Table 1 shows the potency and selectivity of each antagonist.

Table 1. Antagonist potencies[a] (95% CI) and M_3 selectivity ratios for antimuscarinic agents in isolated tissues.

	(M_3)	(M_1)	(M_2)
Darifenacin	8.9 (8.3-9.5)[b]	7.2 (6.6-7.9)	7.2 (6.8-7.5)
M_3 selectivity	*1*	*50*	*50*
Tolterodine	8.8 (8.5-9.1)	8.6 (8.3-8.9)	8.6 (8.3-8.9)
M_3 selectivity	*1*	*2*	*2*
Oxybutynin	8.6 (8.2-9.0)	7.6 (7.4-7.9)	7.0 (6.7-7.3)
M_3 selectivity	*1*	*10*	*40*
Atropine	9.2 (8.8-9.6)	9.6 (8.9-10.3)	9.1 (8.8-9.4)
M_3 selectivity	*1*	*0.4*	*1.3*

[a] pK_B values except for [b], which is apparent pA_2 at 10 nM darifenacin; data are the mean of 4-8 experiments

These data demonstrate that in functional muscarinic receptor systems, darifenacin is more selective for the M_3 receptor than the M_1 or M_2 receptors. Overall, these M_3 selectivity profiles are consistent with binding data generated at human recombinant muscarinic receptor subtypes.

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125P INTERACTION OF DARIFENACIN AT THE HUMAN RECOMBINANT M_3 RECEPTOR IS COMPETITIVE AND REVERSIBLE

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The muscarinic M_3 receptor subtype is the primary receptor in the pathophysiology of overactive bladder (OAB) (Newgreen *et al.*, 2002; Napier & Gupta, 2002). Darifenacin is an M_3-selective antagonist that is currently undergoing Phase 3 clinical testing for the treatment of OAB. This study used radioligand-binding techniques to determine whether darifenacin is a competitive and reversible antagonist of the human recombinant M_3 receptor.

Membrane homogenates of Chinese hamster ovary cells expressing recombinant human M_3 receptors were pre-incubated (1.5 h) with antagonists prior to incubation with [^{3}H]-*N*-methyl scopolamine ([^{3}H]-NMS, 0.1-12 nM) for 3 h at room temperature. Non-specific binding was defined by 1 μM atropine. The effects of the test antagonists on the binding parameters K_D and B_{max} for [^{3}H]-NMS were determined from a 12-point saturation analysis. [^{3}H]-darifenacin was used to determine the dissociation half-life ($t_{½}$) from M_3 receptors. Membrane homogenates were equilibrated for 1.5 h at 37°C with 0.4 nM [^{3}H]-darifenacin and specific binding was determined at various time points up to 3 h following initiation of dissociation by addition of 10 μM atropine. Data were analysed using GraphPad PRISM curve-fitting programs with K_D and B_{max} derived from the Langmuir equation and $t_{½}$ derived directly from a first-order dissociation curve.

B_{max} values were not significantly altered for darifenacin, atropine, tolterodine and oxybutynin ($p>0.05$). 4-DAMP mustard significantly reduced B_{max} values ($p<0.001$) (Table 1).

Table 1. Effects of muscarinic antagonists on mean K_D (nM) and B_{max} (fmole/mg protein) for [^{3}H]-NMS binding to the human M_3 receptor (n=2-4)

		nM					
	Control	**0.3**	**1**	**3**	**5**	**10**	**100**
Atropine							
K_D	0.12	0.33	0.70	1.9	-	-	-
B_{max}	1454	1526	1495	1678	-	-	-
4-DAMP mustard							
K_D	0.25	-	0.68	-	4.84	8.52	-
B_{max}	1665	-	943*	-	642*	524*	-
Darifenacin							
K_D	0.35	-	0.56	0.88	-	1.42	-
B_{max}	1513	-	1598	1637	-	1624	-
Tolterodine							
K_D	0.20	-	0.19	0.32	-	0.42	3.08
B_{max}	1591	-	1505	1469	-	1582	1557
Oxybutynin							
K_D	0.18	-	0.21	0.42	-	0.48	3.10
B_{max}	1565	-	1603	1497	-	1618	1533

*$p<0.001$

[^{3}H]-darifenacin binding to the human M_3 receptor was completely reversible ($t_{½}$=5.32 min at 37°C).

The data obtained in these studies demonstrate that darifenacin binds to the human recombinant M_3 receptor in a competitive and reversible manner, providing further support for its use in the treatment of OAB.

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126P CHARACTERISATION OF A NOVEL FLUORESCENT AGONIST FOR THE HUMAN A_1-ADENOSINE RECEPTOR

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We have previously reported the synthesis and characterisation of xanthine amine congener-BODIPY 630/650 as a fluorescent antagonist at the human A_1-adenosine receptor (A_1-AR) (Briddon *et al.*, 2002). Here we report the synthesis and characterisation of a functional fluorescent agonist at this receptor, based on 5'-*N*-ethylcarboxamidoadenosine (NECA).

N^6-aminobutyl-5'-deoxy-5'-oxo-5'-ethylaminoadenosine (ABEA) was synthesised from commercially available reagents in 6 steps. The primary amine group of ABEA was acylated with the fluorophore BODIPY®630/650-X-succinimydyl ester (BY-630, Molecular Probes) as previously reported (Briddon *et al.*, 2002) to afford BY630-ABEA, which was purified by RP-HPLC. Functional studies were performed in CHO-K1 cells expressing both the human A_1-AR and a c-fos-pGL3 reporter vector (CHO-A1fos cells, Megson *et al.*, 1998). Cells were incubated for 24h in serum-free DMEM/F-12 medium, then stimulated with agonist for 5h, in some cases following 30 min incubation with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Luciferase expression was quantified using a Luclite® kit according to manufacturer's instructions, and pEC_{50}s calculated using a 4 parameter logistic equation. Live cell confocal imaging was carried out at 22°C (Briddon et al., 2002) on CHO-A1 cells or CHO cells expressing the A_1-AR tagged on the C-terminus with a green fluorescent protein (CHO-A1Tpz).

In CHO-A1fos cells, both BY630-ABEA and the A_1-AR agonist N^6-cyclopentyl adenosine (CPA) stimulated luciferase expression in a dose-dependent manner (pEC_{50}'s of 7.01±0.04 (n=6) and 6.76±0.18 (n=5) for CPA and BY630-ABEA, respectively, mean±s.e.mean). Stimulation was mediated by the A_1-AR receptor, since the concentration response curves were shifted to the right in a competitive manner by 10nM DPCPX, yielding apparent pK_ds of 8.72±0.03 and 9.05±0.10 vs. CPA and BY630-ABEA, respectively (n=3). A higher dose of DPCPX (100nM), gave an apparent pK_d of 8.62±0.02 for CPA stimulation, but completely blocked the response to BY630-ABEA (n=3). For receptor visualisation, CHO-A_1 cells were incubated with 100nM BY630-ABEA for up to 60min. Binding of ligand to the membrane was detectable after 5 min, and was substantial after 30 min (n=3). Binding was to the A_1-AR, since it was substantially reduced by preincubation with DPCPX (1μM, 30 min). In addition, experiments in CHO-A1Tpz cells, showed co-localisation of ligand fluorescence at the membrane with that from the fluorescently tagged A_1-AR.

In conclusion, we have successfully synthesised a novel fluorescent agonist ligand for the human A_1-AR, which will be useful in monitoring the localisation of the endogenous A_1-AR receptor in both acutely dispersed cells and cell lines.

We thank the Wellcome Trust for financial support

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127P TIME COURSE OF AGONIST-STIMULATED CRE-MEDIATED REPORTER GENE TRANSCRIPTION IN CHO CELLS

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Cyclic AMP response element (CRE)-reporter assays usually involve incubation with agonist for 4-5 hours (Hill *et al.*, 2001), even though a 10 minute incubation with agonist is sufficient to produce a large increase in intracellular cAMP levels (McDonnell *et al.*, 1998, Baker *et al.*, 2002a). The time taken for the generation of CRE-mediated reporter products and the duration of agonist stimulation required to initiate this process is largely unknown. The aims of this study were to determine: (1) the time taken for the reporter gene product to become detectable and (2) to establish the minimal agonist exposure time to initiate the CRE-gene transcription process.

CHO-K1 cells stably expressing the human β_2-adrenoceptor and a secreted placental alkaline phosphate (SPAP) reporter gene containing a 6 CRE promoter were used. Cells were incubated with agonist for 10min-5hours. The rate of secreted SPAP was measured as previously described (McDonnell *et al.*, 1998) and total cellular content of SPAP was also determined following cell lysis.

A 5 hr incubation with isoprenaline (log EC_{50} -8.52±0.20) stimulated an increase in intracellular SPAP production of 3.7 ±0.08 (n=5) fold over basal. Compared with the 5hr response, the responses seen at 4hr, 3hr and 2hr were 71.1±2.8% (log EC_{50} -7.98±0.26), 46.2±4.3% (log EC_{50} -7.85 ±0.19) and 20.4±1.7% (log EC_{50} -7.80±0.19; all n=5). No significant response was seen after 10 min, 30 min or 1hr of agonist incubation. 5 hours incubation with the direct adenylyl cyclase stimulator forskolin yielded a response 116.7±4.7% of the isoprenaline maximum (log EC_{50} –6.02±0.08) and produced a similar time course for gene transcription with responses at 4 hr being 60.6±2.7% of the 5hr forskolin maximum (log EC_{50} –6.08±0.09), 3hr 33.2±2.43% (log EC_{50} – 6.27 ±0.15), and 2hr 13.4±0.85% (log EC_{50} –6.47±0.11; all n=3). Similar data were also obtained with salbutamol, and labetolol and alprenolol (which we have previously shown to act as β_2-partial agonists, Baker *et al.*, 2002b). The time courses for intracellular generation and secretion of SPAP were very similar in each case.

In view of our previous findings that some ligands dissociate slowly from the β_2-receptor (Baker *et al.*, 2002a), 100nM ICI118551 was added after washout of isoprenaline to ensure agonist-receptor interaction dissociation at set times during a total 5 hour incubation. No response was seen when agonist stimulation was restricted to the first 10 minutes of the 5 hours, but responses were detectable when the agonist was present for the first 30 min (log EC_{50} -8.22±0.24, 28.1±1.7%, n=8), 1 hr (log EC_{50} -8.39±0.32, n=7) 43.1±2.3%, and 2 hr (log EC_{50} - 8.29±0.28, n=4) 66.8±6.7% of the overall 5 hr incubation.

In summary, a minimum of 1-2 hr is required from the addition of agonist for a detectable SPAP response to be observed. Although an initial 30 min agonist exposure is sufficient to initiate a measurable CRE- mediated gene transcription response when monitored 5 hr later, the response is very much larger if agonist is present for the full 5 hr.

JGB holds a Wellcome Trust Clinical Training Fellowship.

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128P EFFECT OF MEK1/2 INHIBITORS ON CRE-MEDIATED GENE TRANSCRIPTION IN CHO CELLS

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Cyclic AMP response element (CRE)-mediated gene transcription is thought to result from the activation of the adenylyl cyclase, cAMP, protein kinase A pathway leading to the phosphorylation of the CRE binding protein CREB (Lali and Sassone-Corsi, 1994). Previous work has, however, suggested an additional signalling pathway may contribute to CRE-mediated responses in CHO cells (Baker *et al.*, 2002).

Experiments were performed on CHO cells stably expressing the human β_1-or β_2-adrenoceptor and either a secreted placental alkaline phosphatase (SPAP) or luciferase reporter gene containing a 6 CRE promoter. MAP Kinase inhibitors (English and Cobb, 2002) (were incubated for 1 hour before the addition of agonist for a further 5hr at 37°C. SPAP and luciferase activity were measured as described previously (McDonnell *et al.*, 1998, Hill and Baker, 2002).

Isoprenaline stimulated an increase in β_2-mediated CRE-SPAP secretion of 6.2 ± 0.03 fold over basal (log EC_{50} -8.16 ± 0.07, n=35). This response was unaffected by pre-incubation with pertussis toxin (Gi inhibitor, Calbiochem, 24hrs, 0.1μg/ml, n=9) and SB 203580 (P38 MAP Kinase inhibitor, Calbiochem, 3μM,n=3). PD 98059 (MEK1 inhibitor, Calbiochem, 50μM) however decreased the maximum to 65.1 ± 2.7% of the original response whilst having little effect on the log EC_{50} (-8.42 ± 0.13, n=12). U0126 (MEK1/ MEK2 inhibitor, Tocris, 10-50μM) decreased the basal CRE-SPAP secretion to 54.8±6.7%,n=20 untreated, and attenuated the isoprenaline-stimulated response to 13.0 ± 1.8%, n=6.

Similar results were seen with forskolin (direct adenyly cyclase activator) salbutamol, salmeterol, and the partial agonist CGP 12177 in these β_2-CRE-SPAP cells. To ensure that the inhibitors were not reducing the secretory part of the cascade, cells were lysed and intracellular SPAP measured. This yielded identical results to the secreted SPAP.

In β_1-CRE-SPAP cells, U0126 also reduced basal (to 30.5±1.6%,n=3) and isoprenaline-stimulated responses (log EC_{50} -8.29±0.07,n=3) to 13.4 ± 1.74%,n=3. CGP 12177 responses were similarly inhibited. To ensure that reporter product itself was not affecting the signalling cascade, a different system with a β_1-CRE-luciferase reporter was used. Here, U0126 caused a similar reduction in basal in β_1-CRE-luciferase cells to 50.3±0.8%, n=4. The isoprenaline (EC_{50} – 7.49±0.13, 2.03±0.05 fold over basal, n=4) and forskolin (EC_{50} –5.32±0.06, 8.9±1.7 fold over basal, n=4) stimulated responses were also attenuated by pre-incubation by U0126 to 34.3±4.9%,n=4 and 21.5±2.9%, n=4 of the original responses.

In summary this data suggests that following an increase in intracellular cAMP, a large part of the subsequent CRE-gene transcription may actually occur via the P42/44 MAP Kinase pathway rather than the PKA pathway.

JGB holds a Wellcome Trust Clinical Training Fellowship.

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129P ANTAGONIST PHARMACOLOGY OF ADENOSINE A_{2B} RECEPTORS FROM RAT, GUINEA PIG AND DOG

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The purpose of our studies was to explore the pharmacological similarities or differences between A_{2B} receptors of different species. To this end we have measured the dissociation constant of the antagonist-receptor complex, K_B, for eight adenosine receptor antagonists from four chemical classes at the native A_{2B} receptors which mediate relaxation of smooth muscle from rat colon, guinea pig aorta and dog saphenous vein.

Longitudinal muscle (LM) strips were obtained from the colons of male Brown Norway rats (ca. 300g), aortas from male Dunkin Hartley guinea pigs (ca. 500g) and saphenous veins from male and female adult beagle dogs. Tissues were set up for isotonic recording of tension and relaxant effects of cumulative concentrations of NECA against tone induced by bethanechol (LM strip) or phenylephrine (aorta, saphenous vein) recorded (for general details see Hannon et al., 2002). Antagonists were used at concentrations which induced a minimum 3-fold shift in the NECA concentration-response curves (CRCs) and incubated with the tissues for 30 min. K_B, values were calculated from shifts in the CRCs (Hannon et al., 2002).

In all three assays, the antagonists caused parallel rightward shifts in the CRCs to NECA and there was no depression of the maximum response. There was generally good agreement between the tissues with respect to the antagonist pK_B values (Table 1) and regression analysis revealed highly significant correlations between each of the three receptors (rat/guinea pig, $r = 0.988$, $P < 0.0001$; rat/dog, $r = 0.903$, $P < 0.002$; guinea

Table 1 pK_B values for blockade of A_{2B} receptors from different species.

	Rat	Guinea Pig	Dog
Aminophylline	5.19 ± 0.16 (4) [5]	4.96 ± 0.11 (3)	5.51 ± 0.34 (5)
8-SPT	5.80 ± 0.06 (3) [5]*	5.66 ± 0.13 (5)*	6.65 ± 0.18 (4)
XAC	7.37 ± 0.15 (4)*	7.75 ± 0.24 (4)	8.45 ± 0.33 (4)
DPCPX	6.70 ± 0.14 (5)	6.84 ± 0.07 (4)	6.88 ± 0.26 (4)
MRS 1754	7.78 ± 0.14 (3) [5]	8.04 ± 0.14 (3)	7.91 ± 0.20 (4)
CGS 15943	7.18 ± 0.08 (5)*	7.63 ± 0.13 (6)*	8.61 ± 0.08 (4)
ZM 241385	7.19 ± 0.11 (3)	7.06 ± 0.04 (5)	7.61 ± 0.27 (4)
CGH 2473[a]	7.51 ± 0.10 (5)*	7.83 ± 0.08 (3) [4]*	8.51 ± 0.24 (4)

Values are means (± s. e. means) of data from the number of animals shown in parentheses. Numbers in square brackets indicate the the total number of tissues used. [a]*N*-[4-(3,4-dichloro-phenyl)-5-pyridin-4-yl-thiazol-2-yl]-acetamide. Chemical names of all other compounds are given in Fredholm et al., (2001). *$P < 0.05$ that the value differs significantly from the dog value (Student's t-test with Hommel-Hochberg correction for multiple comparisons).

However, the correlation between the rat and guinea-pig receptors was clearly stronger than the correlation between the dog receptor and either the rat or guinea pig receptors. Indeed, the pK_B values of 8-SPT, XAC, CGS 15943 and CGH 2473 for the dog receptor exceeded, by at least 0.5 log units, and significantly, the pK_B values at the other sites (Table 1).

The data indicate that the A_{2B} receptors of rat, guinea pig and dog are similar but not identical with respect to antagonist pharmacology. The majority of the antagonists we have investigated are widely used as tools to define the involvement of adenosine receptor subtypes in biological responses. Our data provide reference values for these agents for A_{2B} receptor affinities from species widely used in pharmacological investigations.

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130P EXPRESSION OF ADENOSINE RECEPTORS IN MCF-7 HUMAN BREAST CANCER CELLS

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The adenosine receptor family consists of four G protein-coupled receptors, termed A_1, A_{2A}, A_{2B} and A_3, widely distributed in the human body (Fredholm *et al.*, 2001). Each receptor has its own pharmacological characteristics. Adenosine receptors have been shown to be involved in the proliferation of various human tumour cells (Colquhoun *et al.*, 1997). To our knowledge little work has been done to investigate the presence and function of adenosine receptors in breast cancer tumours. The aim of this study was to demonstrate the presence of mRNA for each receptor in MCF-7 human breast cancer cells and to characterise the A_1 and A_3 receptors by radioligand binding assays.

Studies were performed using MCF-7 cells which had been quiesced by serum starvation for 24 hours prior to RNA extraction. RNA was extracted from the MCF-7 cells using a standardised method. The mRNA corresponding to the four adenosine receptors was amplified by RT-PCR using primers obtained from published sources. The resulting cDNAs were separated by gel electrophoresis and visualised. Radioligand binding assays were performed using the A_1 antagonist [^{3}H]-DPCPX and A_3 agonist [^{125}I]AB-MECA with a modified method of Lohse et al. (1987). Assays were conducted at room temperature for 2 hours using 60µg membranes and a 50mM Tris pH 7.4 buffer with or without 10mM $MgCl_2$ for 0.3nM [^{125}I]AB-MECA or 0.2nM [^{3}H]-DPCPX, respectively. The ability of adenosine analogues to inhibit binding was investigated. Non-specific binding was determined using the respective analogue at its highest concentration. Both the RT-PCR and binding experiments are n=3.

The radioligand binding for the [^{125}I]AB-MECA was unsuccessful with the MCF-7 cells as no specific binding was seen. From the RT-PCR it was seen that there was mRNA expression for the A_1, A_{2B} and A_3 receptors but no mRNA expression for the A_{2A} receptor. Initial binding assays carried out with rat brain membranes (20µg) to characterise [^{3}H]DPCPX gave rank orders of agonist affinity of CPA>R-PIA>NECA>IB-MECA and antagonist affinity of DPCPX >MRS1220>8-PT, comparable with the literature (Fredholm *et al.*, 2001). With the MCF-7 cell membranes a low level of specific binding was obtained (~ 150dpm). An order of agonist affinity of NECA>IB-MECA>CPA (1.74(0.38-5.46) > 2.29(0.42-7.88) > 7.63 (4.74-11.60) µM + 95% confidence limits) was obtained, inconsistent with the literature (Fredholm *et al.*, 2001).

This study shows that there is expression of mRNA for A_1, A_{2B} and A_3 receptors but not A_{2A} in MCF-7 cells. Further investigation by a binding assay with a highly A_1 selective antagonist radioligand [^{3}H]-DPCPX showed the presence of A_1 receptors although their binding affinities were atypical of transfected human A_1 receptors. This suggests that the A_1 receptors on MCF-7 cells, which are native receptors, may have different pharmacological characteristics to transfected human A_1 receptors. We tried to check this by repeating the binding assays using adenosine receptor antagonists. However, the vehicle (DMSO) for the antagonist reduced the specific binding to such an extent that it was not possible to perform experiments. The presence of A_1 receptors confirms our previous findings indicating a functional role in MAPK activation (Clark *et al.*, 2002).

In conclusion the presence of A_1, A_{2B} and A_3 receptor mRNA has been demonstrated in MCF-7 cells, although only A_1 receptors could be identified by radioligand binding and agonist affinity was atypical.

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131P LYSOPHOSPHOLIPIDS STIMULATE PROLIFERATION OF RAT PRIMARY AIRWAY FIBROBLASTS

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The lysophospholipid receptors [previously named EDG (endothelial differentiation gene) receptors] were identified as a family of G-protein coupled receptors for which either lysophosphatidic acid (LPA) or sphingosine 1-phosphate (S1P) are specific agonists (Chun *et al.*, 2000). There is evidence that extracellular levels of lysophospholipids may be elevated during inflammatory reactions (for review see Racké *et al.*, 2000). Since growth stimulation is a common cellular response to lysophospholipids, it appeared to be an interesting idea that lysophospholipids could play a role in remodelling processes associated with chronic inflammatory reactions (Racké *et al.*, 2000). Since fibroblasts appear to play a significant role in the remodelling observed in asthmatic airways (Wilson *et al.*, 2001), it was tested whether LPA and S1P might affect the proliferation of airway fibroblasts.

Primary airway fibroblasts were obtained from isolated trachea of Sprague Dawley rats of either sex (180-240 g) by an outgrowth technique in the presence of fetal calf serum (FCS, 10%). Confluent cultures were passaged 1:4 and passage 3-4 used for the following experiments. After trypsinisation the cells were disseminated ($0.25*10^6$ cells $well^{-1}$) and cultured for 24 h in DME/F-12 medium in the presence of 10% FCS followed by 24 h culture in the absence of FCS. Thereafter, the cells were cultured for additional 24 h (test period) in the absence or presence of FCS, LPA or S1P and additional presence of [^{3}H]-thymidine (10 µM, 1 µCi).

When serum was present during the test period, the incorporation of [^{3}H]-thymidine amounted to 11 526 ± 681 d.p.m. (mean ± s.e.mean, n=12). When the fibroblasts were cultured during the test period in serum free medium, the incorporation of [^{3}H]-thymidine was reduced to 668 ± 93 d.p.m ($p<0.0001$). In the absence of serum, 0.1 and 1 µM S1P enhanced the incorporation of [^{3}H]-thymidine by 65 ± 16 and 75 ± 11 %, respectively (each n=12, $p<0.01$). Lower concentration of S1P (1 and 10 nM) had no significant effect. LPA also stimulated the incorporation of [^{3}H]-thymidine under serum free conditions, by 95 ± 18% at 10 µM (n=12, $p<0.01$). At the lower concentration of 1 µM, LPA did not exert a significant stimulatory effect. The proliferative effects of LPA and S1P were also seen at the level of total protein content of the culture which was enhanced by about 50% in LPA (10 µM) or S1P (0.1 and 1 µM) treated cultures.

In conclusion, LPA and S1P exerted a clear proliferative effect on primary airway fibroblasts suggesting that these lysophospholids might play a role in remodelling processes in chronic airway diseases. Whether this effect is mediated via LPA and S1P receptors, respectively, will have to be studied in future experiments.

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132P THE PPARγ LIGAND 15-DEOXY-$\Delta^{12,14}$-PROSTAGLANDIN J_2 (15D-PGJ_2) INHIBITS PROLIFERATION AND CYTOKINE RELEASE FROM HUMAN PULMONARY ARTERY SMOOTH MUSCLE

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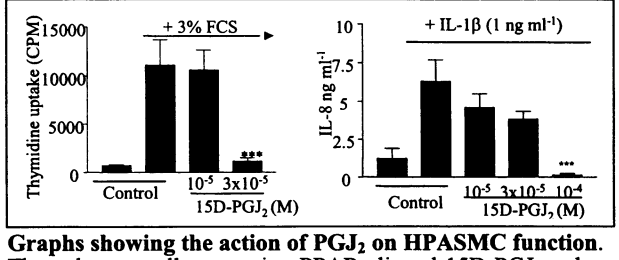
Pulmonary hypertension, either primary or secondary (Strange *et al.*, 2002), is a progressive fatal disease for which there are few therapeutic interventions. Chronic pulmonary hypertension is associated with structural changes in both the pulmonary vasculature and the right ventricle. Reversing or reducing the remodelling of pulmonary vessels is an important therapeutic target for the treatment of pulmonary hypertension. This may be achieved in some patients by the continuous infusion of prostacyclin. Here we have investigated the effects of another cyclo-oxygenase metabolite, 15D-PGJ_2, which activates PPARγ receptors, and has been demonstrated to limit inflammation in other tissues (Bishop-Bailey, 2000).

Human pulmonary artery smooth muscle cells (HPASMC) were obtained and cultured in 96-well plates as described previously (Jourdan *et al.*, 1999). Cell proliferation following incubation with foetal calf serum (FCS, 3 %) was determined by the incorporation of methyl-[^{3}H]thymidine (Wort *et al.*, 2001). Cytokine release was determined by ELISA following incubation (24 h) of cells with interleukin (IL) -1β (1 ng ml^{-1}) in the presence of 15D-PGJ_2 (10^{-5}-10^{-4} M), cicaprost (10^{-7} M) or vehicle (0.1% DMSO) (Stanford *et al.*, 2000). Cell viability, measured by the conversion of MTT to formazan (Stanford et al., 2000), were not affected by 15D-PGJ_2. Data are presented as mean ± S.E.M. and was compared using ANOVA with the appropriate post test (GraphPad, Prism 3.0).

Cicaprost induced small but non significant inhibition of proliferation, with a maximum effect of 32.7± 6.5% at 10^{-7} M. By contrast, 15D-PGJ_2 3 x 10^{-5} M completely blocked FCS-induced proliferation (Figure 1; n=9). Cicaprost up to 10^{-5} M had no effect on the release of either IL-8 or G-CSF by IL-1β treated cells (n=9) whereas 15D-PGJ_2 induced concentration-dependent reductions in the release of IL-8 (Figure 2; n=9). Similarly, 15D-PGJ_2 ($3x10^{-5}$M) reduced the release of G-CSF by IL-1β treated cells from 364 pg ml^{-1} ± 99pg ml^{-1} to 157pg ml^{-1} ± 53 pg ml^{-1} (n=9).

Figure 1 **Figure 2**



Graphs showing the action of PGJ_2 on HPASMC function.

Thus, the naturally occurring PPARγ ligand 15D-PGJ_2 reduces both the proliferation of HPASMC and the release of cytokine markers of remodelling to a much greater degree than the prostacyclin-mimetic cicaprost. These data are the first to indicate that PPAR ligands may have some therapeutic use in the treatment of human pulmonary hypertension.

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This work was supported by the MRC and the BHF

133P EFFECTS OF L-ARGININE ON NEUTROPHIL INFLUX AND AIRWAY HYPERREACTIVITY 1H AFTER LIPOPOLYSACCHARIDE CHALLENGE

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The combined levels of nitric oxide (NO) metabolites, nitrate and nitrite, in bronchoalveolar lavage fluid (BALF) are reduced 1h after lipopolysaccharide (LPS) challenge. This is associated with neutrophil influx and airway hyperreactivity (AHR) to histamine and may be used as a model for COPD (Toward et al, 2000). The administration of the stereospecific precursor of NO, L- but not D-arginine to rats reduced lung neutrophil levels 4h following LPS challenge (Sheridan et al, 1998). This study investigated whether a deficiency in L-arginine and therefore NO contributes to neutrophil influx and AHR in our model for COPD.

Specific airway conductance (sGaw) was measured in groups of conscious Dunkin-Hartley guinea-pigs (male, 300-350g) by whole body plethysmography. Baseline sGaw values were obtained and 30min later they received a nose-only exposure to a threshold dose of nebulized histamine (1mM nose-only for 20s) and sGaw was recorded at 0, 5 and 10min afterwards. 24h later, the animals were box-exposed (60min), to either nebulized LPS (30$\mu g.ml^{-1}$) or vehicle (0.9% LPS-free saline) for 1h and sGaw measured at 0, 15, 30, 60 min afterwards. Airway reactivity to histamine was re-assessed 1h post exposure. Other animals received L-arginine or D-arginine (10$mg.kg^{-1}$ in 1ml, i.p.) or vehicle (saline) 1h before LPS exposure. Immediately after re-assessing histamine, all animals were overdosed with pentobarbitone sodium (200$mg.kg^{-1}$, i.p.), the lungs lavaged (saline 1$ml.100g^{-1}$, twice) to determine leukocyte numbers expressed as 10^6ml^{-1}. Mean values ±SEM are shown (n=6). Statistical anyalysis was by analysis of variance followed by Dunnett's test for cell numbers and paired Student's *t*-test for AHR, $P<0.05$.

There was no change in sGaw following LPS or saline challenge. 1h after LPS, histamine caused significant bronchoconstriction (-23.9±5.13% reduction in sGaw) compared with no response (-5.6±4.1%) before LPS, indicating AHR to histamine. There was no AHR in saline challenged animals. All groups had similar levels of macrophages and eosinophils as saline challenged animals (1.3±0.1 and 0.07±0.02, respectively). There was increased total cells (2.8±0.2) in LPS compared with saline challenged animals (1.4±0.1) and unlike saline challenge there was neutrophil influx (1.4±0.1). Similar to untreated LPS challenged animals, saline-treated animals gave AHR to histamine (-28.0±5.2%), increased total cell (2.5±0.1) and neutrophil numbers (1.3±0.07). L-arginine abolished AHR and reduced total cell (1.5±0.05) and neutrophil (0.7±0.03) numbers towards saline challenged levels. D-arginine did not, however, affect AHR (-19.3±3.4%), total cell levels (2.5±0.3) or neutrophil (1.2±0.1) influx.

These results suggest that the AHR and neutrophilia after LPS in our model of COPD is due to reduction of NO levels arising from a deficiency of the NO precursor, L-arginine.

Supported by a Nicox Studentship to BJN

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134P ROLE OF ENDOGENOUS ADENOSINE IN THE EARLY AND LATE RESPONSE TO ALLERGEN CHALLENGE IN ACTIVELY SENSITISED, BROWN NORWAY RATS

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Endogenous adenosine has been suggested to amplify the response of airway mast cells to allergen *in vivo* (Meade et al., 2001). We have sought evidence for this by monitoring the acute and late response to allergen in Brown Norway (BN) rats actively sensitised to ovalbumin (OA) in animals treated with adenosine deaminase linked covalently to polyethylene glycol (PEG-ADA; Adagen®) to decrease adenosine availability and in animals treated with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an inhibitor of adenosine deaminase, plus S-(4-nitrobenzyl)-6-thioinosine (NBTI), an inhibitor of facilitated adenosine transport, to increase adenosine availability.

Male BN rats (250-300g), sensitised to OA (Hannon et al., 2001) were pretreated 20-24 h prior to experimental intervention with PEG-ADA (500 Units kg^{-1} i.m) or 1 h previously with EHNA (10 mg kg^{-1} i.p.) plus NBTI (1 mg kg^{-1} i.p.) or vehicle (saline i.m. or i.p.). Animals from each group were anaesthetised (sodium pentothal, 70 mg kg^{-1} i.p.) and set-up for measurement of airway resistance, blood pressure (BP) and heart rate (HR) (Hannon et al., 2001). The cardiovascular sensitivity to adenosine (0.01-3 mg kg^{-1} i.v.) was determined in each group. In a second series, a sequence of i.v. injections of 5-HT (3-30 µg kg^{-1}), methacholine (3-30 µg kg^{-1}) and OA (0.1-10 mg kg^{-1} i.v. or 5-45 mg kg^{-1} intratracheal, i.t.) was established. In a third series, rats were challenged either with OA (0.3 mg kg^{-1}) or saline i.t. and 24 h later bronchoalveolar lavage (BAL) performed. Differential leukocyte cell counts, eosinophil peroxidase, myeloperoxidase and protein concentrations were measured in BAL fluid as described (Beckmann et al. 2001).

The cardiovascular effects of adenosine were significantly reduced in animals treated with PEG-ADA and augmented in animals treated with EHNA plus NBTI (Fig. 1).

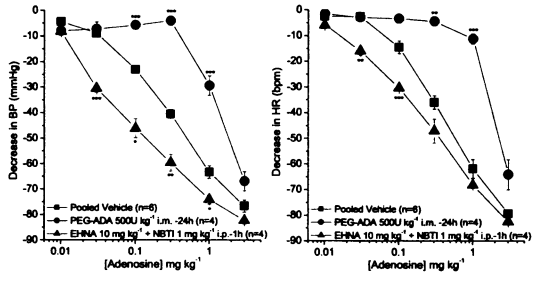

Figure 1. Cardiovascular sensitivity to adenosine in actively sensitised, BN rats treated with PEG-ADA, EHNA/NBTI or vehicle. *$P<0.05$, **$P<0.01$, ***$P<0.001$ that the value differs from the equivalent vehicle control value (Student's *t* test; Hommel-Hochberg correction).

Despite this, there were no differences between the PEG-ADA- and the EHNA/NBTI-treated animals in the acute bronchoconstrictor response to allergen administered either i.v. or i.t, or in the BAL fluid parameters of inflammation 24 h after OA challenge.

Thus, despite evidence of a substantial difference in adenosine availability following pretreatment with PEG-ADA or EHNA/NBTI, there was no difference in either the acute or late response to allergen in the actively sensitised BN rat. Our data suggest no role for endogenous adenosine in determining the response to allergen under our experimental conditions.

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135P INFLUENCE OF POLYMORPHISMS IN THE β_2-ADRENOCEPTOR GENE ON THE EXPRESSION OF β-ADRENOCEPTORS IN HUMAN LUNG TISSUE

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The gene for the β_2-adrenoceptor (β_2-AR) is polymorphic at a number of loci. Previous studies have shown that a polymorphism at position –47 may affect β_2-AR expression (Drysdale *et al.*, 2000). However, the validity of considering single nucelotide polymorphisms (SNPs) in isolation has been questioned. The aim of the present study, therefore, was to determine whether extended haplotypes of the β_2-AR gene influence the expression of the β_2-AR in human lung tissue.

Human lung tissue was obtained from surgical resections. For genotypic analysis, genomic DNA was extracted from a small amount of the lung tissue and genotype was determined by methods similar to those described (Aynacioglu *et al.*, 1999; Chong *et al.*, 2000; Drysdale *et al.*, 2000). Genomic DNA was amplified by Polymerase Chain Reaction (PCR) and the PCR products were then genotyped either by restriction fragment length polymorphism (RFLP) or sequenced using an ABI sequencer (model 373). Saturation binding assays using ^{125}I-iodocyanopindolol (^{125}I-CYP; 0.03125-2nM) were performed on membrane fractions of human lung tissue as described by Nishikawa *et al.* (1996). Specific binding was determined using the non-selective β-AR antagonist, propranolol (1μM), and discrimination of β_2-AR and β_1-AR densities using the selective β_1-AR antagonist, CGP20712A (2-hydroxy-5-(2-(hydroxy-3-(4((1-methyl-4-trifluoromethyl)-1-*H*-imidazol-2-yl)-phenoxy)-propyl)-aminoethoxyl)-benzamide; 0.01μM). Saturation curves were analysed using GraphPad Prism software. Values are means±s.e.means and statistical significance was determined by the use of Mann-Whitney tests.

72 lung preparations were genotyped at 11 previously reported polymorphic positions of the β_2-AR gene. In this cohort, positions –709 (C→A) and –406 (C→T) were not polymorphic, whereas polymorphisms at positions 100 (G→A) and 491 (C→T) were very uncommon. Considering the remaining 7 positions, the most frequent homozygous haplotypes observed were:

	Nucleotide position						
	-654	*-468*	*-367*	*-47*	*-20*	*46*	*79*
Haplotype 1 (n=12)	A	C	T	T	T	A	C
Haplotype 2 (n=13)	G	G	C	C	C	G	G

Saturation binding assays show that there was no difference (P>0.05) in the K_D values obtained for ^{125}I-CYP (0.05±0.01 and 0.03±0.004 nM for haplotypes 1 and 2, respectively). There was also no difference (P>0.05) in the total β-AR density expressed by human lung (90±13 and 73±8 fmol mg^{-1} protein for haplotypes 1 and 2, respectively). β_2-AR densities (69±10 and 67±7 fmol mg^{-1} protein for haplotypes 1 and 2, respectively) were no different (P>0.05). However, β_1-AR density was significantly higher (P<0.001) for haplotype 1 (21±2 fmol mg^{-1} protein) than for haplotype 2 (6±2 fmol mg^{-1} protein).

These findings suggest that β_2-AR haplotype does not influence β_2-AR density in human lung tissue. However an association between β_2-AR haplotype and β_1-AR density was observed.

Supported, in part, by the National Asthma Campaign.

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136P DESENSITISATION OF β_2-ADRENOCEPTOR-MEDIATED RESPONSES BY SALBUTAMOL AND SALMETEROL IN HUMAN LUNG MAST CELLS

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β_2-adrenoceptor (β_2-AR) agonists are effective inhibitors of the stimulated release of histamine from human lung mast cells (HLMC). However, long-term incubation (24 h) with the non-selective β-AR agonist, isoprenaline (ISO), reduces the subsequent effectiveness of ISO to inhibit the IgE-mediated release of histamine from these cells (Chong *et al.*, 1997). The aim of this study was to determine whether long-term treatment of HLMC with either the short-acting β_2-AR-selective agonist, salbutamol (SALB), or the structurally-related long-acting β_2-AR agonist, salmeterol (SALM), leads to a functional desensitisation to β-AR agonists.

Mast cells were obtained by physical and enzymatic disruption of human lung tissue. For histamine release, HLMC were incubated with or without a β-AR agonist for 10 min before challenge with a maximal releasing concentration of anti-human IgE (1:300) for 25 min. Histamine release was measured by an automated fluorometric technique. For cyclic AMP (cAMP) experiments, cells were further purified by countercurrent elutriation. HLMC (91±6% purity) were incubated (10 min) with β-AR agonists (10^{-5}M) and total cell cAMP was determined using commercially available EIA kits. Results are expressed as means±s.e.mean and data were analysed statistically using ANOVA (GraphPad InStat, version 3.0a).

The effects of ISO, SALB and SALM (all 10^{-10}-10^{-5}M) on the inhibition of IgE-mediated histamine release from HLMC were evaluated (n=10-23). SALB was less potent than ISO (pD_2 values 7.7±0.3, and 8.6±0.2, respectively) and was a partial agonist compared to ISO (E_{max} values 35±4 and 49±3% inhibition of histamine release, respectively). pD_2 and E_{max} values for SALM could not be accurately determined as SALM was a weak inhibitor of histamine release. The effects of the agonists on the inhibition of histamine release were mirrored by the ability of the compounds to increase intracellular cAMP. In purified HLMC, ISO, SALB and SALM elevated cAMP levels over basal by 361±90% (P<0.001), 150±37% (P<0.05), and 64±24% (P>0.05), respectively. In further studies, we determined whether long-term treatment with SALM or SALB leads to desensitisation of β_2-AR-mediated responses in HLMC. Cells were incubated with SALM or SALB (10^{-6} M for 24 h) and then washed thoroughly. The subsequent ability of ISO (10^{-10}-10^{-5} M) to inhibit histamine release was determined (n=7). Treatment with both compounds significantly (P<0.05, at least) reduced the effect of ISO as an inhibitor of histamine release, although SALM was significantly (P<0.05) more effective than SALB. As SALM is a weak agonist in this system, it is possible that, rather than causing desensitisation, it may be acting as an antagonist. To address this, cells were incubated with or without SALM (10^{-6} M, 20 min) and either washed thoroughly or not washed and then incubated (10 min) with ISO (10^{-10}-10^{-5} M) (n=4). In the unwashed system, the presence of SALM caused an approximately 300-fold rightward shift of the ISO inhibition of histamine release. Washing the cells reversed the antagonism by SALM of the ISO inhibition. This suggests that SALM, in the desensitisation experiments, acts to desensitise rather than to antagonise the ISO response in HLMC.

These data indicate that, although SALB is a more efficacious agonist than SALM, SALM causes greater levels of functional desensitisation than SALB.

This work was supported by the National Asthma Campaign.

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137P ROLE OF ENDOTHELIN-1 AND ATP-SENSITIVE K^+ CHANNELS IN HYPOXIC RESPONSE OF RAT INTRAPULMONAL ARTERIES

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The mechanism(s) underlying the hypoxic vasoconstriction response (HPV) in the pulmonary circulation are poorly understood. In vitro, hypoxia evokes a transient contraction followed by a sustained contraction. The purpose of the present study was to investigate the role of endothelin-1 (ET-1) and of nitric oxide (NO) in the hypoxic vasoconstriction in rat intrapulmonary arteries.

Intrapulmonary arteries from adult male Wistar rats (300-350 g) were mounted in a wire-myograph for isometric tension recordings and kept in PSS at 37 ^{O}C. The arterial segments were adjusted to a passive load of ~30 mmHg. Experiments were performed in the presence of a β-adrenoceptor agonist, propranolol (1 μM). Vessels were contracted by the α-adrenoceptor agonist, phenylephrine (10 μM) and hypoxia was induced by changing from 5% CO_2 in air to 5% CO_2 in N_2. Oxygen levels were simultaneously measured with an oxygen sensitive electrode. Glibenclamide, an ATP-sensitive K^+ channel blocker, was studied to investigate the role of these channels in the hypoxic response. N^G-nitro-L-arginine (L-NOARG), an inhibitor of NO synthase (100 μM) was added, to study the role of the NO in HPV. The integrity of the endothelium was tested by assessing the relaxant response to acetylcholine (10 μM). To evaluate the role of ET-1 in the hypoxic response, pulmonary arteries were incubated with the combination of ET_A and ET_B antagonists, BQ-123 (3 μM) and BQ-788 (3 μM) (Ishikawa et al., 1994) for one hour and hypoxia was induced. Results are expressed as means±sem and evaluated by t-test and ANOVA followed by Bonferroni method.

In endothelium-intact preparations contracted with phenylephrine, hypoxia evokes a transient contraction. In endothelium-denuded vessels (n=27), hypoxia evoked relaxation. In endothelium-intact arteries contracted with L-NOARG (100 μM), the response to hypoxia during 60 min was unaltered compared to phenylephrine-contracted preparations (162± 24% *vs* 180± 27%, n=23), respectively. Glibenclamide (10 μM) increased the contraction to hypoxia in phenylephrine-contracted endothelium-intact preparations (n=8) and also inhibited the hypoxic relaxations in endothelium-denuded vessels (90 ±4% *vs* 17±9% $P<0.05$, n=4) as well as relaxations evoked by the K^+ channel opener, pinacidil (10^{-8}-$3*10^{-5}$ M, n=10). Only the combination of the ET-1 receptor antagonists, BQ-123 and BQ-788, shifted to the right the concentration-response curve for ET-1 (10^{-10}-$3*10^{-8}$ M). In the absence of glibenclamide, incubation with the combination of BQ-123 and BQ-788 had little effect on the response to hypoxia. However, in the presence of glibenclamide, incubation with the combination of the two ET-1 receptor antagonists abolished the sustained hypoxic contraction (n=8).

The present results suggest that, in rat intrapulmonary arteries, hypoxia causes the release from the endothelial cell layer of a contractile factor, which might be ET-1. Furthermore, with some delay, hypoxia opens smooth muscle ATP-sensitive K^+ channels.

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Work supported by the Danish Lung Foundation

138P SILDENAFIL AND 3-MORPHOLINOSYDNONIMINE (SIN-1) RELAX RAT PULMONARY ARTERIES

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In vascular smooth muscle cells, cyclic GMP (cGMP) is produced by the guanylyl cyclase and hydrolysed by phosphodiesterases (PDEs). Increased cGMP leads to vasodilatation. In the lung, the most abundant phosphodiesterase that breaks down cGMP is PDE5 (Jeffery et al, 1998). Sildenafil is a potent selective inhibitor of PDE5 (Ballard et al, 1998) and SIN-1 releases nitric oxide that stimulates the soluble guanylyl cyclase to produce cGMP. The present study was designed to investigate the relaxant effect of sildenafil and SIN-1 in rat intrapulmonary arteries.

Pulmonary arteries were dissected from the lungs of male Wistar rats aged 11-13 weeks and mounted in wire myographs containing physiological salt solution (PSS) (37°C), and stretched stepwise to a passive transmural pressure of 30 mmHg. All arteries were tested regarding contractile and endothelial cell function. To investigate the relaxant effect of sildenafil and SIN-1, the arteries were contracted with the thromboxane A_2 analogue U46619 ($3*10^{-8}$ M) and increasing concentrations of sildenafil (10^{-9}-10^{-6} M) or SIN-1 (10^{-8}- $3*10^{-4}$ M) were added. To test for a synergistic vasodilator effect, the arteries were contracted with U46619 ($3x10^{-8}$ M) and treated with single doses of sildenafil and SIN-1 alone and in combination. In both experimental series some of the arteries were incubated with an inhibitor of soluble guanylyl cyclase, ODQ ($3x10^{-6}$ M), to evaluate the role of guanylyl cyclase. We analysed the data using a two- or one-way ANOVA followed by a Bonferroni test. All results are shown as mean ± SEM.

In endothelium-intact pulmonary arteries, sildenafil evoked maximal relaxations of 40±7% at 10^{-6} M (n=10) with pD_2=7.74±0.25. Mechanical removal of the endothelium caused a small rightward shift of the curve (n=4). In the presence of ODQ, sildenafil did not evoke relaxation. SIN-1 relaxed the arteries maximally by 71±9%, pD_2=5.37±0.05 (n=7). In the presence of ODQ, low concentrations (<10^{-5} M) of SIN-1 did not evoke relaxation, but at the highest concentration ($3x10^{-4}$ M) applied, SIN-1 relaxed the pulmonary arteries 53±10% (n=6). Application of single concentrations of sildenafil (10^{-7} M) and SIN-1 ($3x10^{-6}$ M) relaxed U46619-contracted pulmonary arteries by 58±9% (n=7) and 38±6% (n=7), respectively. The combination of sildenafil and SIN-1 exerted a relaxation of 71±2% (n=7, $p<0.01$ compared to SIN-1 alone). All three results were significantly different from a time control ($p<0.01$). In the presence of ODQ, the combination of sildenafil (10^{-7} M) and SIN-1 ($3x10^{-6}$ M) did not induce relaxation (n=7).

In isolated pulmonary arteries, sildenafil has both direct vasodilatory effects and enhances SIN-1 evoked vasorelaxation by cGMP-dependent mechanisms. Since both sildenafil and SIN-1 are registered for human use it would be of interest to test the combination of these drugs, in chronic pulmonary hypertension.

Ballard *et al*, 1998 *J.urol*;. 159, 2164-2171

Jeffery *et al*, 1998 *Cardiovasc. Pharmacol;* 32, 213-219

139P THE SELECTIVE REDUCTION OF 5HT-LINKED PULMONARY VASOCONSTRICTION BY DEXFENFLURAMINE IS PREVENTED BY PRE-TREATMENT WITH CITALOPRAM IN THE WISTAR RAT LUNG

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Chronic use of the weight-loss drug, dexfenfluramine (dFEN), is linked to the development of pulmonary hypertension (PH; Abenhaim *et al.*, 1996). We have previously shown dFEN (100 μM) to selectively attenuate the acute changes in pulmonary arterial pressure (ΔPpa) evoked by 5-hydroxytryptamine (5HT) rather than hypoxic ventilation (HV; 2% O_2 + 5% CO_2), in the blood-perfused isolated lung (IPL) preparation (Sisodiya *et al.*, 2000). As dFEN is a substrate for the 5HT transporter (Rothman *et al.*, 1999), we have questioned whether pre-treatment with citalopram (Cit), a selective serotonin reuptake inhibitor (SSRI), will modulate the pulmonary activity of dFEN in the Wistar (W) rat IPL. Furthermore, we have investigated the pulmonary responses to Cit on basal Ppa and the ΔPpa caused by 5HT and HV.

Male W rats (8-12 weeks) were terminally anaesthetised with sodium pentobarbitone (Sagatal, 60 $mg.ml^{-1}$, intraperitoneally). Lungs were isolated *in situ*, ventilated with air + 5% CO_2 and perfused with autologous blood at a constant flow of 20 $ml.min^{-1}$ (39°C) (IPL). Ppa was recorded continuously. The effect of cumulative doses of dFEN (100-400 μM) on the ΔPpa produced by 5HT (20 μg) and HV was tested after initial pre-treatment with 0.3 μM Cit (n=6), a dose based on its affinity for the 5HT transporter (Owens *et al.*, 1997). The pulmonary vascular reactivity to cumulative doses of Cit (0.3-1 μM; n=6) or volume-control saline (n=6-7) were followed by treatment with 5HT and HV in the W rat IPL. Results are presented as the mean ΔPpa ± SEM, from the pre-dose baseline. Statistical analysis employed a one-way ANOVA with Tukey's post hoc test or Student's t-test.

Table 1 illustrates the dose-dependent sustained ΔPpa to cumulative dFEN (100-400 μM) and its influence on the transient responses of 5HT and HV, following Cit treatment. Whilst Cit did not alter the responses to 100 μM dFEN (data not shown), the subsequent ΔPpa to 5HT and HV were significantly increased. A higher dose (400 μM dFEN) reduced the responses to 5HT and HV to levels recorded prior to dFEN treatment.

Table 1 Vasoconstrictor responses to dFEN and subsequent responses to 5HT and HV after pre-treatment with Cit

dFEN (μM)	ΔPpa to dFEN	ΔPpa	
		20 μg 5HT	HV
0	0.0 ±0.0	4.0 ±0.5	10.3 ± 1.6
100	4.7 ±0.4	10.0 ±1.6**	22.3 ± 2.6**
400	10.0 ± 1.1	2.4 ±0.7	10.5 ± 2.0

Values are mean ΔPpa ± SEM mmHg. ** $p < 0.01$, compared to values obtained prior to dFEN.

Table 2 shows the dose-dependent sustained increases in Ppa to Cit (0.3-1 μM) and saline and the subsequent responses to 5HT and HV. ΔPpa evoked by 5HT and HV were significantly increased by 0.3 μM Cit ($p < 0.05$).

Table 2 Cit significantly increases the ΔPpa evoked by 5HT and HV

Cit (μM)	ΔPpa to Cit/Saline		ΔPpa to 20 μg 5HT		ΔPpa to HV	
	Saline	Cit	Saline	Cit	Saline	Cit
0	0.0±0.0	-0.1±0.0	4.9±0.7	6.2±1.2	9.8±1.9	10.7±2.5
0.3	0.4±0.2	1.0±0.2	6.6±0.9	16.3±2.9*	14.7±2.3	23.5±2.7*
1	0.7±0.3	3.6±0.9*	8.6±1.7	17.0±3.3	24.0±3.6	35.5±2.0*

Values are mean ΔPpa ± SEM mmHg. * $p < 0.05$, compared to values obtained after treatment with saline.

In agreement with earlier findings (Sisodiya *et al.*, 2002), Cit did not modulate dFEN-evoked vasoconstriction but significantly increased the ΔPpa generated by 5HT or HV. Further, Cit prevented the selective attenuation of 5HT-evoked pulmonary vasoconstriction caused by 100 μM dFEN. This suggests that the indirect but not direct effects of dFEN may require the 5HT transporter.

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140P THE VASORELAXANT EFFECTS OF SIMVASTATIN IN THE RAT AORTA

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Statins, which are HMG-CoA reductase inhibitors, are widely used in patients with hypercholestolaemia and are associated with a significant reduction in the risk of cardiovascular events (Heart Protection Study Collaborative Group, 2002). Statins may also exert vascular effects, independent of their action on cholesterol synthesis and Glorioso *et al.* (1999) reported that pravastatin lowered blood pressure in hypertensive patients. Furthermore, simvastatin has been shown to cause endothelium-dependent and independent relaxations in rat conduit and resistance vessels (de Sotomayor *et al.*, 2000). We have now investigated the vasorelaxant mechanisms of simvastatin in rat aortic rings.

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

Simvastatin induced concentration-related relaxations (pEC_{50}=7.57±0.40, mean±s.e.mean; maximum relaxation (R_{max})=32.6±3.6%, n=6). The relaxant responses were abolished by the presence of indomethacin (n=6). In the presence of L-NAME the responses to simvastatin were converted to a modest contractile response, described by pEC_{50}=7.16±0.72 and R_{max}=-14.2±2.0% (n=4). However, in the additional presence of indomethacin, the contractile response revealed by L-NAME was abolished. In 6 preparations, removal of the endothelium significantly ($P<0.05$) reduced relaxations to simvastatin, except at concentrations >3μM.

The results of the present study confirm that simvastatin acts as a potent vasorelaxant via both endothelium-dependent and independent mechanisms. These mechanism involve the action of both NO and prostanoids. Following inhibition of NO synthesis, a prostanoid-dependent contractile response was uncovered.

We thank Merck Sharp and Dohme for the gift of simvastatin.

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141P THE EFFECT OF ADENOSINE A_3 RECEPTOR ACTIVATION ON THE RECOVERY FROM SIMULATED ISCHAEMIA OF GUINEA PIG LEFT ATRIA

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Myocardial stunning is ventricular dysfunction following acute cardiac ischaemia and reperfusion (Braunwald & Kloner 1982). Endogenous adenosine is released during ischaemia and protects against the various consequences of myocardial ischaemia and reperfusion (Bolli 1995). This study examined the effect of endogenous adenosine and an adenosine A_3 agonist IB-MECA (N^6-(3-iodobenzyl)adenosone-5'-N-methylcarboxamide) on the recovery of contractile tension following simulated ischaemia.

Left atria from male Dunkin-Hartley guinea pigs (250-300g) were arranged in 50ml organ baths containing Krebs bicarbonate solution at 37±0.5°C gassed with 5% CO_2 in oxygen. Atria were paced throughout at 2Hz with threshold voltage+50%. Resting tensions of 0.5-1g were applied and isometric tension recorded. Simulated ischaemia was induced after 30min equilibration by gassing with 5% CO_2 in nitrogen and substituting the glucose with choline chloride (7mM). After 30min simulated ischaemia, atria were reoxygenated and glucose was reintroduced. Mean developed contractile tension±S.E.M was expressed as a percentage of the pre-ischaemic developed contractile tension expressed in grams. Statistical comparisons were made using ANOVA or the Bonferroni multiple comparisons test with $P<0.05$ indicating a significant difference. There were no significant differences between the pre-ischaemic contractile tensions for the different experimental groups.

Following 30min simulated ischaemia the contractile tension returned to 33.3±2.1% of the pre-ischaemic value (0.24±0.03g) after 10min reoxygenation. Adenosine deaminase (AD, $1Uml^{-1}$) added at the onset of simulated ischaemia and during reoxygenation did not significantly affect the recovery of contractility. The contractile tension after 10min reoxygenation recovered to a value of 31.7±3.7% of 0.44±0.09g. Subsequent experiments were conducted in the absence of AD. When IB-MECA (3×10^{-7}M) was added at reoxygenation, the contractile tension after 10min significantly improved to 61.2±4.0% of 0.45±0.11g. This improvement in contractile tension was significantly blocked by MRS-1220, (N-[9-Chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-benzeneacetamide) (1×10^{-6}M), an adenosine A_3 antagonist. A higher concentration of IB-MECA (1×10^{-6}M) added at reoxygenation had no significant effect on the recovery of contractility after 10min reoxygenation compared to the vehicle control (34.7±5.4% of 0.59±0.10g Vs 31.3±2.8% of 0.57±0.05g). However, 10min after several changes of IB-MECA-free bathing solution, contractile tension significantly improved compared to washout of the vehicle control (80.5±8.9% of 0.59±0.10g Vs 35.1±3.5% of 0.57±0.05g). IB-MECA (3×10^{-7}M) also significantly improved contractility to 78.3±4.8% of 0.32±0.06g following washout, in addition to the initial significant improvement in contractile tension before washout (60.0±5.1% of 0.32±0.06g)

In conclusion, endogenous adenosine does not appear to protect against myocardial stunning in atria since its removal with AD does not worsen recovery from simulated ischaemia. The adenosine A_3 agonist IB-MECA protects atria from myocardial stunning when added at reoxygenation at 3×10^{-7}M but not 1×10^{-6}M. On washout of IB-MECA during reoxygenation both concentrations significantly improved developed contractile tension. This suggests that IB-MECA may activate A_1 receptors at high concentrations causing negative inotropy which counteracts the improved contractility mediated via A_3 receptors until IB-MECA is washed out.

Supported by a British Heart Foundation studentship to L.Y.

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142P 8sPT RESISTANT COMPONENT OF A_1 MEDIATED NEGATIVE INOTROPIC RESPONSE IN PRESENCE OF 4-AP IS PERTUSSIS TOXIN SENSITIVE

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Previous studies from this laboratory showed that when K^+ channels are blocked by 4-aminopyridine (4-AP), the residual response is resistant to antagonism by the P_1 purinoceptor antagonist, 8 (p-sulfophenyl) theophylline (8sPT) (Gardner and Broadley,1999). This residual component may involve Ca^{2+} channels, the adenosine A_1 receptor possibly being coupled to two transduction pathways for negative inotropism via the different G-protein components (receptor-transducer promiscuity) (Kenakin, 1993).The present study investigated the effect of pertussis toxin (PTX), a G-protein inactivator, on this resistant component of the A_1 receptor-mediated negative inotropy of N^6-cyclopentyladenosine (CPA)

Male Dunkin-Hartley guinea-pigs (250-350g) received either PTX (35μg/kg, i.p.) or saline (control). 48h later the animals were killed, the left atria removed and mounted in 50ml organ baths containing Krebs solution gassed with 5% CO_2 in O_2 at 37°C. They were electrically paced (2Hz, 5ms, threshold voltage + 50%) and isometric tension recorded. Following 1h equilibration under 1g tension, a cumulative concentration-response curve (CRC) to CPA (0.01nM-10μM) was elicited in the absence or presence of 4-AP (10mM) or 8sPT (10μM) or both. All tissues were incubated with propanolol (1μM) added 10mins before the CRC.

In control atria, 4-AP failed to antagonize the negative inotropic response to CPA but there was a parallel shift of the CRC by 8sPT (dose-ratio at IC_{50}: 19.2 ± 2.68). In the presence of 4-AP, there was a biphasic rightward shift of the CRC by 8sPT with a greater shift at higher concentrations of CPA (5.3±1.03 fold at IC_{65}) and a non-significant shift (1.57±0.91) at the IC_{35} (Table 1). PTX pretreatment significantly attenuated the inhibition of developed tension by CPA in the absence or presence of 4-AP with mean dose ratios at IC_{50} of 84.6±6.4 and 16.6±3.11, respectively. In PTX pretreated atria in the presence of 4-AP and 8sPT, the CRC was monophasic and at the same position as with 8sPT in controls.

Treatment	IC_{35}	IC_{65}
4-AP (Sal)	7.08 (3.1-16)x1nM	0.21(0.20-0.23)x1μM
8sPT+4-AP (Sal)	14.5(12-17.3)x 1nM	1.02 (0.68-1.55)x1μM*
8sPT+4-AP (PTX)	2.46 (1.35-4.47)x 0.1μM*	5.89(2.9-12)x 1μM

Table1. Concentrations of CPA for 35 or 65% inhibition of developed tension with 95% confidence limits. * $p<0.05$ significantly different from control in the presence of 4-AP.

The present result suggests that the 8sPT-resistant component of CPA-induced negative inotropy in the presence of K^+ channel blockade is coupled to a PTX sensitive G-protein.

Supported by a Welcome Trust Travelling Fellowship to S.C.Parija

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143P INVESTIGATION OF THE ANTIFIBRILLATORY DRUG INTERACTIONS BETWEEN PROPRANOLOL AND BRETYLIUM IN PERFUSED RABBIT HEARTS

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In view of the reliability of the serial-shock method of measuring ventricular fibrillation threshold (VFT) in assessing antifibrillatory potency of many antiarrhythmic drugs (Almotrefi & Baker, 1981) and the alarming reports of the proarrhythmic effects of several antiarrhythmic agents (Peters et al., 1994) we decided to use the above technique to study interactions that may occur when antiarrhythmic drugs from different classes are combined. Recently, we have presented the antifibrillatory interactions between lidocaine and propranolol (Almotrefi et al., 1999) and that between lidocaine and bretylium (Almotrefi et al., 2001). In this abstract we report the antifibrillatory interactions between propranolol and bretylium.Studies were carried out on hearts isolated from New Zealand white rabbits of either sex weighing 1.5 to 2 Kg. The method used has been given previously (Almotrefi & Baker, 1981). Perfusion with propranolol produced significant, dose-dependent increase in VFT while perfusion with bretylium did not cause any significant change (Table1). In addition, there was no significant difference in VFT with the combined infusion of 0.34 µmol of propranolol and 9.65 µmol of bretylium, in contrast to a synergistic antifibrillatory effect of the combined use of lidocaine and propranolol (Almotrefi et al., 1999).These results may suggest that bretylium, unlike class I antiarrhythmic drugs such as lidocaine, does not potentiate the antifibrillatory effect of propranolol.

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Table 1. Effect of propranolol, bretylium and their combination on (VFT) in isolated-perfused rabbit hearts.

Drug	%change in *VFT* after exposure for			%change in *VFT* after washout for		
(µmol)	15min	30min	60min	15min	30min	60min
Control	6.87±3.13	3.49±3.42	-3.47±3.33	0.60±3.84	0.94±4.35	0.32±3.44
Propranolol						
(0.34)	40.18±3.67**	62.43±4.22**	97.61±6.39**	48.54±8.51**	19.73±6.50	11.60±3.96
(0.68)	57.84±5.57**	104±8.62**	183.75±9.06***	81.92±12.99**	32.25±6.86**	16.88±4.89*
(1.35)	88.23±12.69**	204.56±16.15**	272.21±15.83**	98.50±16.70**	48.25±9.57*	28.40±9.99*
bretylium						
(9.65)	1.75±7.65	-7.65±5.28	-6.14±7.57	-1.62±9.74	-7.03±6.78	-8.16±6.61
(19.30)	4.49±7.13	3.66±6.23	-1.17±5.13	-3.71±5.02	-8.46±6.45	-6.96±5.27
Propranolol+ bretylium						
(0.34+9.65)	17.32±4.65*	35.94±5.86*	61.10±6.34*	10.74±5.71	8.64±6.31	4.99±5.64

Values are means ± s.e.m , comparing VFT value to that measured before drug perfusion in each heart (acting as its own control)
* $P< 0.05$, ** $P<0.005$,*** $p<0.0005$, using Student's paired *t* test

144P PROTECTIVE ROLE OF MELANOCORTIN TYPE 3 RECEPTOR IN MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY IN MICE

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The melanocortin peptides (e.g. ACTH and gamma melanocyte-stimulating hormones, γ-MSH) (Guarini *et al.*, 2002) exert protective actions in experimental models of rat myocardial I/R. Five melanocortin receptors (MC-R) have so far been cloned (Getting, 2002). In this study we adapted a protocol used for the rat (D'Amico *et al.*, 2000) to the mouse, to identify the MC-R responsible for the protective actions of melanocortins.

Wild type (WT) and recessive yellow (e/e) mice (strain of mice with a non-functional MC1-R due to a frameshift mutation in amino acid 549) (23-25g body weight in both cases) were anaesthetized with Inactin™ (100 mg kg^{-1}, i.p.; RBI) and subjected to the following procedures: cannulation of the left jugular vein (drug administration) and the right carotid artery (blood pressure measurement); thoracotomy and exposure of the heart; placement of a silk ligature around the left anterior descending coronary artery (LADCA) close to its origin. After a 30 min stabilisation period, LADCA was occluded for 25 min, and then a 2 h reperfusion period allowed. Either PBS (100 µl), the MC3-R agonist MTII (10 µg/mouse), alone or in combination with the mixed MC3/4 antagonist SHU9119 10 µg/mouse) (Fan *et al.*, 1997) or the selective MC4-R antagonist HS024 (10 µg/mouse) were administered s.c. 30 min prior to ischaemia. Doses used were previously shown to exert anti-inflammatory activities (Getting *et al.*, 2001). Post-reperfusion, the LAD was re-occluded and Evans Blue dye (1 ml of 2% wv^{-1}) injected to determine the area at risk (AR). The heart was excised, sectioned and incubated in 2,3,5-triphenyltetrazolium chloride. The sections were then photographed and the left ventricular (LV) area, AR and the infarct size (IS) for each tissue slice determined by computer planimetry (NIH Image Sofware). Data (mean ± s.e.mean) were analysed by ANOVA and post-hoc Bonferroni test taking a P value <0.05 as significant.

In vehicle-treated animals, occlusion and reopening of LADCA produced an IS/AR ratio of 37 ± 2% (n=6) and 39 ± 2 % (n=6) in WT and recessive yellow (e/e) mice, respectively. This figure was reduced by administration of 10 µg MTII to 19 ± 2% (n=6) and 18 ± 1% IS/AR ratios (n=6) in WT and recessive yellow (e/e) mice. MTII caused a slight increase in systemic blood pressure in recessive yellow (e/e) mice but not WT, whilst heart rate was unaltered (not shown). To investigate the possible involvement of MC3-R in the action of MTII a dual MC3/4-R and a selective MC4-R antagonist were administered at 10 µg per mouse alone or in combination with the agonist MTII (10 µg). The antagonist SHU9119 did not modify the IS/AR ratio when administered alone in either WT (43 ± 2% IS/AR, n=6) or recessive yellow (e/e) mice (42 ± 2% IS/AR, n=6) which were not significantly different from control. A similar observation was noted with the selective MC4-R antagonist HS024) Importantly, SHU9119 but not HS024 attenuated the protective effects afforded by MTII both in WT (35 ± 2% n=6) and recessive yellow (e/e) mice (32 ± 1%, n=6).

In conclusion, a synthetic melanocortin peptide that displays higher selectivity towards MC3-R attenuates experimental myocardial I/R in the mouse. An integrated pharmacological (antagonists) and genetic (recessive yellow e/e mouse strain) approach indicates that MC-1R is superfluous to bring about this protective action.

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This work was supported by the Arthritis Research Campaign (grant G0571)

145P CHARACTERISATION OF THE CONTRACTILE PROPERTIES OF AN EQUINE AORTIC VALVE PREPARATION

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Aortic valve (AoV) disease is a common problem of ageing people and horses. Recently, porcine AoV cusps have been shown to have contractile properties (Chester *et al*, 2000). The aim of this study is to define receptor-mediated contraction of normal equine aortic valves

AoVs were obtained from healthy adult mixed breed horses, humanely killed at an equine abattoir. Valve cusps were harvested within 30 min of death. The right coronary cusp was sectioned transversely and half cusps were suspended under 2.5 g tension in organ baths bathed in a modified Krebs Henseleit solution (KHS) maintained at 37°C, aerated with 95% O_2 and 5% CO_2 and isometric tension was recorded. Following 60 min equilibration, valve segments were contracted to a 118 mM potassium containing solution (depolarising Krebs solution; DKS) and tension generated was recorded over 1 h then washed in KHS and all re-equilibrated.

Cumulative concentration response curves (CRC) were obtained from valves to 9,11-Dideoxy-9α,11α-epoxymethano-prostaglandin F2α (U44069; 10^{-9} to 10^{-5} M; n=10), to phenylephrine (10^{-6} to 10^{-2}M; n=10) and to 5-hydroxy-tryptamine (5-HT; 10^{-10} to 10^{-2}M; n=6). Paired sections of AoV were also incubated in 10^{-6} M yohimbine for 30 minutes prior to administration of phenylephrine. Finally, relaxation of U44069 constricted valves was assessed using [1*S*- [1α,2*α* (*Z*), 3*α*,4*α*]] -7-[3-[[2 [(phenyl amino) carbonyl] hydrazino] methyl] -7- oxabicyclo [2 . 2 . 1] hept – 2 – y l] – 5 - Heptanoic acid (SQ29548; 10^{-6}M ; n=6). Contractile responses were expressed in terms of percentage of DKS response. CRCs were fitted using non-linear regression and values expressed as mean ± s.e.m. and as geometric means with 95% confidence intervals for logarithmic data. The effects of yohimbine and SQ2958 on responses to phenylephrine and U44069 respectively were assessed using a Wilcoxon Sign Rank test or paired t-test as appropriate (P<0.05 taken to indicate significance).

Equine aortic valves contracted in response to U44069 and phenylephrine as shown in Table 1. At concentrations above 10^{-4}M, phenylephrine caused relaxation of valve segments. No contraction was recorded in response to 5-HT. The thromboxane receptor antagonist SQ2958 relaxed pre-constricted valve segments by 66.6% (± 15.7%; P=0.018). Yohimbine caused a 28 fold shift in the CRC to phenylephrine (PE) (P=0.005) giving an apparent pKB of 6.413 (± 0.27).

These preliminary results indicate that thromboxane receptor and α-adrenoceptor agonists cause receptor-mediated contraction in equine AoV. The phenylephrine responses are consistent with α_2-adrenoceptor mediated-contraction; further work is required to confirm this. In contrast to other species, 5-HT does not cause contraction of the equine AoV. This preparation will enable quantification of responses from normal and diseased tissue to increase our understanding of naturally occurring aortic regurgitation in the horse.

Agonist	Maximum %	$LogEC_{50}$	Hill Slope
U44069	127 (±27.1)	-8.36 (-8.70 to – 7.96)	1.34 (±0.23)
Phenylephrine	74.9 (±32.3)	-5.31 (-5.66 to –4.32)	1.25 (± 0.20)

Table1. CRC parameters for equine aortic valves.

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146P CHARACTERISATION OF ENDOTHELIUM-DEPENDENT VASODILATORY RESPONSES IN THE EQUINE ISOLATED PERFUSED DIGITAL VASCULAR BED

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Equine laminitis is thought to be the result of ischaemia and reperfusion injury of the sensitive dermal lamellae (Hood et al., 1993). The vasoactive mediator(s) triggering this disease remain unknown. Factors, which regulate blood flow through the equine digit, are important in understanding the patho-physiology of this disease. The aim of the present study was to examine the contribution of nitric oxide, prostacyclin and EDHF to the regulation of equine digital vascular resistance.

Hind limbs were obtained from mixed breed healthy adult horses killed at an abattoir. The digital circulation was perfused (100ml min^{-1}) with Krebs-Henseleit (30°C) solution and the peak increase in pressure was recorded in response to bolus doses of 5-HT (6 nmol) alone or 5-HT (6 nmol) co-administered with carbachol (CCh; 0.2 μmol), bradykinin (BK; 0.2 nmol), substance P (SP; 0.2 nmol) and sodium nitroprusside (SNP; 0.2 μmol). The percentage reduction of the 5-HT pressor response by the vasodilator compounds was calculated. The vasodilator responses were also examined in the presence of: (i) N^{ω}-Nitro-L-Arginine methyl ester (L-NAME, 100 μM); (ii) L-NAME and ibuprofen (10 μM); (iii) Krebs containing 30 mM potassium and nifedipine (1 μM) (high K^+ Krebs); (iv) high K^+ Krebs containing L-NAME and ibuprofen in separate preparations. The effect of the treatment on responses to each vasodilator was determined using a paired Student's *t*-test.

Table 1. Percentage inhibition of 5-HT-induced vasoconstriction in the equine isolated perfused digital vascular bed.

Agonist	Control 1	L-NAME	Control 2	L-NAME + Ibuprofen	30mM K^+ + L-NAME +Ibuprofen
Carbachol	54.6 ± 2.8	13.0 ± 2.1**	72.8 ± 7.7	34.9 ± 4.4*	3.5 ± 1.2**
Bradykinin	49.4 ± 6.0	30.8 ± 3.9	64.4 ± 4.7	26.3 ± 7.4*	8.1 ± 3.5**
Substance P	47.2 ± 4.5	23.8 ± 7.4	70.9 ± 11.1	41.2 ± 13.7	0.8 ± 4.1***
SNP	ND	ND	78.2 ± 6.3	81.9 ± 5.9	97.2 ± 1.6

*P<0.05, **P<0.01, ***P<0.0005 Vs. control compared by paired *t*-test (mean ± S.E.M. n=4 – 5). SNP – Sodium nitroprusside, ND – not determined.

The results are presented in Table 1. The chosen doses of CCh, BK and SP were equi-effective in reducing 5-HT-induced contraction by 47 to 76%. L-NAME alone significantly but partially inhibited the responses to CCh. A large proportion of the response to BK and SP-induced was resistant to L-NAME. Ibuprofen plus L-NAME did not cause further inhibition of the CCh and SP-induced relaxations, but did significantly reduce the responses to BK. High K^+ Krebs caused a partial but significant reduction in the response to the BK and SP, but did not inhibit the CCh-induced relaxation. L-NAME and ibuprofen in high K^+ Krebs virtually completely abolished the relaxations to CCh, SP and BK. The responses to SNP did not change significantly following any of the treatments tested.

These data indicate that CCh, BK and SP-induced relaxations involve combinations of the NO, EDHF and prostacyclin pathways. CCh responses were mainly dependent on the NO pathway whereas BK and SP vasodilation depended on both NO and EDHF pathways, with, in the case of BK some evidence for involvement of the prostacyclin pathway. These results contrast with those reported previously for large digital arteries and veins (Elliott et al., 1994) and suggest EDHF is an important factor in regulating the resistance of the equine digital vascular circulation.

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147P MEASUREMENT OF ENDOTHELIAL FUNCTION IN THE RAT EAR ARTERY *IN VIVO*

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Nitric oxide (NO) is a key vasodilator in large arteries, but has a diminishing role as vessel calibre decreases (Urakami-Harasawa *et al.*, 1997). We have previously non-invasively assessed endothelial function in the rabbit ear artery using photoplethysmography (Weinberg *et al.*, 2001). In the current study we investigated the extent NO contributed to blood flow in the rat ear artery *in vivo*, using laser-Doppler flowmetry, for its potential use in non-invasive measurement of endothelial (dys)function.

Male Wistar rats (200-300 g) were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.) and the trachea cannulated to facilitate respiration. Body temperature was maintained at 37°C. The carotid artery was cannulated for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The jugular and femoral veins were cannulated for drug administration. The ear was shaved and a flow probe placed in close proximity with the ear artery. ACh (0.001-10 nmol kg^{-1}) and SNP (0.01-30 nmol kg^{-1}) dose response curves were generated following a 30 min stabilisation period. L-NAME was given as a bolus (50 mg kg^{-1}) followed by a 10 min infusion (100 mg kg^{-1} hr^{-1}). All data are expressed as % SNP (10 nmol kg^{-1}).

ACh dose-dependently elicited a decrease in MAP with a concomitant increase in blood flow from baseline values of 126.28±5.76 mm Hg (n=12) and 4.42±0.16 V (n=12) respectively. Similarly, SNP increased blood flow and decreased MAP dose-dependently. However, ACh or SNP did not alter the basal HR (380±11 beats min^{-1} (bpm), n=12). The correlation coefficient (r) for ACh dose responses was r=0.71 (P<0.05, n=6). However, SNP showed a correlation between flow and MAP of r=0.88 (P<0.05, n=6). ACh and SNP, in comparable doses, induced similar changes in MAP (P>0.05). Changes in blood flow, were more sensitive to ACh than SNP (P<0.05). ACh (1 and 10 nmol kg^{-1}) responses were not affected by L-NAME (Vehicle; n=5, L-NAME; n=6) (Figure A). In contrast the decrease in MAP elicited by ACh was impaired by L-NAME (n=5-6) (Figure B). Furthermore SNP-induced (10 nmol kg^{-1}) changes in basal blood flow (0.89±0.26 V, n=6), MAP (33.96±3.40 mm Hg, n=6), and HR (21±13 bpm, n=5) were unaffected by L-NAME (flow; 1.46±0.21 V, n=6, MAP; 31.97±8.9 mm Hg, n=6, HR; 2.76±4.56 bpm, n=5). MAP increase, and decreases in HR and flow by L-NAME alone were not statistically significant.

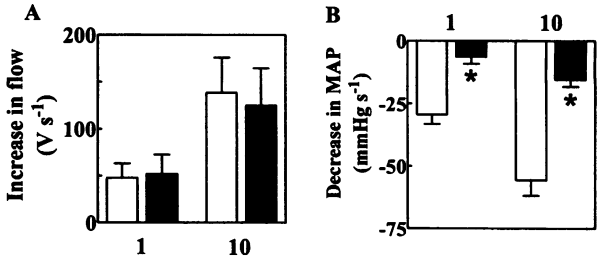

Figure 1. ACh-induced (1 and 10 nmol kg^{-1}) changes in (A) Blood flow (B) MAP in vehicle (□) and L-NAME (■) treated rats. *, P<0.05 vs. vehicle using unpaired t-test.

Our study shows a NO-independent action of ACh regulating blood flow in the rat ear artery *in vivo*, consistent with an involvement of EDHF. Similar effects have been shown *in vitro* in smaller arterioles (Berman & Griffith, 1998). This system may be a suitable method in which to non-invasively assess endothelial function in small arteries and arterioles in the rat where repeat measurements of vascular/endothelial activity in the same animal are desirable over an extended period of time.

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148P THROMBIN INCREASES THE EXPRESSION OF ADIPOCYTE FATTY-ACID BINDING PROTEIN aP2 mRNA IN HUMAN VASCULAR ENDOTHELIAL CELLS

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Damage to and subsequent inflammation of endothelium plays a significant role in the development of atherosclerosis. In the course of developing a model of endothelial inflammation *in vitro*, we have applied a highly sensitive technique to evaluate the effects of thrombin and lipopolysaccharide (LPS) on the expression of several genes. These genes included aP2 (adipocyte fatty acid binding protein), a gene recently implicated in metabolic disorders and atherosclerosis (Makowski *et al.*, 2001), but which has not previously been studied in human vascular endothelium.

Human umbilical vein endothelial cells (HUVECs) (n = 6, passage 2) grown on collagen coated 96 well plates were incubated with endothelium basal media-2 (2% FCS), containing LPS (10 μg/ml) or thrombin (6 U/ml) for 2, 4, 12 and 24 hours. Each condition was studied in triplicate. At the end of the incubation period the media was removed, the cells were lysed and the total RNA was extracted. The mRNA levels were determined using quantitative real-time reverse transcriptase-polymerase chain reaction (Bowen *et al.*, 2000).

As anticipated, the addition of both LPS and thrombin up-regulated the expression of mRNA for IL-8 and MCP-1. Induction of IL-8 and MCP-1 gene expression was rapid, with a significant increase in the expression observed at 2h and an apparent peak between 4 and 12h. Interestingly, considerable expression of aP2 was observed in non-stimulated HUVECs. Moreover, aP2 expression was significantly up regulated following 12 and 24h exposure to thrombin but not LPS.

Target / Treatment		Target copy number x 10^{-4} (95% CI) Time point (h) 2	4	12	24
IL-8	Cont	0.5 (0.4-0.6)	0.4 (0.3-0.6)	0.1 (0.0-0.1)	0.0 (0.0-0.1)
	LPS	1.8* (1.6-2.2)	2.6* (1.8-4.0)	1.3* (0.9-1.9)	0.9* (0.5-1.4)
	Thro	3.0* (2.1-4.3)	5.0* (3.7-6.8)	2.6* (1.8-3.8)	0.8* (0.4-1.6)
MCP -1	Cont	4.7 (3.6-6.3)	3.7 (1.0-6.3)	2.5 (2.0-3.1)	3.1 (2.6-3.7)
	LPS	12.8* 10.0-16.3)	16.9* (12.0-23.9)	17.1* (12.1-24.3)	14.4* (9.7-21.3)
	Thro	18.5* (11.6-29.8)	23.7* (16.2-34.9)	18.9* (15.5-23.0)	19.4* (15.8-23.7)
aP2	Cont	1.2 (0.8-1.7)	0.9 (0.7-1.3)	0.7 (0.4-1.1)	0.7 (0.5-0.9)
	LPS	0.9 (0.5-1.6)	0.8 (0.4-1.8)	0.8 (0.4-1.7)	0.8 (0.5-1.3)
	Thro	0.7 (0.5-1.0)	0.7 (0.4-1.0)	1.6* (1.1-2.4)	3.9* (2.4-6.3)

Table 1. Expression of IL-8, MCP-1 and aP2 following the addition of LPS and thrombin (Thro) (*-P<0.05 vs. control; ANOVA/Dunnett's).

These data demonstrate for the first time that aP2 mRNA is expressed in human endothelial cells. The data also show that aP2 is up regulated by thrombin, further adding to the emerging evidence suggesting aP2 may play a role in the development of atherosclerosis. In addition, these data provide further evidence of thrombin's involvement in atherosclerosis.

Bowen W *et al.* (2000) *Drug Metab. Dispos.* **28**, 781-788.
Makowski L *et al.* (2001) *Nature Medicine.* 7, 699-705.

149P ENDOTHELIAL HYPERREACTIVITY IN THE PERFUSED OBESE ZUCKER RAT HINDQUARTER IS ABOLISHED BY PRO-OXIDANT TREATMENT

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Pro-oxidant challenge with hydroquinone (HQ) and buthionine sulfoximine (BSO) provokes the onset of type II diabetes mellitus in a model of insulin resistance, the obese Zucker rat (Laight *et al.*, 1999a). Interestingly, the obese rat has previously been shown to have an enhanced endothelial vasodilator function compared to its lean littermate. The current study further investigates the relationship between oxidant stress and hyper-endothelial function in this model (Andrews *et al.*, 2000).

12-week old Male Zucker rats (lean 250-300 g, obese 300-350 g) were injected daily with HQ and BSO (each at 50 mg kg^{-1} i.p. for 7 days. Control animals received saline (2 mg kg^{-1} i.p.) daily. Rats were anaesthetised (pentobarbitone sodium, 60 mg kg^{-1} i.p.) and heparinized (200 U kg^{-1} i.v.). The aorta was cannulated for perfusate inflow at the bifurcation point of the iliac arteries, the vena cava was cannulated for effluent outflow. Rats were exsanguinated and temperature maintained at 37°C. Hindquarters were perfused with physiological salt solution (PSS) gassed with 95 % O_2 and 5 % CO_2. Flow was 7 mL min^{-1}.

Basal perfusion pressures (PP) in obese preparations were control, 27.7±3.1 mm Hg, (n=5); HQ, 33.5±3.9 mm Hg, (n=6); BSO, 30.3±8.8 mm Hg, (n=4); HQ+BSO, 35.8±4.5 mm Hg, (n=5). Lean basal values were unaffected by pro-oxidant treatment (Lean control, 31.2±2.5 mm Hg, n=6; HQ, 31.3±3.8 mm Hg, n=4; BSO, 46.4±9.6 mm Hg, n=5; HQ+BSO: 28.2±1.5 mm Hg, n=5) (P>0.05). Preparations were stabilised for 20 min before a phenylephrine (Phe) ED-85 (100 nmol min^{-1}) infusion was started in order to constrict the vascular bed. Phe-induced PP in obese preparations were not significantly affected by pro-oxidant treatments (control, 140.8±18.3 mm Hg, n=5; HQ, 176.5±11.0 mm Hg, n=4; BSO, 134.9±18.1 mm Hg, n=4; HQ±BSO, 167.7±15.6 mm Hg, n=5). Similarly, lean values were unaffected by pro-oxidant treatment (Lean control, 175.0±13.5 mm Hg, n=6; HQ, 201.5±23.2 mm Hg, n=4; BSO, 209.2±54.1 mm Hg, n=5; HQ±BSO, 165.7±22.6 mm Hg, n=5: P>0.05). After stabilisation, dose-responses to acetylcholine (ACh) or sodium nitroprusside (SNP) were generated. Data are mean±s.e.mean, compared by 1-way ANOVA followed by Dunnett's test.

Table 1. Effects of HQ and BSO on vasodilation to ACh (0.0001-10 nmol) standardised with respect to vasodilation to SNP (100 nmol).

	LEAN		OBESE	
Treatment	pD_2	E_{max} (% SNP)	pD_2	E_{max} (% SNP)
Control	10.69±0.01	111.9±3.6	10.95±0.06*	132.6±6.3*
HQ	10.59±0.01	120.2±8.9	10.70±0.02"	121.6±5.5
BSO	10.58±0.02	114.4±3.7	11.07±0.04	127.2±12.3
HQ+BSO	10.81±0.02	115.8±2.5	10.74±0.01"	107.1±3.4"

*P<0.05 with respect to lean; "P<0.05 with respect to obese control

Vasodilator responses to ACh were significantly enhanced in obese relative to lean animals. This difference was abolished following pro-oxidant treatment with HQ, BSO, and HQ+BSO. This effect in obese rats was associated with a decrease in the magnitude of vasodilator responses to ACh with HQ and HQ+BSO treatment, and small but significant increases in lean responses. BSO alone did not significantly change the responses in lean or obese rats.

Pro-oxidant treatment abolished the hyperreactivity of endothelial-dependent responses in obese rats, likely due to HQ generating $O_2^{\cdot-}$ and scavenging NO. Lean rats showed small but significant increases in reactivity with pro-oxidant treatment commensurate with their higher anti-oxidant status. The higher oxidant levels in the obese rat (Laight *et al.*, 1999b) may thus reduce their ability to counteract excess free radical generation, resulting in an apparent normalization of endothelial function.

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150P TESTOSTERONE DOES NOT INCREASE THE INTRACELLULAR CONCENTRATION OF cGMP IN A7r5 AORTIC SMOOTH MUSCLE CELLS

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Testosterone is a coronary vasodilator, and improves myocardial ischaemia in men with coronary artery disease (Webb *et al.*, 1999; English *et al.*, 2000). This endothelial-independent action is proposed to occur either via potassium channel opening through increased production of cGMP (Deenadayalu *et al.*, 2001) or via calcium channel antagonism (English *et al.*, 2002, Jones et al., 2002). The aim of this study was to determine whether testosterone increases cGMP production in A7r5 vascular smooth muscle cells.

A7r5 rat aortic smooth muscle cells (ECACC) were grown in DMEM media supplemented with glutamax and 10% foetal calf serum at 37°C, 5% CO_2. Once confluent, the cells were detached from the flask using trypsin, washed and re-seeded into 12 well plates. Once confluent, the cells were exposed to IBMX (10^{-3}M) for 30min, prior to addition of either sodium nitroprusside (SNP, 10^{-5} – 10^{-3}M) (positive control), testosterone (10^{-7} – 10^{-4}M), or ethanol or distilled water (dH_2O) (negative controls) for 2h. The cells were then fixed in ethanol and the intracellular cGMP level measured via commercial assay kits (Amersham).

Whilst the positive control SNP (10^{-4} – 10^{-3}M) resulted in a significant increase in the intracellular concentration of cGMP, testosterone had no effect (Table 1).

Table 1. The effect of 2hr incubation with SNP (10^{-5} – 10^{-3}M) or testosterone (10^{-7} – 10^{-4}M) upon the intracellular concentration of cGMP in A7r5 aortic smooth muscle cells. The mean results (S.E.M) from three experiments conducted in triplicate are displayed.

	Concentration of cGMP (pmol / well)	p
dH_2O	0.18 (0.01)	
SNP (10^{-5}M)	0.18 (0.04)	
SNP (10^{-4}M)	0.80 (0.18)	<0.01
SNP (10^{-3}M)	1.01 (0.22)	<0.01
Ethanol	0.15 (0.01)	
Testosterone (10^{-7}M)	0.13 (0.07)	
Testosterone (10^{-6}M)	0.12 (0.04)	
Testosterone (10^{-5}M)	0.14 (0.04)	
Testosterone (10^{-4}M)	0.20 (0.07)	

(p calculated via Student's unpaired t test)

Testosterone does not increase the intracellular concentration of cGMP in A7r5 aortic smooth muscle cells. Testosterone-mediated vasodilatation is therefore unlikely to occur via this mechanism.

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