152P CHARACTERISATION OF A DISSOCIATED GLUCOCORTICOID IN THE A549 CELL LINE

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The anti-inflammatory properties of glucocorticoids (GCs) in represssing genes such as COX-2 and GM-CSF are thought to result from transrepression of transcription factors such as NF-κB, where-as side effects are often attributed to transactivation of metabolic genes. We have used A549 cells to investigate RU24858 (Vayssière *et al*, 1997), a dissociated GC reported to cause transrepression, but not to induce transactivation. The ability of RU24858 to drive glucocorticoid response element (GRE)-dependent transcription (transactivation), inhibit κB driven transcription (transrepression) and suppress IL-1β-induced release of PGE_2 and GM-CSF was compared to dexamethasone.

A549 cells stably transfected with a luciferase reporter containing a minimal promoter driven by either two copies of a GRE site (2 x GRE.BG) or 6 copies of an NF-κB binding site (6κB.tk) were used to assess GRE- and κB-dependent transcriptional activation. Cells were growth arrested for 24 h prior to treatment. 2xGRE cells were harvested 6 h after the addition of various concentrations of dexa-methasone, RU24858 or 0.1% (v/v) DMSO. 6κBtk cells were treated for 1 h with steroids before stimulation with 1 ng/ml IL-1β and harvested after 6 h. The GRE reporter was highly activated by dexamethasone (25.0 ± 3.6 fold, n=10) and only weakly activated by RU24858 (6.5±2.2 fold, n=9). Dexamethasone suppressed κB-de-pendent transcription in a concentration dependant manner (EC_{50} = 3.5 ± 1.7 nM, n=7) with a maximal inhibition of 40% at 10^{-5}M. RU24858 also inhibited κB-dependant transcription (EC_{50}=1 ± 0.38 μM, n=8) but maximal suppression was 30% (10^{-5}M; figure 1A).

In order to determine whether these effects were due to changes in glucocorticoid receptor (GR) expression binding studies were performed using ^{3}H-dexamethasone. Bound ligand was separated by filtration and radioactivity measured. Scatchard analysis revealed 16500 ± 2700 (n=6), 8500 ± 1000 (n=6) and 5500 ± 1200 (n=5) binding sites/cell in wild type A549, 2xGRE and 6κBtk cells respectively.

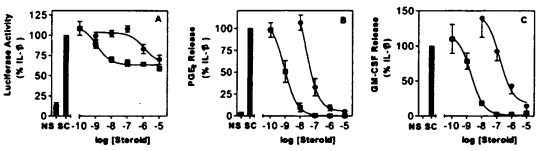

Figure 1. The effects of dexamethasone and RU24858 on 6κB.tk cells (A) and IL-1β induced release of PGE_2 (B) and GM-CSF (C), where NS represents unstimulated cells, SC 1ng/ml IL-1β, ■ dexamethasone and ● RU24858

Growth arrested cells were pre-treated with dexamethasone or RU24858 for 1h prior to addition of 1ng/ml IL-1β. After 24h culture media was assayed for PGE_2 and GM-CSF by radioimmunoassay and enzyme-linked immunoabsorbent assay respectively. Dexamethasone totally suppressed the release of both PGE_2 (n=6, EC_{50} = 1.8 ± 0.8 nM) (figure 1B) and GMC-SF (n=7, EC_{50} = 2.1 ± 0.6 nM) (figure 1C). The dissociated steroid RU24858 also suppressed PGE_2 and GM-CSF release in a concentration dependant manner, but with reduced potency (EC_{50} = 53.9 ± 12.8 nM, n=5 and 1.0 ± 0.4 μM, n=3 respectively) (figures 1B and 1C).

In summary RU24858 is a weak activator of GRE-dependent transcription and only represses κB-dependant transcription at high doses in this model. However, RU24858 represses cytokine induced PGE_2 and GM-CSF release. Dexamethasone represses κB dependant transcription by 40% yet results in total suppression of PGE_2 and GM-CSF. We therefore conclude that transrepression of NF-κB-dependent transcription does not fully explain the effect of glucocorticoids on COX-2 and GM-CSF expression.

Vayssière, B.M., Dupont, S., Choquart, *et al.*, (1997) *Mol. Endocrinol.*, **11(9)** : 1245-55

153P MECHANISM OF THE BRONCHOCONSTRICTOR RESPONSE TO BRADYKININ AUGMENTED FOLLOWING ALLERGEN CHALLENGE IN ACTIVELY SENSITISED BROWN NORWAY RATS

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In Brown Norway (BN) rats actively sensitised to ovalbumin (OA) it has been shown that the bronchoconstrictor response to bradykinin (BK) is potentiated 24 h after OA challenge (Huang et al. 1999; Hannon et al., 2001). This study defines the time course, the receptor mediating the effect and mechanism(s) involved in the potentiated bronchoconstrictor response to BK following i.t. OA challenge in actively sensitised (AS) BN rats.

Male BN rats (250-300g) were sensitised to OA, anaesthetised using sodium pentothal (70 $mgkg^{-1}$, i.p.) and prepared for recording of blood pressure, heart rate and lung function as previously described (Hannon et al., 2001). Bronchoconstrictor responses to bolus i.v. injections of adenosine (Ado), methacholine (MCh) and BK were established in AS animals 3, 12, 24, 48 or 96 h after i.t. challenge with saline (0.2 ml) or OA (0.3 $mgkg^{-1}$). Ado (1 $mgkg^{-1}$) was administered first, followed fifteen min later with a dose-response curve to MCh (5-20 μgkg^{-1}). Finally two doses of BK were administered (100 & 300 μgkg^{-1}) with 15 min between doses.

Responses to BK were potentiated at each time point to a maximum at 24 h after challenge. In contrast, responses to Ado were maximally potentiated 3 h after challenge whereas responses to MCh were little changed over the time period (Figure 1).

Pharmacological analysis implicated the involvement of the BK B_2 receptor in the potentiated response to BK 24 h after challenge with OA. Thus, the selective B_1 agonist, Lys-[desArg^9]-BK (100 & 300 μgkg^{-1}, i.v., n=4), had no bronchoconstrictor effects; whereas, the response to BK was completely inhibited by the selective B_2 antagonist, HOE 140 (200 μgkg^{-1} i.v., 5 min before agonist sequence, n=3) 24 h after OA challenge.

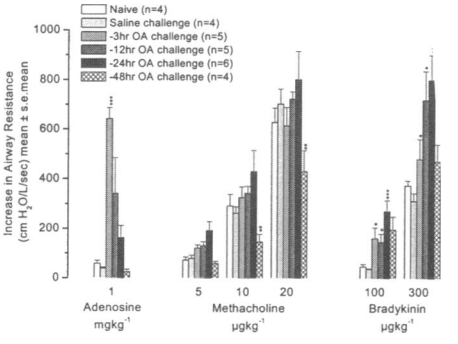

Figure 1: Bronchoconstrictor responses to adenosine, methacholine and bradykinin in naïve and actively sensitised BN rats challenged previously with OA or saline. Means ± s.e. mean are presented. *p<0.05, **p<0.01, ***p<0.001 indicates significant difference between saline and OA challenged animals using Student's *t* test for paired data assuming unequal variances.

Neither disodium cromoglycate (40 $mgkg^{-1}$ i.v., 5 min before BK, n=4) nor methysergide (10 μgkg^{-1} i.v., 5 min before BK, n=4) inhibited the potentiated response to BK 24 h after OA challenge, thus excluding a role for mast cells in this response. Similarly indomethacin (1 $mgkg^{-1}$ i.t., 30 min before BK, n=4) did not inhibit the potentiated response to BK 24 h after OA challenge, thus excluding a role for prostanoids in this response. Conversely, atropine (30 μgkg^{-1} i.v., 5 min before BK, n=4), maximally inhibited the response to BK by 61.3 ± 3.8% demonstrating a major role for cholinergic nerves.

Huang, T.-J. et al. (1999). Am. J. Respir. Crit. Care Med., 160, 1717-1723.

Hannon, J.P. et al. (2001). Br. J. Pharmacol., 132, 1509-1523.

154P INTERLEUKIN (IL)-13 ENHANCES A CALCIUM ACTIVATED ANION CONDUCTANCE IN HUMAN BRONCHIAL EPITHELIAL CELLS (HBECS) THAT IS SENSITIVE TO DIDS AND DNDS BUT NOT TO TAMOXIFEN.

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IL-13 has been implicated in asthma (Huang *et al.* 1995), chronic sinusitis (Hamilos *et al.* 1996) and allergic rhinitis (Pawankar *et al.* 1995) conditions where an imbalance in fluid secretion / absorption could impact upon the disease progression. To this end we have investigated the effects of IL-13 on the ion transport characteristics of the human bronchial epithelium modelled *in vitro* at an apical air interface to form a differentiated mucociliary phenotype.

HBECs (Clonetics) were cultured on Snapwell permeable supports for 21 days, the final 14 days at an apical air interface. These conditions provided a differentiated transporting epithelial structure. HBECs were then treated for 48h with either vehicle or IL-13 ($10ngmL^{-1}$) added to the basolateral media. HBECs were then placed in Ussing chambers bathed in Ringer solution containing (mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose (37°C, 5%CO_2:O_2) and voltage clamped at 0mV. Basal characteristics were assessed in addition to the amiloride (10μM)-sensitive short circuit current (ISC) and the subsequent increase in ISC induced by UTP (30μM). In additional studies the basolateral membrane of IL-13 treated cells was permeabilised with α-toxin ($200UmL^{-1}$) following which the apical [Cl^-] was reduced to 20mM. Under these conditions the sensitivity of the ionomycin (1μM)-stimulated ISC response to DIDS (300μM), DNDS (300μM) and tamoxifen (30μM) was determined. All compounds were added to the apical membrane except ionomycin which was added to both membranes and α-toxin (basolateral side only). Data are expressed as absolute changes in ISC (mean±s.e.mean) and significance assumed when $P<0.05$ (paired Student t-test).

IL-13 treatment reduced the basal ISC from 7.0±0.3μAcm^{-2} to 3.3±0.2μAcm^{-2} ($P<10^{-5}$; n=6) and the amiloride-sensitive component of this current from 1.9±0.1μAcm^{-2} to 0.1±0.1μAcm^{-2} ($P<10^{-5}$; n=6). The subsequent addition of UTP (30μM) induced a peak increase in ISC of 5.2±0.2μAcm^{-2} in the control cells that was significantly enhanced in the IL-13 treated cells to 33.9±1.3μAcm^{-2} ($P<10^{-9}$; n=6). We next investigated the sensitivity of this enhanced Ca^{2+}-activated current to chloride channel blockers under conditions where the observed ISC is exclusively regulated by the apical membrane conductance. Following IL-13 treatment for 48h the basolateral membrane was permeablised with α-toxin and a basolateral to apical Cl^- gradient was established. Under these conditions ionomycin induced a mean increase in ISC of 83.6±12.7μAcm^{-2} (n=6) that was reduced to 30.0±3.9μAcm^{-2} ($P<0.005$; n=5) in the DIDS treated cells. In the presence of tamoxifen, ionomycin induced an increase in ISC of 64.3±10.8μAcm^{-2} ($P=0.32$; n=4) that was not significantly different from the control group. In a separate experiment, DNDS also reduced the ionomycin stimulated increase in ISC from 92.9±8.8μAcm^{-2} (n=7) to 44.0±7.3μAcm^{-2} ($P<0.001$; n=6).

This study indicates that IL-13 can convert the human bronchial epithelium from an absorptive to a secretory phenotype. This is due in part to the induction of an enhanced Ca^{2+}-activated anion conductance that is sensitive to DIDS and DNDS, recognised blockers of Ca^{2+}-activated Cl^- channels, but not to tamoxifen a blocker of volume regulated Cl^- channels. The phenomena described in this study may represent novel pharmacological approaches to the treatment of respiratory diseases associated with imblances of fluid secretion.

Hamilos *et al.* (1995) Am. J. Resp. Cell Mol. Biol. 15:433-450
Huang *et al.* (1995) J. Immunol. 155:2688-2694
Humbert *et al.* (1997) J. Allergy Clin. Immunol. 99:657-665

155P PROSTAGLANDIN E_2 INHIBITS GM-CSF RELEASE FROM INTERLEUKIN-1β-STIMULATED HUMAN AIRWAY SMOOTH MUSCLE CELLS BY INTERACTING WITH PROSTANOID RECEPTORS OF THE EP_2-SUBTYPE

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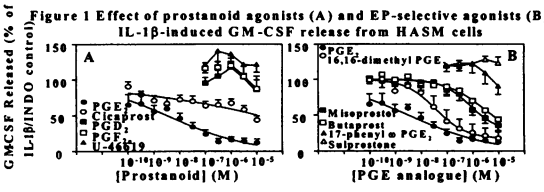
We have shown previously that human airway smooth muscle (HASM) cells generate granulocyte/macrophage-colony stimulating factor (GM-CSF) when exposed to interleukin-1β (IL-1β). Moreover, this response is negatively regulated in an autocrine manner by prostaglandin E_2 (PGE_2), which is also released from HASM cells following induction of the cyclo-oxygenase-2 gene (Clarke *et al.*, 2000). Here we report the characterisation of the prostanoid receptor that mediates the inhibition of GM-CSF generation by PGE_2 from IL-1β-stimulated HASM cells.

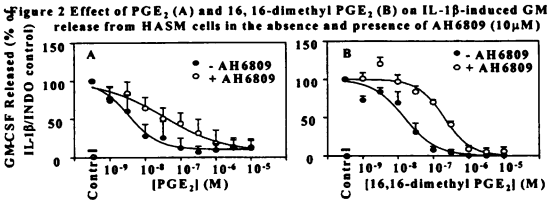
HASM cells from donor tracheae (4 female, 13 male, aged 17-52 years) were cultured in DMEM containing 3% FCS in 96 well plates. At sub-confluence cells were growth-arrested for 24 h, treated (5 min) with the drugs/vehicle under investigation before being exposed to IL-1β (0.1 ng ml^{-1}; ~EC_{90}). After 24 h the supernatants were assayed for GM-CSF by ELISA. Indomethacin (10 μM) was present throughout to prevent the production of PGE_2.

None of the drugs tested affected cell viability and neither DMSO nor ethanol (both 0.01% v/v), which were used to dissolve all drugs, affected GM-CSF release.

PGE_2 and the IP-receptor agonist, cicaprost, suppressed GM-CSF release in a concentration-dependent manner with pIC_{50} values (log_{10} concentration of drug that suppressed GM-CSF release by 50%) of 8.88 ± 0.33 and approximately 5 respectively (n = 12-33 determinations from 4 -11 donors Fig. 1). PGD_2, $PGF_{2\alpha}$ and U-46619 (synthetic TP-receptor agonist) did not inhibit IL-1β-induced GM-CSF release at any concentration examined. 16,16-Dimethyl PGE_2 ($EP_2 = EP_3 = EP_1$), misoprostol ($EP_2 = EP_3 > EP_1$) and butaprost ($EP_2 >> EP_1 > EP_3$) also suppressed GM-CSF release but were less potent than PGE_2 with pIC_{50}'s of 7.13 ± 0.37, 5.64 ± 0.25 and 5.43 ±0.26 respectively (n=12-21 determinations from 4-7 patients).



As neither PGD_2 nor the EP_1-agonist, 17-phenyl-ω-trinor PGE_2, had any effect on GM-CSF release, the EP_1/EP_2/DP-receptor antagonist, AH 6809, was used to determine if EP_2-receptors mediate the inhibitory effect of PGE_2. Pre-treatment of HASM cells with AH6809 (10 μM; 30 min) caused a rightwards shift of the PGE_2 and 16,16-dimethyl PGE_2 concentration-response curves, from which pA_2's of 5.85 ± 0.31 and 6.09 ± 0.22 (n = 9-12 determinations from 3-4 donors) were derived (Fig. 2). These values are similar to the affinity of AH 6809 at EP_2-receptors in a number of other preparations (Woodward *et al.*, 1995).



We conclude that PGE_2 inhibits GM-CSF release from HASM cells through prostanoid receptors of the EP_2-subtype.

Woodward, D.F., Pepperl, D.J., Burkey, T.H., et al., (1995) *Biochem.Pharmacol.* **50**, 1731-1733.
Clarke, D.L., Patel, H.J., Mitchell J.A *et al.*, (2000). *Br. J. Pharmacol.*, **133**, 40P.

156P DIVERGENT MECHANISMS OF ACTION OF THE INFLAMMATORY CYTOKINES INTERLEUKIN 1-β (IL-1β) AND TUMOUR NECROSIS FACTOR-α (TNFα) IN MOUSE CREMASTERIC VENULES.

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IL-1β and TNFα are pro-inflammatory cytokines, often considered to act via common mechanisms. However, we have previously found differences in the mechanisms by which these cytokines elicit neutrophil transmigration (Thompson *et al.*, 2001). We now report further differences in these mechanisms, specifically, the synthesis of new proteins and the lipid mediators, platelet activating factor (PAF) and leukotriene B_4 (LTB_4).

Intravital microscopy was used to measure leukocyte responses within cremasteric venules of fully anaesthetised C57BL/6 mice (20-25g; groups of 5) (Thompson *et al.*, 2001). Briefly, 400μl of saline, IL-1β, or TNFα was injected intrascrotally (i.s.), 4h prior to surgical exteriorisation of the cremaster muscle. Post-capillary venules (20-40μm diameter) were identified and leukocyte firm adhesion and transmigration quantified (Thompson *et al.*, 2001). Results are given as mean±s.e.mean of adherent or transmigrated leukocytes/100μm vessel segment, analysed using ANOVA and Newman-Keuls post test with $P<0.05$ taken as significant. In different groups of mice, the protein synthesis inhibitor actinomycin D (Act D, 0.2mg/kg) was co-administered i.s. with saline/cytokines; the PAF receptor antagonist UK-74,505 (Pons *et al.*, 1993) (0.5mg/kg) was administered i.v. 10 min before i.s. injections; the LTB_4 receptor antagonist CP-105,696 (Koch *et al.*, 1994) (100mg/kg) was administered p.o. 19 hrs prior to the i.s. injections. Some groups of mice were pre-treated with both UK-74,505 and CP-105,696.

IL-1β (30ng) or TNFα (300ng) increased leukocyte firm adhesion (IL-1β: 9.02±1.05, TNFα: 9.68±0.64) and transmigration (IL-1β: 4.12±0.55, TNFα: 4.42±0.70), relative to saline (adhesion: 0.52±0.20, transmigration: 0.34±0.07). Act D decreased the number of adherent and transmigrated leukocytes induced by IL-1β, to 49.0±13.6% and 67.2±4.2%, respectively, of values with cytokine alone ($P<0.05$). UK-74,505 inhibited IL-1β induced transmigration (66.5±4.5% inhibition, $P<0.05$) without affecting firm adhesion, whilst CP-105,696 inhibited both IL-1β-induced adhesion (81.4±15.2%, $P<0.05$) and transmigration (58.7±7.2%, $P<0.05$). Combined treatment with the two antagonists did not result in further suppression of responses induced by IL-1β, relative to their inhibition when injected alone. In contrast to their effects on IL-1β-induced responses, Act D, and the antagonists, UK-74,505 and CP-105,696, when administered alone or in combination, did not affect responses elicited by TNFα. None of the treatments had any effect on leukocyte blood counts or blood flow, quantified as previously detailed (Thompson *et al.*, 2001).

In conclusion, the results indicate that leukocyte firm adhesion and transmigration in mouse cremasteric venules stimulated by IL-1β, but not TNFα, is protein synthesis dependent and mediated by endogenous generation of PAF and LTB_4. These findings further illustrate the divergent mechanisms of actions of two cytokines that are often considered to act via common molecular/cellular pathways.

This work was supported by the BHF, MRC and The Wellcome Trust. REY is a BHF PhD student.

Koch K., *et al*, (1994). *J. Med. Chem.* 37, 3197-3199.

Pons F., *et al*, (1993). *Br. J. Pharmacol.* 109, 234-242.

Thompson R.D., *et al*, (2001). *Blood.* 97, 1854-1860.

157P NEUTROPHIL STIMULATING ACTIVITY IN INDUCED SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD): CONTRIBUTION OF GRO AND IL-8

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COPD is a chronic inflammatory condition characterised by increased macrophages, $CD8^+$ T cells and neutrophils in the lung. We investigated the chemotactic factors that attract neutrophils to the lung. We recruited 9 patients with COPD with a mean age 62 (range, 44-71), FEV_1/FVC ratio of 50 (32-72) and smoking history of 71 packyears (34-112), and 8 healthy non-smokers with a mean age 34 (24-57) and FEV_1/FVC ratio 87 (78-92). Sputum was induced, a small portion was used to prepare cytospins to count neutrophils as % of total leukocytes and the remainder was homogenised in saline with 0.2% trifluoroacetic acid. The supernatant was concentrated on 3.5 kDa filter membranes and reconstituted with PBS/0.1% BSA (1ml/g sputum). Neutrophil stimulating activity was measured in a flow cytometry based cell shape-change assay using blood granulocytes from healthy donors (Sabroe *et al.*, 1999). The contributions of IL-8 and GRO were assessed using neutralising antibodies. Chemokines were measured by ELISA. Statistical analysis: Mann Whitney test.

Sputum neutrophils were increased in COPD patients (fig. 1A). IL-8 (1.79±0.52 vs. 0.32±0.12 nM), GRO (20.9±3.4 vs. 12.6±2.9 nM) and eotaxin (52.0±9.0 vs. 3.0±2.0 pM) were all increased in COPD samples (mean±SEM, $p<0.05$). Neutrophil stimulating activity was increased in COPD sputum extracts (fig. 1B). Neutrophil stimulating activity in a pool of sputum extracts was reduced to 54, 50 & 20% of total by an excess of antibodies to IL-8, GRO or both antibodies (fig. 1C).

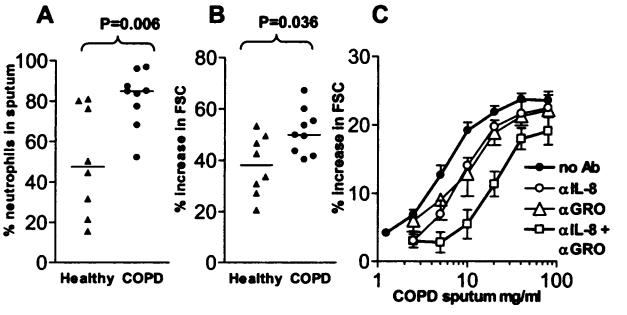

Figure 1. A) Sputum neutrophils as % of >500 leukocytes counted. B) Neutrophil stimulating activity (as % increase in forward light scatter (FSC)) in sputum extract of each subject, at 30mg sputum/ml (bars = median). C) Pre-incubation of a pool of sputum extracts from 9 COPD patients, with neutralising antibodies against GRO and IL-8 (mean±SEM of n=4 separate experiments with cells of different blood donors).

We conclude that neutrophil stimulating activity in sputum is increased in COPD and is attributable to IL-8 and GRO, together with an unidentified mediator(s). These investigations suggest that receptors for IL-8 and GRO (CXCR -1 and -2) may be effective targets for therapy to reduce excessive neutrophil accumulation in patients with COPD.

This work was funded by a bursary from Imperial College.

Sabroe I *et al.* (1999) *J Immunol* **162**:2946-55.

158P ACTIVATION OF MELANOCORTIN TYPE 3 RECEPTOR AS A NOVEL MECHANISM FOR ACTH EFFICACY IN GOUTY ARTHRITIS

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Adrenocorticotrophic hormone (ACTH) was successfully used to control human gouty arthritis (Gutman & Yu, 1950) but no mechanism of action was identified, besides classical adrenal gland stimulation. We have tested here the hypothesis that a melanocortin receptor (MC-R) could be targeted by ACTH to modulate experimental joint inflammation.

Intact or adrenalectomized male Sprague-Dawley rats (220-270 g body weight) were anaesthetised with halothane and injected intra-articular (i.a.) with 1 mg monosodium urate (MSU) crystals. Animal sacrifice occurred at 16 or 96 h post injection and the joint size and arthritic score calculated. Knee joints were then lavaged and neutrophil accumulation measured by staining in Turk's solution and light microscopy. Specific ELISA were used to quantify interleukin(IL)-1 and IL-6 concentrations. $ACTH_{1-39}$, alone or with the MC3-R antagonist SHU9119 (10 μg, 9 nmol) (Fan et al., 1997), was given s.c. (20-100 μg, -30 min) or i.a. (1-5 μg). Some naïve rats were treated with $ACTH_{1-39}$ and plasma corticosterone levels measured by radioimmunoassay 2 h. FACS analysis was used to detect MC-R expression on rat knee joint macrophages using rabbit polyclonal antibodies from Santa Cruz (1:50 final dilution) and an anti- goat IgG conjugated to Alexa48™ (Molecular Probes, Leiden, The Netherlands), measuring median fluorescence intensity (MFI) units in the FL1 channel. Values were analysed by ANOVA and Bonferroni test with *$P<0.05$ taken as significant.

MSU crystals provoked an intense neutrophil accumulation at 16 h ($1.93 \pm 0.11 \times 10^5$ joint^{-1}) inhibited by ACTH given either i.a. (-42% at 5 μg) or s.c. (-74% at 100 μg) (n=6, $P<0.05$, in both cases). Similar degrees of inhibition were observed for arthritic score, joint size and cytokine release. The MC3-R antagonist SHU9119 abrogated these effects. ACTH 100 μg s.c., but not i.a. at 5 μg dose, caused a significant increase in plasma corticosterone levels (ng ml^{-1}): 33 ± 5, 44 ± 20 and 214 ± 43 for vehicle, i.a. and s.c. ACTH, respectively, n=5, $P<0.05$). ACTH (5 μg i.a.) maintained its anti-inflammatory activity also in adrenalectomized rats (n=5, $P<0.05$), indicating the existence of corticosterone independent mechanism(s). FACS analysis showed that joint macrophages expressed MC3-R (42 ± 2 MFI units, $P<0.05$, n=4), but MC1-R or MC5-R, with punctuate pattern of localization as visualised by immunofluorescence. A second injection of MSU crystals (time 72 h) led to an increased neutrophil influx as measured 24 h later. ACTH (5 μg i.a. given only at 72 h) inhibited also this second influx of PMN by 63% ($P<0.05$, n=6), and this effect was again blocked by co-injection with SHU9119. A similar pattern of inhibition was also determined for joint size and cytokine levels.

In conclusion, this study indicates the existence of a novel molecular mechanism for ACTH therapy, and it suggest that MC3-R agonists could be novel anti-inflammatory drugs.

Fan, W. *et al.*(1997) *Nature* **385,** 165-168.
Gutman and Yu (1950) *Am. J. Med.* **9,** 24-30.
This work was supported by the Arthritis Research Campaign (grant P0562).

159P A NEW ANTI-NOCICEPTIVE PATHWAY MEDIATED BY CYCLOOXYGENASE-2 IN RAT PAW INFLAMMATION INDUCED BY CARRAGEENAN

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Inflammation induced by carrageenan in rat hind paws exhibits two characteristic features, oedema and hyperalgesia. Both of these inflammatory responses can be modified by preventing prostaglandin biosynthesis through inhibition of cyclooxygenase (COX) with non-steroidal anti-inflammatory drugs (NSAIDs). There are two isoforms of COX and the NSAIDs are either non-selective or selective inhibitors of COX-2, the isoform induced by inflammatory stimuli (Vane et al, 1998). We have assessed both types of NSAID against the hyperalgesia and oedema induced in rat paws by carrageenan.

Male Holtzmann rats (150-200g) were given carrageenan (250 μg/paw). Mechanical hyperalgesia (Randall & Selitto, 1957) and oedema (plethysmography) were measured hourly, for 4h subsequently. Commercial preparations (tablets or capsules) of piroxicam (Feldene, Pfizer, Brazil), a non-selective COX inhibitor, and of celecoxib, (Celebra, Searle, Puerto Rico), a selective COX-2 inhibitor (Penning et al, 1997), were crushed in isotonic saline to give a fine suspension, containing a dose of NSAID calculated from the amount of active substance in the tablet. Indomethacin (Sigma) was dissolved in buffer and diluted in saline. All NSAIDs were given as subcutaneous injections (1ml/kg), 30 minutes before carrageenan, to groups of at least 4 rats.

Carrageenan-induced hyperalgesia (-50±8g (mean±s.e.mean); below control threshold, at 3h) was reduced by indomethacin (0.5mg/kg; -20±17g) or piroxicam (3mg/kg; -20±15g). The carrageenan-induced oedema (0.6±0.06ml at 3h) was either not affected (indomethacin, 0.72±0.07ml) or mildly decreased (piroxicam, 0.43±0.03ml; $p<0.05$; Anova *t*-test) by these NSAIDs. Celecoxib (3-12mg/kg) successively decreased hyperalgesia and then raised the pain threshold (hypoalgesia) above that in the test paw (160±50g at 3h: $p<0.05$; Anova *t*-test), without any reduction in oedema. At the highest dose used (30mg/kg), celecoxib did reduce oedema at 3h to 0.42±0.06ml; however, at this level, hypoalgesia was lost although hyperalgesia was still reduced (-34±18g). Celecoxib (12mg/kg) did not modify the pain threshold in (non-inflamed) paws injected only with saline, instead of carrageenan, over the 4h of observation.

From these results we conclude that, although oedema and hyperalgesia were induced together by the same level of carrageenan, hyperalgesia was more sensitive to inhibition of COX. Only selective inhibition of COX-2 raised nociceptive thresholds in the paw above normal levels (hypoalgesia). This effect was not associated with decreased oedema and was not exhibited in the absence of inflammation.

We thank FAPEMIG, CNPq and CAPES for support

Randall, L. O. & Selitto, J. J. (1957). *Arch. Intl. Pharmacodynam.*, **111**, 409-419
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160P EFFECT OF GALECTIN-1 ON NEUTROPHIL CHEMOTAXIS *IN VITRO* AND MIGRATION *IN VIVO*

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Galectin 1 (Gal-1, 14 kDa) belongs to a family of proteins defined by their affinity for β-galactoside and by conserved sequence elements (Rabinovich *et al.*, 1999) with at least 10 members identified so far. Gal-1 has recently been found to possess anti-inflammatory activity in the model of collagen type II induced murine arthritis (Rabinovich *et al.*, 1999), as well as during an acute inflammation model of the rat hind paw (Rabinovich *et al.*, 2000). The aim of the present study was to investigate the potential effect of Gal-1 on neutrophil migration using *in vitro* and *in vivo* models of inflammation.

Western Blotting analysis: expressions for Gal-1 were examined in several human primary cells (neutrophil and monocytes) and an endothelial hybrid cell line (EA.hy 926/B3). In brief, cells were lysed in 50 mM Tris HCl buffer together with the appropriate proteinase inhibitors. Protein extracts (30 μg) were loaded onto a 12 % SDS-PAGE for electrophoresis and transferred to nitrocellulose membrane. Membrane was blocked overnight in 5 % non-fat dry milk followed by incubation with a rabbit serum raised against full-length Gal-1 (1:5000) in phosphate buffer saline with 0.1 % Tween 20. This was followed by washing in and incubation with a second goat-anti-rabbit HRP (Dako). After further washing, immunoreactive proteins were detected using a chemiluminescence ECL kit (Amersham). Under resting condition, Gal-1 is expressed by EA.hy 926/B3 cells, human monocytes but not by neutrophils.

Chemotaxis assay: Direct effect of Gal-1 on neutrophil migration was first examined using a chemotaxis assay. Neutrophils ($0.1x10^6$ well^{-1} in RPMI-1640 media) were incubated with the Gal-1 (0.04, 0.4 4 μg ml^{-1}) for 10 min at 37°C before being added to the upper chamber of a 96-well ChemoTx plate (3 μm pore; Neuro Probe Inc) with 30 ng ml^{-1} of IL-8 in the lower chambers as a chemoattractant. After 2 h incubation in a humidified incubator at 37°C, neutrophils in the bottom wells were collected, counted and expressed as the % control of IL-8 induced migration. Gal-1 markedly reduced the IL-8-induced neutrophil chemotaxis in a concentration dependent manner. Neutrophil migration was reduced by Gal-1 (n=3) at 0.04, 0.4 and 4 μg ml^{-1} to 60 ± 13 %, 40 ± 7 % and 7 ± 3 % (P<0.001), of control response to IL-8 (10±2 $x10^4$ cells), respectively.

In vivo model of inflammation. Male Swiss Albino mice (20-22 g body weight) were injected i.p. with IL-1β (5 ng) alone or together with the indicated doses of Gal-1 (0.01, 0.03, 0.1, 0.3 and 1 μg per mouse). Animals were sacrificed 4 h later and the peritoneal cavities were washed with PBS and heparin (25 U/ml). Aliquots of the lavage fluid were stained with Turk's solution (0.01% crystal violet in 3 % acetic acid) and neutrophil counted and expressed as % control of IL-8 induced migration. Data (mean ± s.e.mean) were analysed by one way ANOVA + Dunnett's test taking a P value <0.05 as significant. In this model of peritonitis, influx of neutrophil was reduced by Gal-1 at the doses of 3, 1 and 0.3 μg to 60 ± 12 % (n=6), 56 ± 7 % (n=6, P<0.05) and 45 ± 8 % (n=6, P<0.001) of control IL-1β responses ($3±0.9x10^6$ neutrophil per mouse, n=10), respectively. Doses of Gal-1 of 0.001 and 0.003 μg were not effective.

These data indicate that Gal-1 is a novel inhibitor of neutrophil migration. In addition, the expression of Gal-1 by endothelial cell such as EA.hy 926/B3 under resting condition, suggests possible role as an endogenous mediator of inflammation.

Rabinovich G. A. *et al.* (1999) *J. Exp. Med.*.**190**, 385-398.
Rabinovich G. A. *et al.* (2000) *E. J .Immunol.*, **30**, 1331-1339.

This work was supported by the Wellcome Trust (project grant 06236/Z/00)

161P INCREASED MIGRATION OF MONOCYTES FROM COPD PATIENTS TOWARDS GROα IS NOT MEDIATED BY AN INCREASE IN $CXCR_2$ RECEPTOR EXPRESSION

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Chronic obstructive pulmonary disease (COPD) is associated with an increase in leukocyte migration, which may be due, in part to the chemokine growth-related oncogene alpha (GROα), which exerts its effects via the $CXCR_2$ receptor. We have previously shown that GROα is significantly elevated in induced sputum from COPD patients when compared to smokers and non-smokers (Traves *et al.*, 2000). This study determined whether (i) the migration of PBMC is directed by GROα and (ii) the $CXCR_2$ receptor on monocytes is responsible for the observed migration.

Migration of PBMC or monocytes from non-smokers (n=8), smokers (n=8) and patients with COPD (n=11) was determined using a standard Boyden chamber. PBMC or monocytes were allowed to migrate through a cellulose nitrate filter for 90 min towards GROα or suspension buffer control. The number of cells present in the filter from 20μm onwards were counted. PBMC migrated towards GROα with a maximal response observed at 50ng/ml for COPD patients and at 10ng/ml for non-smokers and healthy smokers. PBMC from COPD patients migrated in higher numbers when compared to cells from non-smokers (73 ± 11 vs. 54 ± 3 vs. 43 ± 3 cells/field, p<0.05), (Figure 1), similar responses were observed with monocytes. Significant differences were assessed using Kruskal-Wallis analysis followed by Dunns post test for ANOVA analysis.

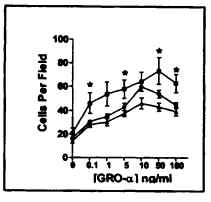

Figure 1 Concentration response of migrating PBMC to GROα. Data represents 8 non-smokers ( ), 8 healthy smokers (●) and 11 COPD patients (■). Each point is expressed as the mean ± sem. * = P<0.05

Monocyte migration to GROα (10ng/ml) was significantly inhibited with a combined $CXCR_1$ and $CXCR_2$ antagonist SB468477 (100ng/ml) (39 ± 2 vs. 22 ± 2 cells/field, p<0.05) (Figure 2a). The $CXCR_2$ specific antagonist SB332235 also inhibited GROα in a concentration dependent manner (Figure 2b).

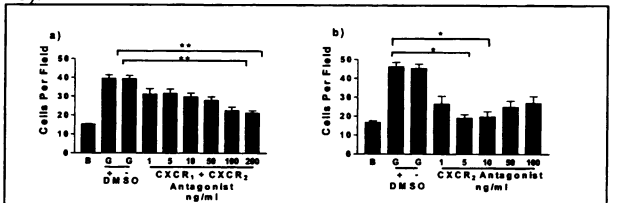

Figure 2 Migration of Monocytes to GROα in the Presence of a) SB468477 and b) SB-332235. Monocytes that had been incubated with SB-468477 or SB332235 for 1 h prior to chemotaxis were allowed to migrate towards GROα (10ng/ml). Each panel is representative of 6 experiments, and each point is the mean ± sem (n=6). * = p<0.05. B=suspension buffer, G=GROα.

Since GROα induced migration was $CXCR_2$ dependent, the level of receptor expression on monocytes was examined to determine whether differences in receptor expression could account for the increased migration of monocytes from COPD patients towards GROα. However, FACS analysis of $CXCR_2$ receptor expression on monocytes from these subject groups showed that there was no difference in the level of $CXCR_2$ receptor expression.

These results indicate that increased monocyte migration to GROα in COPD is not due to an increase in $CXCR_2$ receptor expression on monocytes.

Traves, SL, Culpitt, SV, De Matos, C, *et al* (2001) Am.J.Crit.Care.Med, **163**; A987.

162P DETERMINATION OF PHOSPHODIESTERASE 7A PROTEIN EXPRESSION IN HUMAN IMMUNE AND PRO-INFLAMMATORY CELLS

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Phosphodiesterase (PDE) 7 is a generic term that describes a novel family of enzymes that hydrolyse cAMP with high affinity. Two human genes (*HSPDE7A* and *HSPDE7B*) encode PDE7 and, to date, three isoenzymes have been identified that are derived from *HSPDE7A* by alternative mRNA splicing. We have previously shown that mRNA transcripts for PDE7A1 and PDE7A2 are expressed in human primary cells that have been implicated in the pathogenesis of airway inflammatory diseases including chronic obstructive pulmonary disease and asthma. (Smith *et al.*, 2000). Here we have investigated PDE7A protein expression by immuno-cytochemistry (ICC) and Western blot analysis using a rabbit polyclonal antibody that is specific for a 15 amino acid sequence (CELNSQLLTQENRLS) at the C-terminus of PDE7A, which is common to both PDE7A1 and PDE7A2.

Leukocytes (n=4 for each cell type), sputum (n=4) and bronchoalveolar lavage (BAL) fluid (n=3) were collected from normal healthy individuals using standard techniques. ICC was performed on cells spun on to cytospins. Briefly, slides were fixed in 4% paraformaldehyde, washed and incubated for 1 h with either 1 μg/ml of the anti-PDE7A antibody or a rabbit immunoglobulin (Ig) control antibody. The slides were washed again and a biotin-conjugated goat anti-rabbit Ig was used in conjunction with an avidin-biotin-alkaline phosphatase detection system. The reaction was visualised using fast red in the presence of levamisole. The slides were subsequently counter-stained with haematoxylin. Using this technique PDE7A protein was detected in smooth muscle (SM) cells (vascular and airway), lung fibroblasts, peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes and monocytes. Immunoconfocal microscopy (Leica TCS; x 40 magnification) also identified PDE7A in blood neutrophils and mixed cell populations from sputum and BAL fluid. In the later experiments cells were prepared as cytospins and labelled with the rabbit anti-PDE7A antibody and avidin-biotin alkaline phosphatase detection kit with fast red substrate as described above. Mouse monoclonal antibodies specific for neutrophil elastase or CD68 were incubated the cytospins for 1 h to label neutrophils and macrophages respectively. A Bodipy-conjugated goat anti-mouse IgG antibody was used to detect the phenotypic markers in cells from sputum and BAL fluid. The slides were washed, incubated with diaminidino phenylindol and mounted. Sections (0.7 μm) were analysed by confocal microscopy and confirmed that PDE7A was expressed in airway neutrophils and alveolar macrophages.

Western blotting reproducibly detected PDE7A1 in T-cells, smooth muscle (airway and vascular) and monocytes but not in neutrophils. PDE7A1 was also found in human epithelial (BEAS-2B, 16HBE14, A549), T-cell (Jurkat, HUT-78), monocytic (U-937) and eosinophilic (AML-1) cell lines as well as MRC-1 fibroblasts. In none of the cell types studied was PDE7A2 protein detected despite the unambiguous identification of HSPDE7A2 mRNA (Smith *et al.*, 2000). In contrast, PDE7A2 protein was identified in human ventricular cardiomyocytes.

These results demonstrate that PDE7A1 is ubiquitously expressed in human immune and pro-inflammatory cells that have been implicated in the pathogenesis of respiratory diseases. PDE7A could, therefore, provide a target for novel anti-inflammatory drugs. Our failure to detect PDE7A2 protein suggests that translation or stability this isoenzyme may be highly regulated in pro-inflammatory and immune cells.

Smith, S.J., Staples K.J., Donnelly L.E., *et al.*, (2000) *Am.J.Resp.Crit.Care Med.*, **163**, A221.

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163P THE LEUKOTRIENE SYNTHETIC PATHWAY IN HUMAN PERIPHERAL BLOOD T-LYMPHOCYTES.

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Leukotrienes (LT) generated by the 5-lipoxygenase (5-LO) pathway are implicated in asthma and other allergic diseases. In activated leukocytes, arachidonic acid is converted by 5-LO and its activating protein FLAP into LTA_4, and then by LTC_4 synthase into the bronchoconstrictor and vasoactive cysteinyl-LT, LTC_4, or by LTA_4 hydrolase into LTB_4, a potent leukocyte chemoattractant. In allergic inflammation, T-lymphocytes generate cytokines that regulate inflammatory leukocyte activity, including LT synthesis, but their capacity to generate LTs directly is controversial. The effects of LTs on T-cell function are also poorly understood, but LTs modulate cytokine synthesis and apoptosis in other cell-types and a sub-population of T-cells expresses the cysteinyl-LT type 1 receptor ($CysLT_1R$) [Figueroa et al.2001]. We hypothesised that human blood T-cells may be able to generate LTs, and that LTs may act on T-cells to modify their activity. We aimed to assess the capacity of human blood T-cells to express 5-LO pathway enzymes and synthesise LTB_4 and LTC_4, and to quantify the effect of a cysteinyl-LT on apoptosis and synthesis of interleukin-5 in T-cells.

Peripheral blood from normal adult volunteers (age 21-40yrs; n=20) underwent Lymphoprep centrifugation to provide mononuclear cells, and negative immunomagnetic selection to deplete contaminating monocytes, basophils, NK cells, B-cells, and platelets. Flow cytometry and immunocytochemistry were used to assess the expression of 5-LO pathway enzymes and $CysLT_1R$, and enzyme immunoassays to quantify release of LTB_4 and LTD_4. T-cells were also cultured in the presence of LTD_4 (10nM), followed by EIA and immunocytochemistry to quantify IL-5 synthesis. Effects of LTD_4 on T-cell apoptosis were explored using the TUNEL technique.

FACS showed that T-cells expressed 5-LO (median 44.9% positive, IQR 10.4-68.7, n=7), FLAP (41.4%, 21.9-63.0, n=7), and LTA_4 hydrolase (48.2%, 28.6-67.0, n=6), but not LTC_4 synthase (<0.5%). Immunocytochemical data were similar. EIA showed median LTB_4 levels of $24pg/10^6$ cells (IQR 10-41), but undetectable LTC_4 after 24h culture (n=10). By FACS, a sub-population of T-cells (0.2-5.0%) expressed surface $CysLT_1$ receptors, but culture with LTD_4 for 24h had no effect on release of IL-5 (median 3.2 $pg/10^6$ cells) versus control (2.4 $pg/10^6$ cells, n=11) or on intracellular IL-5. Preliminary TUNEL experiments showed no marked effect of LTD_4 on apoptosis over 48h.

In conclusion, human T-cells constitutively express a complete pathway for the synthesis of LTB_4, but not of LTC_4, perhaps suggesting involvement of T-cell derived LTB_4 in leukocyte migration. Although some T-cells express the $CysLT_1$ receptor, activation of this receptor by LTD_4 over 24-48h appears to have no marked effects on T-cell apoptosis, or on the synthesis of a cytokine (IL-5) associated with allergic inflammation.

Figueroa DJ, Breyer RM, Defoe SK et al., 2001, Am J Respir Crit Care Med; 163(1):226-233

164P EFFECT OF A NOVEL LOW MOLECULAR WEIGHT INHIBITOR OF THE INTEGRIN ALPHA$_V$ BETA$_3$ (CT6725) ON BONE RESORPTION *IN VITRO*

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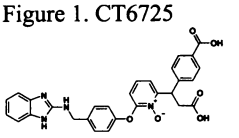
Bone resorption occurs when osteoclasts bind to bone creating an acidic environment capable of degrading bone. This binding is believed to be mediated by specific matrix proteins and integrins. Osteoclast cells express Alpha$_v$ Beta$_3$ ($\alpha v\beta 3$) that recognises a number of proteins e.g. saloprotein and osteopontin (Ross *et al.*, 1993). Therefore, inhibition of $\alpha v\beta 3$ represents a potential therapeutic target for the treatment of osteoporosis. This study examined the role of $\alpha v\beta 3$ in bone resorption using the mouse calvaria assay *in vitro*.

Bone resorption was measured by the release of calcium from explants of neonatal mouse calvaria in culture (Ljunggren *et al.*, 1991). Calvaria were removed from 8 day old BK:W mice and divided in half, then cultured *in vitro* for 24 hours in 2ml of Biggers medium containing, 5% heat inactivated foetal calf serum, 5mg/100ml L-ascorbic acid, 1% L-glutamine, 1% penicillin-streptomycin and $1x10^{-6}$ M indomethacin. After 24 hours medium was removed and replaced with new medium (2ml) without indomethacin. Calvaria were then cultured for a further 72 hours with 1,25 dihydroxyvitamin D_3 (Vit D_3; $1x10^{-9}$ M) to stimulate bone resorption in the presence or absence of test substance. Vit D_3 has been shown to increase $\alpha v\beta 3$ expression on osteoclast cells (Medhors *et al.*, 1992). Medium was analysed for calcium concentration colorimetrically on a spectrophotometer (PYE Unicam PU 8600 UV/VIS, ATI Unicam).

Echistatin, a snake venom $\alpha v\beta 3$ inhibitor ($1x10^{-10}$ - $1x10^{-7}$ M), F11, a monoclonal antibody against the $\beta 3$ chain (supplied by Dr M. Horton; 10-100ug/ml) and a low molecular weight inhibitor of $\alpha v\beta 3$, CT6725, (IC_{50} 32nM in cell based assay; $1x10^{-7}$ - $1x10^{-5}$ M) were examined for effects on calcium release into the medium (Figure 1).

Figure 1. CT6725



Data was analysed using one-way ANOVA and Bonferroni's multiple comparison post test. All data is expressed as mean ± s.e.m. (mg/dL) with $p<0.05$ considered significant.

The basal level of calcium release into the medium over 72 hours was 5.39±0.07 mg/dL (n=40 from 8 experiments). Vit D_3 ($1x10^{-9}$ M) increased calcium release to 6.86 ± 0.16 mg/dL; $p<0.001$ (n=40 from 8 experiments). The increase in calcium release was significantly reduced by echistatin ($1x10^{-7}$ M) from 6.59±0.13 to 5.89±0.07mg/dL ($P<0.01$) and by the antibody F11 (100µg/ml) from 6.96±0.22 to 5.90± 0.12 mg/dL, $P<0.001$). CT6725 caused a concentration dependent inhibition of Vit D_3–induced calcium release with an IC_{50} of 1.0±0.11 µM. At the highest concentration ($1x10^{-5}$ M), CT6725 reduced the Vit D_3–induced increase in calcium release from 6.76±0.27 to 5.14± 0.19 mg/dL ($P<0.001$).

These data indicate that the calvaria assay is $\alpha v\beta 3$ dependent and can be used to evaluate novel low molecular weight inhibitors *in vitro*.

Ljunggren, Ransjo and Lerner, 1991. Journal of bone and mineral research. 6 (6) 543-550.
Medhors, Teitelbaum and Chappel *et al*, 1993. Journal of biological chemistry. 268 (2) 1456-1461.
Ross, Chappel and Alvarez *et al*, 1993. Journal of biological chemistry. 268 (13) 9901-9907.

165P ANTISENSE TO STAT1/IRF-1 AND NF-κB INHIBITS CYTOKINE-STIMULATED ET-1 RELEASE FROM HUMAN VASCULAR SMOOTH MUSCLE CELLS

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Endothelin-1 (ET-1) mRNA expression and peptide production in human vascular smooth muscle cells (HVSMCs) are markedly increased by exposure to TNF-α and IFN-γ (Woods *et al.*, 1999). Synergistic effects of these cytokines are known to occur in a number of cell types (Lee *et al.*, 2000). The aim of this particular study was to investigate the molecular mechanisms underlying this synergy. As transcription factors STAT1, IRF-1 and NF-κB often mediate the effects of IFN-γ and TNF-α in target cells we used antisense oligonucleotides (ODNs) to determine whether these transcription factors may be acting in synergy to stimulate ET-1 release from HVSMCs.

Saphenous vein (SV) was obtained from patients undergoing coronary artery bypass graft surgery. Explants of HVSMCs were grown in DMEM supplemented with MEM non-essential amino acids and 10% foetal calf serum (37°C; 5% CO_2/95% air). HVSMCs were identified by α-actin staining. The phosphorothioate oligonucleotides (ODNs) used as antisense were as follows: STAT1 5'GGTGCAGGATGTCTCAGTGG-3', IRF-1 5'-TCCGAGTGATGGGCATGTTGG-3' and NF-κB 5'-GGGGAACAGTTCATGGC-3'. HVSMCs cultured on 24 well plates were transfected with either STAT1, IRF-1 or NF-κB antisense or sense ODNs (0.75µM) using Transfast reagent (Promega) at a charge ratio of 3:1 (Transfast:ODN) in serum free medium. At the end of a 1 h incubation period DMEM containing 10% FCS was added. After 24 h the medium was replaced and cells were treated with a mixture of TNF-α ($10ng.ml^{-1}$) and IFN-γ ($1000U.ml^{-1}$) for 24 h. Medium was removed and ET-1 levels were measured by specific sandwich ELISA (R&D Systems). Cell viability was assessed by the ability of cells to reduce MTT to formazan (Mosmann, 1983). Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA) and compared by Repeated Measures Analysis of Variance followed by a Dunnetts Multiple Comparison Test.

Treatment of SV HVSMCs with either STAT1, IRF-1 or NF-κB antisense ODNs reduced TNF-α and IFN-γ stimulated ET-1 release by 67±7% (n=3; $p<0.05$), 65±8% (n=4; $p<0.05$) and 68±7% (n=4; $p<0.05$) respectively when compared to cytokine-stimulated sense ODN control. Basal release of ET-1 was not significantly altered by treatment with sense or antisense ODNs.

These results indicate that both STAT1/IRF-1 and NF-κB transcription factors are involved in the signal transduction events leading to cytokine-stimulation of ET-1 peptide release. As up-regulated production of ET-1 within VSMCs may underlie the causative role of ET-1 in a number of disease states this finding indicates that NF-κB and STAT1/IRF-1 within HVSMCs could be central to a number of vascular pathologies. Inhibition of this pathway could be of therapeutic benefit.

Lee *et al.*, 2000. *Biochem. J.*, 350, 131-138.

Woods *et al.*, 1999. *Mol.Pharmacol.*, 55, 902-909.

166P FURTHER INVESTIGATIONS ON EDHF IN THE RAT MESENTERIC ARTERY: THE EFFECT OF HEPES ON ACETYLCHOLINE-INDUCED HYPERPOLARIZATION

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When stimulated by certain agonists the vascular endothelium induces smooth muscle hyperpolarization in the presence of inhibitors of NO and prostacyclin synthesis. A diagnostic criterion for this endothelium-derived hyperpolarizing factor (EDHF) response is its inhibition by apamin plus charybdotoxin but not by apamin plus iberiotoxin (IbTX). Recent reports that IbTX alone inhibits EDHF liberated by bradykinin (BK) in the porcine coronary artery (PCA) (Fisslthaler *et al.*, 2000) result largely from the use of HEPES-buffered Tyrode solution (Edwards *et al.*, 2001). In the presence of HEPES, EDHF-mediated responses induced by BK are abolished revealing an additional IbTX-sensitive, endothelium-dependent hyperpolarization (Edwards *et al.*, 2001). The effects of HEPES on EDHF responses have now been examined in rat mesenteric arteries.

Male Sprague-Dawley rats (150-200g) were killed by stunning and cervical dislocation. *Micro-electrode recordings*:- mesenteric arteries incubated at 20-25°C for 16-24h in Krebs or Tyrode's solution (gassed with 95%O_2/5%CO_2 or 100% O_2, respectively) were pinned to the base of a 10ml bath and superfused with the appropriate solution. Smooth muscle cells were impaled via the adventitia using micro-electrodes filled with 3M KCl (40-80MΩ) (Edwards *et al.*, 1999). *Western blotting*:- arteries from mesenteric beds were dissected and divided into 2 groups. One was processed immediately (t=0) and the other incubated as above in bicarbonate-buffered Krebs or Krebs plus 10mM HEPES (pH 7.4).

Following overnight incubation, resting membrane potentials remained unchanged (n=4) (Krebs, -59.4 ± 0.4mV; Tyrode's, -59.7 ± 0.7mV) as did the hyperpolarizations induced by levcromakalim (Krebs, to -84.8 ± 0.5mV; Tyrode's, to -83.3 ± 0.3mV) indicating that this prolonged incubation *in vitro* did not modify the tissue non-selectively. Levels to which acetylcholine hyperpolarized vessels were significantly reduced ($p<0.01$) in those vessels incubated in Tyrode's compared to vessels in Krebs solution (Krebs, -81.7 ± 0.2mV; Tyrode's, -74.7 ± 0.3mV; Tyrode's plus 100nM IbTX, -75.2 ± 0.8mV). Western blots using antibodies against connexins (Cx) 37, 40 and 43 showed no changes in protein levels of Cx37 and Cx43 after incubation in Krebs or Krebs plus HEPES compared to that at t=0. However, bands corresponding to Cx40 were reduced after incubation with a greater reduction seen with tissue incubated in Krebs plus HEPES compared to tissue incubated in normal bicarbonate-buffered Krebs (see Fig. 1).

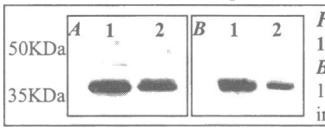

Fig. 1 A; Bicarbonate-buffered Krebs 1) t=0 2) 24h incubation *B*; Bicarbonate-buffered Krebs plus 10mM HEPES 1) t=0 2) 24h incubation

In the rat mesenteric artery, HEPES had a small inhibitory effect on EDHF which may be associated with the observed loss of Cx40. Thus, in contrast to the PCA, gap junctions probably have a less significant role in the EDHF response in rat mesenteric vessels.

Edwards, G. *et al.* (1999) *Br. J. Pharmacol.* **128**, 1788-1794.
Edwards, G. *et al.* (2001) *Br. J. Pharmacol.* **133**, 1145-1153.
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167P ASCORBIC ACID ATTENUATES EDHF-MEDIATED VASODILATATION IN THE BOVINE ISOLATED PERFUSED EYE

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We have recently shown (McNeish *et al.* 2001) that endothelium-derived hyperpolarizing factor (EDHF) is the major mediator when acetylcholine (ACh) dilates the ciliary vascular bed in the bovine isolated perfused eye. We now investigate the effect on this dilatation of ascorbate, an anti-oxidant which is highly concentrated during formation of aqueous humour by the ciliary body.

Bovine eyes, obtained from a local abattoir, were perfused using the constant flow method of Wilson *et al.* (1993). Briefly, eyes were cannulated through a long posterior ciliary artery and perfused at 2.5 ml min^{-1} at 37°C with Krebs solution gassed with O_2 containing 5% CO_2. Perfusion pressure was raised (to ~120 mmHg) with the thromboxane A_2-mimetic, U46619 (300 nM), and vasodilator responses to bolus doses of ACh (10 nmol), injected immediately proximal to the cannulae, were elicited. The effects of ascorbate were studied using two different protocols. In some experiments, eyes were perfused with Krebs containing ascorbate (10-150 M) from the outset. In other experiments, control vasodilator responses were obtained to ACh at 15 min intervals, before ascorbate (50 M) was infused and its subsequent effects studied. Vasodilator responses are given as % reduction of U46619-induced perfusion pressure and vasoconstrictor responses are given in mmHg. Data are expressed as mean ± s.e. mean, n≥6. Statistical differences were determined by one-way ANOVA with Bonferroni's post-test, or by an unpaired *t*-test, as appropriate.

In control eyes ACh (10 nmol) produced solely a vasodilator response (Table 1). Responses were, however, quite different in eyes perfused from the outset in Krebs containing ascorbate (50 μM): these consisted of an initial powerful vasoconstriction (87.0 ± 16.8 mmHg) followed by a small vasodilator response (17.3 ± 6.3 %). A time course study revealed that ascorbate (50 μM) produced a progressive reversal of ACh-induced vasodilatation to vasoconstriction such that at 120 min vasodilator responses were virtually abolished (5.8 ± 3.0 %, $P<0.001$) and vasoconstrictor responses had stabilised (88.1±13.7 mmHg). This ability of ascorbate to reverse ACh-induced vasodilatation to vasoconstriction was concentration-dependent (Table 1).

Table 1. Effects of ascorbate on responses to ACh (10 nmol; **P<0.01 and *** P<0.001 indicate a significant difference from control).

ascorbate (μM)	Vasodilatation (%)	Constriction (mmHg)
0	59.5 ± 4.5	0.0 ± 0.0
10	44.4 ± 4.2	32.0 ± 13.1**
50	26.5 ± 7.5**	76.2 ± 25.5***
150	15.0 ± 5.9***	133.0 ± 25.3***

The data show that reversal of ACh-induced vasodilatation to vasoconstriction in the bovine isolated perfused eye by ascorbate is both time- and concentration-dependent. This action is similar to that previously seen with the EDHF blocker, charybdotoxin (McNeish *et al.* 2001), suggesting that ascorbate blocks EDHF-dependent vasodilatation thus revealing a normally masked vasoconstrictor response to ACh. The mechanism and physiological significance of this action of ascorbate remain to be determined.

McNeish A.J., Wilson W.S., Martin W. (2001) *Br. J. Pharmacol. (in press).*
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168P METHYLTETRAHYDROFOLATE AND TETRAHYDROBIOPTERIN INHIBIT METHIONINE -INDUCED ENDOTHELIAL DYSFUNCTION IN RABBIT AORTIC RINGS: AN ANTIOXIDANT-INDEPENDENT EFFECT?

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Folic acid (FA) has been demonstrated to reverse methionine (METH)-induced endothelial dysfunction (Erhorn *et al.*, 2000). Whether the mechanism underlying this response involves an antioxidant or other more specific effect of FA or its metabolites remains unclear. In the present study we have investigated the effects of FA, methyltetrahydrofolate (MTHF), the active circulating form of folate, and tetrahydrobiopterin (BH_4), an essential co-factor for nitric oxide (NO) synthesis, on endothelial dysfunction produced either by METH or the Cu/Zn superoxide dismutase inhibitor diethyldithiocarbamate (DETCA) in isolated aortic rings from male New Zealand White rabbits (2 to 2.5kg).

Endothelium-intact rings (2-3mm) were mounted in Krebs buffer (with 10μM indomethacin) and gassed with 95%CO_2/ 5%O_2 at 37°C for isometric tension recording (resting tension 2g). Following preconditioning to phenylephrine (PE, 1μM), tissues were incubated at 37°C for 3 hours in the absence or presence of either METH (500μM) or METH with either FA (0.5mM), MTHF (0.5mM), or BH_4, (0.5mM). In a separate experiment tissues were first incubated at 37°C for 90 minutes in the absence or presence of DETCA (3mM). Following washing to remove DETCA, tissues were incubated for a further 3 hours in the absence or presence of either FA, MTHF or BH_4 as above. At the end of the appropriate incubation all rings were constricted with PE, (1μM) before exposure to acetylcholine (Ach, 1nM to 10μM). After washing, rings were reconstricted with PE followed by exposure to sodium nitroprusside (SNP, 1nM to 10μM). Relaxation responses are expressed as a percentage of the PE-induced constriction. Data are expressed as mean±s.e.m. (n=4) and maximum relaxation (Rm) responses compared by ANOVA and Student Newman Keuls multiple range test where $p<0.05$ was considered significant.

PE-induced constrictions in control tissues from the METH and DETCA experiments (4.4±0.8 and 4.0±0.5g respectively) were unaffected by any intervention. METH alone caused a significant ($p<0.01$) inhibition of the relaxation response to ACh (Rm 68.2±1.9 cf. 87.5±4.1% for control). Concomitant incubation with FA, MTHF or BH_4 significantly ($p<0.05$) reversed this inhibiton (Rm 89.2±6.1, 80.4±1.2 & 89.4±9.0% respectively). DETCA alone also caused a significant ($p<0.01$) inhibition of the relaxation response to ACh (Rm 48.5±3.2 cf. 90.7±1.3% for control). Subsequent incubation with FA, MTHF or BH_4 had no effect on this inhibiton (Rm 45.5±8.3, 54.4±5.0 & 38.5±2.3% respectively). No differences in Rm values were observed between any of the groups with SNP.

These data demonstrate that FA, MTHF and BH_4 do not reverse endothelial dysfunction induced by oxidative stress *per se*. The effect on METH-induced dysfunction is more likely to involve other specific actions, possibly augmentation of NO production due to the increased bioavailability of BH_4.

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Erhorn S. *et al.*, (2000) Br. J. Pharmacol. **131**, 100P.

169P METHIONINE-INDUCED OXIDATIVE STRESS IN ISOLATED AORTIC RING PREPARATIONS IS INHIBTED BY METHYLTETRAHYDROFOLATE AND TETRAHYDROBIOPTERIN

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The endothelial dysfunction associated with methionine (METH) loading *in vivo* is thought to involve increased oxidative stress. However, the source of the latter and the role of folic acid (FA) in inhibiting this process remain unclear.

In the present study we have therefore investigated the acute effects of METH and FA, either alone or in combination, on oxygen free radical (OFR) generation in isolated aortic rings from male New Zealand White rabbits (2 to 2.5kg). 2-3mm wide endothelium-intact rings were prepared and incubated in Hepes buffer (pH 7.4, with 10μM indomethacin) at 37°C for 3 hours in the absence or presence of either METH (500μM) or METH with either FA (0.5mM), methyltetrahydrofolate (MTHF, 0.5mM), the active circulating form of folate, or tetrahydrobiopterin (BH_4, 0.5mM), an essential co-factor for nitric oxide (NO) production. In separate tissues FA, MTHF or BH_4 were added acutely 10 minutes before the end of the METH incubation.

Following these various incubations, individual tissues were removed into fresh Hepes containing the lipophilic luminophore coelenterazine (5μM) then placed into measuring chamber of a tube luminometer at 37°C. Coelenterazine-enhanced chemiluminescence was then used to measure OFR production over a 10 minute period (as previously described for lucigenin-enhanced chemiluminescence, Lang et al., 2000). The area under the curve of the response represented the OFR generated and was expressed as mV.s/mg of tissue (wet weight). All data are expressed as mean±s.e.m. (n 9) and responses compared by ANOVA followed by Student Newman Keuls multiple range test. Significant differences are identified where $p<0.05$.

Exposure to METH alone caused a significant ($p<0.01$) increase in OFR production (193.6±15.8 cf. 94.6±8.5mV.s/mg for control). Concomitant incubation with FA, MTHF or BH_4 significantly ($p<0.01$) inhibited this effect of METH (118.0±16.7, 140.6±6.5 & 115.6±14.7mV.s/mg respectively). However, following the acute addition of these compounds only MTHF or BH_4 significantly ($p<0.01$) inhibited the effect of METH (140.7±5.6 & 137.3±8.9mV.s/mg respectively).

These data demonstrate that FA, MTHF and BH_4 can reverse the METH-induced increase in oxidative stress. It is possible that by providing more active BH_4, FA and MTHF prevent NO synthase from generating superoxide anions, thereby inhibiting oxidative stress and increasing NO bioavailibility. However, a direct antioxidant effect of MTHF and BH_4 cannot be ruled out.

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Lang D. *et al.*, (2000) Art. Thromb. Vasc. Biol. **20**, 422-427.

170P THE SENSITIVITY OF CARDIAC AUTONOMIC RECEPTORS AND VASCULAR ENDOTHELIAL FUNCTION TO OXIDATIVE STRESS: A COMPARISON BETWEEN HYPOCHLORITE AND HYDROGEN PEROXIDE

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Reactive oxygen species (ROS) are known to be involved in the progression of various cardiovascular diseases. One source of ROS is the myeloperoxidase, an enzyme which is released by activated neutrophils during inflammatory processes, reperfusion injury and atherosclerosis. The myeloperoxidase catalyzes the oxidation of chloride by hydrogen peroxide yielding hypochlorite, an extremely potent oxidant. The aim of the present study was to evaluate the oxidative effects of hypochlorite in rat isolated thoracic aorta and rat left atria and to compare these results with the phenomena observed after incubation with hydrogen peroxide. For this purpose male Wistar rats (240-280g) were killed by stunning and decapitation. The left atria, the thoracic aorta and the portal veins were isolated, mounted into organ baths and connected to a force transducer.

In isolated left atria the positive inotropic response of α_1 -adrenoceptor stimulation by means of methoxamine (300μM) was converted into a negative inotropic response (16.8 ± 3.0% vs. -48.5 ± 7.9%, n= 4-8 $p<0.05$) after a 30 min incubation with hypochlorite (300μM). This inversion was not obtained in the presence of hydrogen peroxide (500μM) (7.7 ± 5.3%, n = 5) and seems to be specific for α_1- adrenoceptor stimulation since the positive inotropic effects of endothelin-1 (27.4 ± 7.1% vs. 30.2 ± 5.3%, 4-7, n.s.) and of the β_1/β_2-adrenoceptor agonist isoprenaline (17.3 ± 1.5% vs. 18.7 ± 3.4%, n=4-8,n.s.) remained largely unaffected.

Furthermore, the effect of cardiac M_2-receptor stimulation was studied after the incubation with hypochlorite (200μM) and hydrogen peroxide (200μM) for 20 min in left rat atria. The negative inotropic response to acetylcholine was significantly enhanced in forskolin (10μM)-stimulated left rat atria due to hypochlorite incubation when compared to control (32.7 ± 5.4 % vs. 73.2 ± 4.2% of initial contractile force, n=6, $p< 0.05$). The concentration response curve for adenosine remained unaffected when incubated with hypochlorite (73.8 ± 2.5 vs. 73.6 ± 2.7, n=4-5, n.s.). In contrast, preincubation with hydrogen peroxide did not influence the response to cardiac M_2 receptor stimulation (72.6 ± 4.5%, n=5). In addition, the muscarinic M_3-receptor in the portal vein was studied under similar circumstances, but no enhancement of the M_3-receptor-mediated vasoconstriction was established. In phenylephrine (1μM)-precontracted rat thoracic aorta rings, the endothelial function (evaluated by means of acetylcholine-induced vasodilation) was completely abolished after 30 min of incubation with hypochlorite (100μM). However, 100μM hydrogen peroxide hardly affected the endothelial function (-100.0 ± 1.1% vs. -13.2 ± 0.9%, n=4-5, $p<0.05$). From these data we conclude that the myeloperoxidase-driven formation of hypochlorite represents an amplification of the toxic properties of hydrogen peroxide leading to specific alterations in the contractile and relaxing properties of cardiac and vascular tissues.

171P ANGIOTENSIN II AND PHENYLEPHRINE INDUCED VASOCONSTRICTION: A POSSIBLE CONTRIBUTION OF REACTIVE OXYGEN SPECIES AND THE MAP KINASE PATHWAY

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In the past decade, it has become apparent that increased formation of angiotensin II (Ang II) as well as formation of reactive oxygen species (ROS) exert adverse effects in essential hypertension. Angiotensin II has, among others, a direct contractile effect on vascular smooth muscle, mediated by the angiotensin I (AT_1) receptor. It has also has been shown that AT_1 receptor stimulation increases superoxide anion formation in the vessel wall, presumably by activation of the NAD(P)H oxidase system. The superoxide anion (O_2^-) is converted to hydrogen peroxide by superoxide dismutase (SOD). Previous studies in our laboratory (Peters *et al.*, 2000) have demonstrated that ROS, in addition to their tissue damaging effects, induce vasoconstriction in isolated rat aortae. The specific mitogen-activated protein kinase ($MAPK^{erk}$) kinase (MKK^{mek}) inhibitor PD98059 blocked this contractile effect, indicating a role for a ROS-activated $MAPK^{erk}$ pathway herein. Consequently, the aim of our study was to determine whether Ang II-induced free radical formation contributes to the contractile effects of this peptide and if so, whether $MAPK^{erk}$ pathways are involved. Furthermore, as our previous studies (Peters *et al.*, 2000) have also shown that $MAPK^{erk}$ pathway inhibition reduces the contractile response to α_1 stimulation, we investigated the role of ROS in phenylephrine-induced vasoconstriction.

For this purpose, experiments were performed in aortic rings isolated from male Wistar rats (240-260g) in an organ bath set-up. AII rings were allowed an equilibration period of 30 minutes and a priming procedure of three potassium (K^+)-induced smooth muscle contractions. The aortic rings were pre-incubated with Euk-8, a salen-manganese complex with high SOD, catalase and oxyradical scavenging activities or to the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI). After 30 minutes a dose response curve for Ang II was constructed. Responses to Ang II were calculated as percentage of the third K^+ -induced contraction. In the highest concentration Euk-8 (0.4 mM) lowered E_{max} from 92.4 ± 2.6% to 57.3 ± 7.1% ($P<0.05$, n=6) and DPI (10 μM) lowered E_{max} from 80.3 ± 3.3% to 34.3 ± 2.8% ($P<0.05$, n=6). Tissue incubation with the MKK^{mek} inhibitor U0126 (0.1 mM) decreased E_{max} from 95.5 ± 4.1% to 45.0 ± 11.2% ($P<0.05$, n=6) and with PD98059 (0.1 mM) from 101.0 ± 6.6% to 74.8 ± 8.5% ($P<0.05$, n=7). Finally, the effects of Euk-8 and DPI on the contractile response to a single dose phenylephrine were studied. Interestingly, Euk-8 almost completely inhibited phenylephrine-induced contraction from 117.0 ± 5.3% to 15.0 ± 3.3% ($P<0.05$, n=6) while DPI lowered the response from 130.4 ± 6.7% to 64.0% ± 1.9% ($P<0.05$, n=6).

These data suggest both an involvement of ROS as well as an activation of the $MAPK^{erk}$ pathway in Ang II-induced vasoconstriction. Furthermore, the present study shows that ROS might also play a role in α_1 adrenoceptor-mediated vasoconstriction.

Peters *et al.* (2000) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 361, 127-133.

172P A NOVEL MECHANISM FOR THE REGULATION OF ENDOGENOUS INHIBITORS OF NITRIC OXIDE SYNTHASE: *S*-NITROSYLATION OF DIMETHYLARGININE DIMETHYLAMINOHYDROLASE.

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Asymmetrically methylated forms of L-arginine are endogenously produced competitive inhibitors of all three isoforms of nitric oxide synthase (NOS). Elevated plasma concentrations of these compounds have been reported in a wide range of human disorders including renal failure, hypertension and hypercholesterolaemia and this would be expected to decrease nitric oxide generation and thereby contribute to disease pathology. Asymmetric methylarginines are metabolised to citrulline and methylamines by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). We and others have suggested that DDAH activity regulates asymmetric methylarginine concentrations and provides a mechanism for modulation of NOS activity *in vivo*.

We have cloned DDAH sequences from a wide range of species ranging from mammals to microbes. Microbial DDAH sequences were expressed as N-terminally polyhistidine tagged fusion proteins in *E. coli*. Recombinant proteins were purified and screened for the ability to form crystals. *P. aeruginosa* DDAH crystallised readily allowing the crystal structure to be solved.

Active site residues were mutated by PCR or nitrosylated by incubation with the nitric oxide (NO) donor 2-(N, N-Diethylamino)-diazenolate-2-oxide (DEA NONOate)

The crystal structure of microbial DDAH revealed a catalytic triad of glutamic acid (114), histidine (162) and a reactive cysteine residue (249). Site specific mutation of this cysteine residue to serine, a single atom change, reduced the conversion of ADMA to citrulline from 33μM/mg/hr to undetectable levels (n=4, $p<0.001$). We hypothesised that this cysteine residue may be a target for *S*-nitrosylation by NO. Consistent with this hypothesis, incubation of purified recombinant DDAH with the NO donor DEA NONOate produced a concentration dependent inhibition of activity with maximal inhibition of 78 ± 3.3% (n=4, $p<0.001$) at 10^{-3}M DEA NONOate.

These findings suggest a novel mechanism for the regulation of NO production. In situations of low NO production from the constitutive NOS isoforms DDAH would not be *S*-nitrosylated and its activity would facilitate optimal NOS activity. In contrast, high levels of NO produced by the inducible NOS isoform would result in *S*-nitrosylation and inhibition of DDAH leading to accumulation of endogenous NOS inhibitors and reduction of NO production.

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173P EVIDENCE FOR THE FORMATION OF VASOACTIVE NITRIC OXIDE STORES BY *S*-NITROSATION OF CYSTEINE RESIDUES IN ISOLATED BLOOD VESSELS EXPOSED TO *S*-NITROSOGLUTATHIONE

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We have previously reported that nitric oxide (NO) production by the inducible NO synthase results in the formation of low molecular weight thiols (LMWT)-releasable NO stores in rat aorta (Muller *et al.*, 1996; 1998). The aim of the present study was to determine whether or not exposure to NO donors can induce the formation of releasable NO stores in blood vessels.

Endothelium-denuded rings (3 mm length) from rat (male Wistar, 10-12 weeks-old) aorta (RA) or from porcine coronary artery (PCA, obtained from local slaughterhouse) were mounted in bath filled with Krebs solution (kept at 37°C and gassed with 95%O_2, 5%CO_2) for isometric tension recording. Rings were exposed for 30 min to 1 μM *S*-nitrosoglutathione (GSNO) or 2-(N,N-diethylamino)-diazenolate-2-oxide (DEA-NO). At this concentration, both GSNO and DEA-NO produced full relaxation of precontracted RA. After a washout period of 60 min (allowing contraction recovery), the effect of cumulative addition of the LMWT *N*-acetylcysteine (NAC) or of $HgCl_2$ (which can displace NO from *S*-nitrosothiols) was determined in rings precontracted with noradrenaline (100 nM, for RA) or the thromboxane mimetic U46619 (10 nM, for PCA). Relaxation was expressed in % of the precontraction level. Analysis of variance was used for statistical comparisons. P values < 0.05 were considered as statistically significant.

In RA pre-exposed to GSNO (1 μM) but not in controls, NAC elicited a relaxant effect (Table 1). However, NAC (1 mM) did not exert a significant relaxation in RA pre-exposed to 1 μM DEA-NO (2.8±1.6%, n=5). Similarly, NAC (1 mM) elicited a marked relaxant effect in PCA pre-exposed to 1 μM GSNO (61.7 ± 4.9 %, n=9), but not in those pre-exposed to 1 μM DEA-NO (8.2 ± 6.9 %, n=4) or in controls (0.7 ± 0.5 %, n=5).

In RA pre-exposed to GSNO (1 μM), the relaxant effect of NAC was attenuated by the NO scavenger oxyhemoglobin (oxy-Hb, 10 μM) or by the inhibitor of cGMP-dependent protein-kinases, Rp-8Br-cGMPS (0.1 mM). Addition of $HgCl_2$ (0.1 mM) elicited by itself a relaxant effect which was significantly larger in RA pre-exposed to 1 μM GSNO (61.1±10.5%, n=8) than in controls (21.8±7.8%, n=8, $P<0.01$). Furthermore, $HgCl_2$ completely abolished the relaxing effect of subsequent addition of NAC (Table 1).

Table 1: Relaxant effect (in % of the precontraction level) of NAC (1 mM) in the absence or in the presence of 10 μM oxy-Hb, 0.1 mM Rp-8BrcGMPS or 0.1 mM $HgCl_2$ in RA pre-exposed or not to GSNO (1μM). Results are expressed as mean ± s.e.mean of (n) experiments. *P < 0.05, ***P < 0.001 *vs* respective controls.

	NAC	oxy-Hb + NAC	Rp-8BrcGMPS +NAC	$HgCl_2$ +NAC
control	1.1 ±0.6% (10)			
GSNO	39.6±3.9% (14)	13.4±4.4% (3)	4.2±1.8%(3)	3.1+0.6% (4)
	*** *vs* control	* *vs* NAC	*** *vs* NAC	*** *vs* NAC

Immunohistological experiments (n=3) using rabbit polyclonal antibodies directed against *S*-nitrosated cysteine residues revealed a diffuse staining in RA pre-exposed to GSNO (0.1 mM). This staining was less intense in RA treated with $HgCl_2$ after exposure to GSNO, in control tissues (not exposed to GSNO) or in those pre-exposed to DEA-NO (0.1 mM).

These data provide evidence that the *S*-nitrosothiol GSNO, but not the free radical donor DEA-NO, induced the formation of NO stores in blood vessels, and that these stores were formed by *S*-nitrosation of cysteine residues in tissue. This mechanism may be involved in prolonged effect of GSNO and some other *S*-nitrosothiols in blood vessels.

Muller, B. *et al.*, (1996) *Br. J. Pharmacol*. 119: 1281-1285.
Muller, B. *et al.*, (1998) *Br. J. Pharmacol.* 123: 1221-1229.

174P VITAMIN C MAY MODULATE NO-DEPENDENT RELAXATION THROUGH DIRECT INTERACTION WITH NO AND NITROSOTHIOLS

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Previous data from our laboratory indicated divergent effects of vitamin C on endothelium-derived NO and exogenous NO. Such effects could be due to interactions between NO, vitamin C, copper and nitrosothiols (RSNO). In the present study we examined effects of vitamin C on relaxation to acetylcholine (ACh), authentic NO and the NO-donors glyceryl trinitrate (GTN), nitroprusside (NP) and S-nitroso-N-acetyl-penicillamine (SNAP) in rabbit aortic rings. We also examined effects of vitamin C on the spontaneous decay of NO in solution and on the release of NO from RSNO.

Organ bath studies: 2 mm wide descending thoracic aortic rings obtained from New Zealand white male rabbits (2–2.5 Kg) were mounted in 3 ml organ baths containing oxygenated Krebs' solution at 37°C. Isometric measurements were recorded via force transducers (Grass FT03). Rings were constricted with phenylephrine to 80% maximum tension and then relaxed with ACh, authentic NO or an NO donor to obtain control relaxation responses. Rings were then washed out and incubated (15 min) with vitamin C (0.1-10.0 mmol/L or vehicle control) and contraction and relaxation to vasodilators repeated. In some experiments rings were incubated (15 min) with $CuSO_4$ (200 μmol/L).

Biochemical studies: Concentrations of NO in solution were measured using an ISO-NO Mark II meter (World Precision Instruments Ltd, UK). Spontaneous release of NO from SNAP (250 μmol/L) was measured and repeat measurements obtained in the presence of EDTA (1 mmol/L) to chelate copper ions and EDTA plus vitamin C (0.1-3.0 mmol/L).

Vitamin C (0.1-10.0 mmol/L) produced a concentration-dependent attenuation of the response to ACh with a decrease in Emax ranging from 0.8±3% to 71±7%, (n=4, $P<0.001$ by analysis of variance for repeated measures). Vitamin C (0.1-10.0 mmol/L) also produced a concentration-dependent attenuation of relaxation to authentic NO with a shift to the right of the log dose-response curve by 1.8±0.2 log units at highest dose (n=5, $P<0.001$). In contrast vitamin C (3.0 mmol/L) potentiated relaxations to NP and SNAP with a shift to the left of each dose-response curve of >0.5 log units (n≥5, each $P<0.01$) but tended to inhibit the response to GTN. $CuSO_4$ (200 μmol/L) had no significant effect on relaxation to NP. Vitamin C enhanced the spontaneous decay of NO in solution but potentiated release of NO from SNAP in the presence of EDTA. Vitamin C produced a dose-dependent increase in the concentration of NO measured in the bath solution 100 seconds after addition of SNAP (250 μmol/L), from 54±16 nmol/L (in the absence of vitamin C) to 779±101 nmol/L in the presence of 3.0 mmol/L vitamin C (n=5, $P<0.01$).

These results suggest that vitamin C, at concentrations within the physiological range, is capable of potentiating the release of NO from RSNO via a copper ion independent mechanism but may also inactivate NO. Pharmacological actions of vitamin C on NO-dependent vasodilation may depend on the NO species involved.

175P INCREASED METABOLISM OF ENDOGENOUS NOS INHIBITORS PROMOTES TUBE FORMATION IN AN *IN VITRO* MODEL

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Nitric oxide (NO) influences vascular remodelling in part by modulation of VEGF expression (Ni *et al* 1997). Inhibition of NOS activity by N^G-monomethylarginine (L-NMMA) has been shown to reduce VEGF expression (Dulak *et al* 2000) and N^GN^G–dimethyl-arginine (ADMA) reduces angiogenesis in an *in vivo* animal model (Jang *et al* 2000). Accumulation of endogenously produced asymmetrically methylated forms of arginine (L-NMMA and ADMA) can inhibit NO production and therefore might indirectly modulate VEGF expression and angiogenesis.

Levels of endogenously produced L-NMMA and ADMA are determined, at least in part, by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), of which there are 2 isoforms (Leiper *et al* 1999), which metabolises asymmetric methylarginine to citrulline and methylamine. We hypothesised that overexpression of DDAH II would lower intracellular ADMA levels and increase tube formation in an *in vitro* model.

ECV304 cells were transfected with the DDAH II expression plasmid and DDAH II overexpressing cell lines were selected. DDAH II and VEGF mRNA was detected by northern blotting. DDAH II protein levels were detected by Western blot using a rabbit antibody raised against the DDAH $II_{241-255}$ peptide. ADMA secreted by the cells into the culture media was determined using HPLC. Cell proliferation was assayed using Aqueous One Solution Cell Proliferation Assay (Promega). Angiogenic tube formation was induced by growth of cells in Matrigel basement membrane (Becton Dickinson) and the resulting tube formation was quantitated using a microscope (Axiovert) coupled to Improvision Apple Macintosh system. Where appropriate differences between ECV304 wild type and the overexpressing cells were compared by students unpaired T-test. Values are given as mean ± SEM and n=3 unless otherwise stated.

DDAH II transfected cells had 2.31 ± 0.89 (n=5) fold higher levels of DDAH II mRNA than ECV304 cells. Overexpression of DDAH II protein was confirmed by western blotting. ADMA levels in culture media fell from 6.31 ± 0.21 μg/ml in ECV304 cells to 5.10 ± 0.07 μg/ml in the DDAH II overexpressing lines (P=0.0052), whilst no changes were detected in symmetric dimethylarginine, which is not a DDAH substrate. These data are consistent with increased DDAH II activity in the overexpressing cells.

No detectable differences were observed in cell proliferation between ECV304 and overexpressing cells. However in tube formation assays the area covered by the DDAH II overexpressing cells was 149.2 ± 23.3 % higher than the ECV304 cells after 7 days (P=0.049). Addition of VEGF to the ECV304 cells increased tube formation by 174.6 ± 14.7 % compared to untreated ECV304 cells (P = 0.0012). Tube formation could be reduced by the flk-1 receptor antagonist oxindole-1 to 36.3 ± 8.1% (P<0.001) and 60.1 ± 9.7 % (P=0.021) for the DDAH II overexpressing and ECV304 cells respectively compared to untreated cells.

Basal levels of VEGF mRNA were 209 ± 27 % higher in DDAH II overexpressing cells than ECV304 (P=0.0062, n=11). A 2h exposure of ECV304 cells to the NO donor SNAP (1000 μM) increased levels of VEGF mRNA to 131.7 ± 12.6 % after 24h (P=0.033).

We have shown that overexpression of DDAH II lowers ADMA levels and increases ability of ECV304 cells to form tubes on matrigel. Basal expression of VEGF mRNA was higher in DDAH II overexpressing cells than ECV304 cells suggesting that increased DDAH activity results in NO mediated induction of VEGF. Consistent with this hypothesis an NO donor increased VEGF mRNA in ECV304 cells. This suggests that the effect of DDAH II overexpression is mediated by NO to enhance VEGF expression.

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176P HEME OXYGENASE INDUCTION IS ASSOCIATED WITH PROTECTION FROM SIN-1 CYTOTOXICITY IN WKY 3M-22 RAT AORTIC SMOOTH MUSCLE CELLS

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Heme oxygenase (HO) metabolises heme to biliverdin with the concomitant production of carbon monoxide and free iron (Maines *et al.*, 1982). Pre-treatment of rat aortic smooth muscle cells (RASMCs) with hemin to induce HO-1 is associated with a reduction in the cytotoxicity of nitric oxide (NO) (Hamilton & Warner, 1999). 3 morpholinosydnonimine (SIN-1) is an NO donor that also produces equimolar amounts of superoxide anion (O_2^-). Together NO and O_2^- can generate peroxynitrite ($ONOO^-$), a highly reactive and cytotoxic free radical species that is an extremely potent inducer of apoptosis. Vascular smooth muscle cell death may play an important role in the stability of atherosclerotic lesions. Here, we have investigated whether induction of HO-1 by hemin pre-treatment can prevent SIN-1 induced smooth muscle cell death.

RASMCs (WKY 3M-22; Gordon *et al.*, 1986; a gift from Dr. David Han, University of Washington, Seattle) were grown to confluence in 6-well plates. Medium was then replaced with fresh serum-free medium and cells incubated for 24 h with hemin (0.1-10 μM) or vehicle, in the presence or absence of cycloheximide (0.3 μM). Cell homogenates were then analysed by western blotting for the expression of HO-1 protein. In parallel experiments to assess the effects of HO-1 induction on cell viability, cells grown in 96-well plates were similarly incubated with 10 μM hemin or vehicle in the presence or absence of cycloheximide (0.3 μM). The medium was then removed, and replaced with fresh serum-free medium before incubation of cells with SIN-1 (0.03-3 mM) for a further 16 h. As a measure of cell death the release of LDH into the medium was then determined.

Hemin caused a concentration-dependent increase in the induction of HO-1 protein, with 10 μM hemin causing a 12 ± 2 fold increase (arbitrary units, n=3) in the amount of HO-1 protein as determined by densitometry. Co-incubation with cycloheximide inhibited the induction of HO-1 protein, such that in its presence 10 μM hemin caused only a 4 ± 0.5 fold increase in HO-1 expression. In experiments using cells grown in 96-well plates, SIN-1 was found to cause a concentration-dependent increase in cell death, with 3mM causing 73 ± 5 % cytotoxicity (n=3) (100 % cytotoxicity = LDH activity in the medium of cells exposed to 1% Triton-X 100). Pre-treatment of cells with 10 μM hemin significantly ($p<0.001$, 2 way ANOVA plus Bonferroni's test) reduced this cell death to 53 ± 2 % (n=3). However, when cells were incubated with hemin in the presence of 300 nM cycloheximide LDH release was not different from that in control conditions (77 ± 2 %, n=3).

HO-1 induction is associated with a significant reduction in the cell death caused by SIN-1, and this protection is largely reversed by the action of cycloheximide. Hence an increase in HO-1 synthesis and activity could reduce the death of smooth muscle cells exposed to endogenous NO and oxidative stress. HO-1 could, therefore, aid plaque stability in atherosclerosis, where there is an increased production of free radical species and levels of smooth muscle cell death are higher than normal.

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177P PHASE 2 ISCHAEMIA-INDUCED VENTRICULAR FIBRILLATION IN THE RAT ISOLATED BLOOD-PERFUSED HEART

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Rats *in vivo* exhibit two phases of ventricular fibrillation (VF) following coronary occlusion, occurring before (phase 1) and after (phase 2) 90 min of ischaemia respectively, whereas isolated buffer-perfused rat hearts do not develop phase 2 VF (Ravingerova *et al.*, 1995). We explored whether a blood-perfused heart could serve as a viable model to investigate the role of blood components in mediating phase 2 VF.

Hearts, excised from male Wistar rats (260-330g) under pentobarbitone anaesthesia, were perfused in the Langendorff mode with blood at 37°C from a support rat (male Wistar, 350-430g; anaesthetised with 60 mg/kg i.p. pentobarbitone) at an initial pressure of 60 mmHg using an extracorporeal circuit aided by a peristaltic pump (Galinanes *et al.*, 1993). Perfused hearts underwent left coronary artery occlusion (Ravingerova *et al.*, 1995) for 240 min or a sham procedure (n=10/group).

Phase 2 VF (90-240 min) occurred in only 10% of ischaemic hearts despite an ischaemic zone size (41±3% of total ventricular weight) similar to that which *in vivo* is associated with a 100% incidence of phase 2 VF (Curtis, 1998). Tissue myeloperoxidase activity (neutrophil accumulation; Mullane *et al.*, 1985) increased during ischaemia from 0.02±0.004 (6 fresh hearts) to 0.06±0.01 units mg $protein^{-1}$, ($p<0.05$) at 240 min but values were similar in sham hearts (0.08±0.01). Likewise, the decline (-1 vs 240 min of ischaemia shown) in circulating total white blood cells from 6.8±0.5 x 10^3 to 1.9±0.2 x 10^3 μL^{-1}, in neutrophils from 316±72 to 159±49 μL^{-1} and in platelets from 441±32 to 274±16 μL^{-1} (measured by a Coulter counter; all $p<0.05$) was similar in time-matched sham hearts (data not shown). Heart rate, measured every 15 min, was well maintained in shams and throughout the 240 min of ischaemia (means of 270 to 310 beats min^{-1}). QT_{90} intervals were similar in sham and ligated hearts (p=NS) and varied little during 90-240 min (means between 60 and 67 ms). Coronary vascular resistance in the non-ischaemic region increased progressively from 31±4 mmHg g min ml^{-1} at 30 min of ischaemia to 67±14 mmHg g min ml^{-1} at 240 min ($p<0.05$). However, a significant ($p<0.05$) and similar increase occurred in shams (from 25±3 to 97±21 mmHg g min ml^{-1}). Surprisingly, only 10% of ischaemic hearts developed phase 1 VF, which contrasts with a 100% incidence *in vivo* and in Krebs-perfused hearts (Curtis, 1998). K^+ values from blood entering ischaemic hearts were 4.2±0.1, 4.5±0.2 and 5.1±0.3 mM 1 min before, and after 120 and 240 min of ischaemia respectively (p=NS vs sham hearts). However, although K^+ can be antiarrhythmic, 4.2 mM is insufficient to suppress phase 1 VF in buffer-perfused hearts (Curtis & Hearse, 1989).

In conclusion, the lack of phase 2 VF in blood-perfused hearts may suggest that blood components are not mediators of phase 2 VF. However, the decline in circulating white blood cells, platelets and neutrophils, the fact that neutrophil accumulation was similar in ischaemic and sham hearts, and the unexpected paucity of phase 1 VF all question the validity of the model, and hence any conclusion about blood components and VF.

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178P D_1-LIKE RECEPTORS STIMULATE PLC ACTIVITY IN OK CELLS INDEPENDENT OF $G_{q/11}$ ACTIVATION

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Dopamine D_1-like receptors are linked via G proteins to the multiple cellular signaling systems, namely adenylyl cyclase (AC) and phospholipase C (PLC) (Jose et al., 1996; Lokhandwala et al., 1998). We have previously shown that D_1-mediated inhibition of Na^+,K^+ ATPase activity in OK cells involves both the AC-PKA system and the PLC-PKC system (Gomes *et al.*, 2001). The present study examined in more detail the role of the PLC-PKC system on D_1-mediated inhibition of Na^+,K^+ ATPase activity in renal OK cells, as well as the coupling of these receptors to G proteins.

OK cells (ATCC 1840-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% foetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein. 24 h before the experiments the cell culture medium was changed to a serum free medium. Na^+,K^+ ATPase activity was determined using the amphotericin B permeabilization technique, as previously described (Gomes *et al.*, 2001). In some studies, cells were treated overnight with specific antibodies raised against $G_s\alpha$ and $G_{q/11}\alpha$ proteins. 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.

The D_1-like receptor agonist SKF 38393 decreased Na^+,K^+ ATPase activity with an IC_{50} value of 130 (91, 186) nM. This effect was prevented either by the D_1-like receptor antagonist SKF 83566 (1 μM), the PKA antagonist H-89 (10 μM), the PKC antagonist chelerythrine (1 μM), or the PLC inhibitor U-73,122 (3 μM) (Jose et al., 1996; Lokhandwala et al., 1998). However, cAMP (500 μM) was found to increase PLC activity, both in membranes and in cytosol from OK cells; PDBu (1 μM) was devoid of effect. Western blot analysis revealed the presence of both $G_s\alpha$ and $G_{q/11}\alpha$ proteins in OK cells. The inhibitory effect of the D_1-like receptor agonist SKF 38393 (300 nM) on Na^+,K^+ ATPase 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. The dopamine (1 μM) stimulated increase in cAMP was abolished in cells treated with the anti-$G_s\alpha$ antibody or the selective D_1-like receptor antagonist SKF 83566 (10 μM), but not by the PLC inhibitor U-73,122 (3 μM).

It is concluded that D_1 agonists stimulate PLC and PKC activity in renal OK cells via $G_s\alpha$ proteins. PLC activation probably occurs as a result of phosphorylation by PKA.

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179P EXPRESSION OF RGS INSENSITIVE $G_{\alpha o}$ ENHANCES μ-OPIOID AGONIST SIGNALLING THROUGH ADENYLYL CYCLASE BUT NOT INTRACELLULAR CALCIUM

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Regulators of G protein signalling (RGS) proteins act as GTPase activating proteins for members of the $G_{\alpha o/i}$ and G_q families of G proteins (Hepler *et al.*, 1999). A glycine to serine point mutation in $G_{\alpha o}$ (G184S) blocks the interaction between G protein and RGS proteins (Lan *et al.*, 1998). In this study we used C_6 glioma cells stably expressing the μ-opioid receptor ($C_6\mu$) and RGS insensitive $G_{\alpha o}$ (RGS-i) to study μ-opioid receptor mediated signalling. RGS insensitive $G_{\alpha o}$ was also pertussis toxin insensitive (C351G; PTX-i), enabling natively expressed $G_{\alpha o}$ to be inactivated by PTX treatment. $C_6\mu$ cells expressing only the PTX-i $G_{\alpha o}$ acted as control.

Cells were cultured in supplemented Dulbecco's modified Eagle medium, differentiated using 5μM forskolin and treated overnight with PTX (100ng ml^{-1}) prior to assays. GTPγ[^{35}S] binding was measured as described previously (Traynor and Nahorhski, 1995), cyclic AMP accumulation was measured in adherent cells using a specific radioimmunoassay (Diagnostic Products Corp). $[Ca^{2+}]_i$ was measured in fura-2 loaded whole cell suspensions as described by Hirst *et al.*, (1999). Data are presented as mean±s.e.mean for n=3-6, statistical comparisons were made by Student's *t*-test where appropriate and considered significant when $p<0.05$.

The selective μ-opioid receptor agonist DAMGO produced a concentration-dependent stimulation of GTPγ[^{35}S] binding in RGS/PTX-i and PTX-i $C_6\mu$ cells, with no difference in pEC_{50} (RGS/PTX-i=6.42±0.06; PTX-i=6.32±0.09) or maximal stimulation (RGS/PTX-i=414±31%, PTX-i=452±31%). DAMGO produced a concentration-dependent inhibition of forskolin stimulated cyclic AMP production, with a significant 40-fold higher potency in the RGS/PTX-i $G_{\alpha o}$ expressing cells (pEC_{50}: RGS/PTX-i=8.42±0.14: PTX-i=6.82±0.10) and increased maximal inhibition (RGS/PTX-i=84±1.2%, PTX-i=69±5.4%). DAMGO afforded stimulation of $[Ca^{2+}]_i$ in RGS/PTX-i and PTX-i cells but with no difference in pEC_{50} values (RGS/PTX-i=7.09±0.24, PTX-i=6.85±0.12) or maximal stimulation (RGS/PTX-i=0.13±00.02, PTX-i=0.10±0.01; Δ340/380 ratio). At 1 μM DAMGO the induced rise in $[Ca^{2+}]_i$ was from intracellular stores since the increase was the same in the presence (RGS/PTX-i=0.11±0.02, PTX-i=0.06±0.01; Δ340/380 ratio) or absence (RGS/PTX-i=0.07±0.01; PTX-i=0.07±0.01; Δ340/380 ratio) of extracellular Ca^{2+}. No stimulation of GTPγ[^{35}S], inhibition of cyclic AMP or stimulation of $[Ca^{2+}]_i$ was seen in PTX treated wild-type $C_6\mu$ cells.

These data show that opioid receptor mediated cyclic AMP signalling is enhanced in cells expressing RGS-i $G_{\alpha o}$, whereas GTPγ[^{35}S] binding and $[Ca^{2+}]_i$ signalling are unaffected. Thus, endogenous RGS proteins may be responsible for differentially modulating opioid signalling by promoting the rapid hydrolysis of G_α-GTP.

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180P EFFECT OF THIMEROSAL ON μ-OPIOID RECEPTOR-EVOKED $[Ca^{2+}]_i$ ELEVATION IN SH-SY5Y NEUROBLASTOMA CELLS

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In SH-SY5Y neuroblastoma cells activation of G_i/G_o-coupled receptors, such as the μ-opioid receptor, does not result in the elevation of $[Ca^{2+}]_i$. However, during concomitant stimulation of the G_q-coupled m3 muscarinic receptor activation of G_i/G_o-coupled receptors now causes a further elevation in intracellular calcium concentration ($[Ca^{2+}]_i$) (Connor & Henderson, 1996). The mechanism underlying this coincident signalling is not fully understood, but in these cells the G_i/G_o-coupled receptor-mediated response is dependent on release of Ca^{2+} from intracellular stores, yet does not appear to be due to elevation of intracellular inositol trisphosphate (IP_3) levels (Yeo *et al.*, 2001). Given that G proteins have been suggested to sensitise the IP_3-receptor (Xu *et al.*, 1996) we have investigated the possibility that activation of G_i/G_o-coupled μ-opioid receptors results in IP_3-receptor sensitisation and thus facilitates G_q mediated Ca^{2+} release from intracellular stores. We have studied the effects of thimerosal, an agent known to sensitise the IP_3-receptor (Bird *et al.*, 1993), on the coincident signalling between the G_i/G_o-coupled μ-opioid receptor and the G_q-coupled m_3 muscarinic receptor.

SH-SY5Y cell monolayers were cultured, loaded with Fura2 and perfused with drugs as described previously (Connor & Henderson, 1996). Fluorescence changes were measured ratiometrically using a spectrophotofluorimeter. Data are presented as the mean ± s.e. mean.

Application of carbachol (1μM) for 1min evoked an elevation of $[Ca^{2+}]_i$ that rapidly returned to basal levels after washout. Cells were then exposed to thimerosal (10μM) for 10min, which alone had no effect on $[Ca^{2+}]_i$. However, a second application of carbachol (1μM) in the continued presence of thimerosal evoked a rise in $[Ca^{2+}]_i$ that was 248 ± 18% (n=9) of that evoked by carbachol alone. On separate monolayers, carbachol (1μM) was applied for 1min both alone and in the presence of the μ-opioid receptor agonist DAMGO (100nM). Co-application of carbachol and DAMGO evoked a rise in $[Ca^{2+}]_i$ 147 ± 5% (n=7) of that evoked by carbachol alone. This experiment was repeated in the presence of thimerosal (10μM) whereupon the rise in $[Ca^{2+}]_i$ evoked by carbachol and DAMGO (245 ± 26%; n=8) was not significantly different ($p>0.05$, Student's t test) from that evoked by carbachol alone.

While thimerosal alone had no effect on $[Ca^{2+}]_i$ it markedly potentiated the elevation of $[Ca^{2+}]_i$ evoked by carbachol. This observation is consistent with thimerosal sensitising the IP_3-receptor. However, in the presence of thimerosal, there was no further increase in $[Ca^{2+}]_i$ in response to DAMGO in the presence of carbachol. This does not result from the release process having already reached a maximum because carbachol (1mM) alone elevated $[Ca^{2+}]_i$ by 383 ± 14% (n=3). That prior exposure to thimerosal, an agent known to sensitise the IP_3 receptor, occludes the μ-opioid receptor-mediated response may indicate that μ-opioid receptor activation also works by sensitising the IP_3 receptor.

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181P SYNERGY BETWEEN P2Y2 NUCLEOTIDE RECEPTOR AND CXCR2 CHEMOKINE RECEPTOR AT THE LEVEL OF CALCIUM SIGNALLING IS DEPENDENT ON THE PLC/INS(1,4,5)P_3 PATHWAY.

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We have shown previously that in HEK-293 cells, the presence of UTP, an agonist at endogenous G_q-coupled P2Y2 receptors, reveals a release of intracellular Ca^{2+} to interleukin (IL)-8 acting through the recombinant G_i-coupled CXC chemokine receptor 2 (Werry et al., 2001). The mechanism of this effect is unknown and here we have investigated the role of phospholipase C/Ins(1,4,5)P_3, and Ca^{2+}-releasing pathways mediated by sphingosine-1-phosphate (SPP) and Ca^{2+}-induced Ca^{2+} release (CICR) via ryanodine receptors (RyR).

Using $[Ca^{2+}]_i$ imaging to study ~20 HEK-CXCR2 cells per experiment, the effects of the phospholipase C (PLC) inhibitor, U73122, and the Ins(1,4,5)P_3 receptor inhibitor, 2-aminoethoxydiphenylborate (2APB) (Maruyama et al, 1997), were studied. Cells on coverslips were loaded with fluorophore (5μM fura-2/AM or fluo-3/AM) for 1hr at RT. Cells were then repeatedly stimulated (in the absence of extracellular Ca^{2+}) with 20s pulses of 100μM UTP to fully deplete UTP-sensitive Ca^{2+} stores. Test agents (U73122, U73343, 2APB) were then applied as required and cells stimulated individually with 100μM UTP then 10nM IL-8 followed by the two in combination. Neither agonist alone gave a response following store depletion. In the absence of a test agent, or in the presence of the negative control for U73122, U73343 (10μM), the co-addition of UTP/ IL-8 gave a $[Ca^{2+}]_i$ response equivalent to 64±3% or 64±8%, respectively compared to the response to 100μM UTP prior to store depletion.

All data are mean±sem, n=4. U73122 (10μM) significantly diminished the UTP/IL-8 response to 9±4% ($P<0.001$). Similarly, a 60s incubation with 100μM 2APB significantly reduced the response to UTP/IL-8 co-addition, from 57±9% to 17±5% ($P<0.05$).

We investigated whether the phenomenon involved SPP using the sphingosine kinase inhibitor, dimethyl sphingosine (DMS) to block production of SPP. Following a 5 minute incubation with 30μM DMS (that fully blocked LPA-induced $[Ca^{2+}]_i$ elevation), the effect of UTP/IL-8 co-addition (57±9%) was not significantly different to controls (54±9%; $P=0.65$). Similarly, blocking RyRs (10 min with 30μM ryanodine) did not affect the UTP/IL-8 response (62±7% following ryanodine treatment *vs* 61±4% in controls).

The accumulation of total [^{3}H]-inositol phosphates ([^{3}H]-IP_x) against a Li^+-block of inositol monophosphatase was also determined in cells pre-labelled for 48hrs with [^{3}H]-*myo*-inositol. Cells were stimulated for up to 30mins with either buffer, 1mM UTP, 100nM IL-8 or UTP/IL-8. IL-8 did not elevate [^{3}H]-IP_x levels while UTP caused a maximal increase of 1.60±0.09 fold of basal. UTP/IL-8 caused an accumulation of 3.0±0.1 fold of basal that was significantly greater than UTP alone ($P<0.001$ by two-way ANOVA).

We conclude that the mechanism by which co-stimulation of P2Y2 nucleotide receptors and CXCR2 causes release of Ca^{2+} from stores other than the normal UTP-sensitive store does not involve SPP or CICR at RyRs but does involve stimulation of PLC, phosphoinositide hydrolysis and the subsequent activation of Ins(1,4,5)P_3 receptors.

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182P AGONIST-SPECIFIC TEMPORAL CHARACTERISTICS OF β_2-ADRENOCEPTOR-STIMULATED GENE TRANSCRIPTION RESPONSE IN CHO-K1 CELLS TRANSFECTED WITH THE HUMAN β_2-ADRENOCEPTOR

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The time allowed for the generation of a reporter protein in response to G-protein-coupled receptor activation in cells transfected with a reporter gene is usually a minimum of 4-5 hours (Hill *et al* 2001). We have previously reported that a variety of β_2-agonists can stimulate cyclic AMP-response element (CRE) - regulated gene transcription in CHO K1 cell transfected with the human β_2-adrenoceptor (CHO-β_2, McDonnell *et al.*, 1998; Baker *et al.*, 2001). In the present study we have evaluated the effect of different times of agonist stimulation on the reporter protein (secreted placental alkaline phosphatase; SPAP) response in these CHO-β_2 cells.

SPAP responses were measured after a 5h incubation as previously described (McDonnell *et al* 1998) during which agnists were present for the first 10min, 30min, 1h, 2h or for the full 5h. ^{3}H-CGP 12177 (1nM) was used to measure the on and off rates of this ligand in whole cell binding assays at 37°C. Non-specific binding was defined with 100nM ICI 118551.

Previously, isoprenaline, salbutamol and salmeterol have been shown to be full agonists and CGP 12177 a partial agonist in this system (McDonnell *et al* 1998; Baker *et al* 2001). A 10 minute agonist incubation was sufficient to induce a gene-transcription response with all four agonists. For isoprenaline and salbutamol, the response at 30 min, 1h and 2h appeared to contain two components. The responses to these two agonists at 10 min appeared to be primarily due to a component with a high EC_{50} value, while those at 5h were best described by a single component with much higher agonist potency (Table).In contrast, gene transcription responses to salmeterol and CGP 12177 both appeared to contain only a single component, although the potency of these responses increased slightly over time (Table). ^{3}H-CGP 12177 binding to intact cells yielded a $t_{1/2}$ of 6.3 ± 0.7min for the observed on rate and 65.4 ± 9.4 min for the rate of dissociation of this ligand.

	5 hour	1hour			10 min	N
	-log EC_{50}	-log EC_{50}1	-log EC_{50}2	% site 1	-log EC_{50}	
isopren	8.4 ± 0.2	8.5 ± 0.3	6.1 ± 0.3	57.7 ± 4.4	6.0 ± 0.1	5
salbut	8.5 ± 0.1	8.4 ± 0.2	5.7 ± 0.2	45.4 ± 6.8	6.3 ± 0.4	3
salmet	10.7 ± 0.2	10.5 ± 0.2 (single site)			10.1± 0.1	3
CGP	9.7 ± 0.1	9.4 ± 0.1 (single site)			8.9 ± 0.1	6

Table. EC_{50} values for agonist stimulated gene transcription at different time points. Isoprenaline (isopren); salbutamol (salbut); salmeterol (salmet); CGP 12177 (CGP).

Salmeterol is known to have a long duration of action because of its ability to bind to an exocite in TM IV of the β2-adrenoceptor (Green *et al* 1996). In contrast to the two component response of the short-acting agonists isoprenaline and salbutamol, the single component response observed for CGP 12177 is likely to be due to the prolonged off rate of this compound. The reason for the two components in the responses to isoprenaline and salbutamol (which may represent signalling via monomer and dimers, different G-proteins or intracellular cascades) remains to be determined.

JGB holds a Wellcome Trust Clinical Training Fellowship.

Baker JG, *et al* (2001) Bristol BPS Meeting
Green SA, *et al* (1996) J Biol. Chem. 271, 24029-24035
Hill SJ, *et al* (2001) Curr. Op. Pharmacol. 1, 526-532
McDonnell J, *et al* (1998) Br. J. Pharmacol. 125, 717-726

183P THE PUTATIVE BETA 4-ADRENERGIC RECEPTOR IS A NOVEL STATE OF THE BETA 1-ADRENERGIC RECEPTOR

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In human cardiac tissue, there is evidence from functional, second messenger and radioligand binding studies of a novel Gs-coupled third cardiostimulatory receptor: the putative beta 4-adrenoceptor (β4AR). β4AR effects are defined by the non-conventional partial agonist CGP 12177 (CGP) and include positive inotropy, lusitropy and chronotropy in heart. Recent evidence from recombinant β-AR subtypes and β-AR knock-out mice suggests that the β4AR may be a novel **state** of the β1AR protein (Granneman, 2001). We have examined the effect of β1AR overexpression in adult rat cardiomyocytes on the inotropic responses of isoprenaline (ISO) and CGP.

Rat ventricular cardiomyocytes were prepared using methods previously described and transfected with adenovirus containing sequence for the human β1AR (Davia *et al.*, 1999). β1AR density was measured by [^{125}I]-iodocyanopindolol binding to ventricular myocyte membranes. Inotropic responses to ISO and CGP (in the presence of **1μM** propranolol) were studied 48 hours after transfection by measuring cell shortening in electrically stimulated ventricular myocytes. The effect of **3μM** bupranolol on CGP inotropic responses was also studied.

Binding confirmed an 18-fold increase in ventricular β1AR density. There was a parallel left shift of the concentration-response curve (CRC) to ISO (control pD_2 7.72 (SD 0.66, n=21), β1AR transfected 8.76 (SD 0.65, n=20), p<0.0005) (Figure 1). There was also a left shift of CRC to CGP (pD_2 6.23 (SD 0.47, n=16) and 7.16 (SD 0.6, n=22) respectively, p<0.005) as well as an increase in maximum response. Bupranolol antagonised the inotropic effect of CGP in control (Basal 4.17% shortening (SD 1.9%), 1μM CGP 7.18% (SD 2.2%), 1μM CGP + 1μM bupranolol 4.52% (SD 2.3%) (n=4), p<0.02) and in β1AR transfected myocytes (4.79% (SD 1.9%), 9.71% (SD 3.49%) and 6.18% (SD 2.35) respectively (n=6), p<0.01).

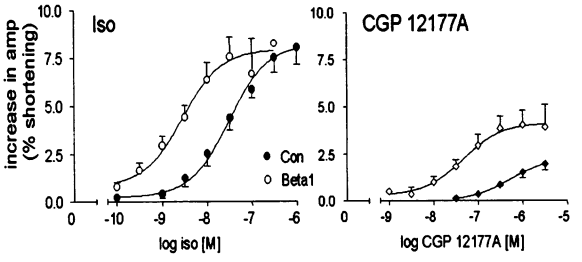

Figure 1. CRCs to ISO and CGP in control and β1AR transfected myocytes

The similar magnitude of the decreases in EC_{50} to ISO and CGP following β1AR overexpression further supports the hypothesis that the β4AR is a novel **state** of the β1AR.

Davia K, Hajjar RJ, Terracciano CM *et al.* Physiol Genomics. 1999 Aug;1(2):41-50.

Granneman JG. Am J Physiol Endocrinol Metab. 2001 Feb; 280(2):E199-202.

184P TESTING THE EXTENDED TERNARY COMPLEX MODEL USING THE DOPAMINE D_{2S} RECEPTOR AS A TEST SYSTEM

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The dopamine D_{2S} receptor belongs to the superfamily of G protein coupled receptors (GPCRs). The interaction between ligand, receptor and G protein may be described by the extended ternary complex model (Samama *et al.*, 1993). The aim of this study was to test the extended ternary complex model, using the dopamine D_{2S} receptor as a test system.

The expression levels of receptor and G protein in the D_{2S} receptor membrane preparation (CHO-D_{2S} membranes purchased from NEN Receptor Biology) were determined. The results obtained experimentally for the binding of the full agonist, dopamine, were then compared with those predicted using an Excel model of the extended ternary complex model.

The binding of dopamine at the D_{2S} receptor was studied by competition against 2 nM [^{3}H]methylspiperone (10 µg membrane, in a total volume of 200 µl, 2 hr incubation, room temperature). The incubation was terminated by filtration of membranes onto GF/C filter plates, using a Brandel harvester. After centrifugation (377 g, 10min) the plate was read on TopCount.

The receptor expression levels were determined using saturation [^{3}H]methylspiperone binding (0.1 nM to 50 nM), by conversion of maximal specific counts into maximal specific [^{3}H]methylspiperone binding using a standard curve for [^{3}H]methylspiperone.

The level of G protein activated by maximal stimulation with dopamine (100 µM), was quantified by [^{35}S]GTPγS saturation analysis (as described for [^{3}H]methylspiperone binding, in the presence of 6 pM to 5 nM [^{35}S]GTPγS, 5 µM GDP, with either vehicle alone or 100 µM dopamine). The maximum dopamine specific counts were converted into maximal specific [^{35}S]GTPγS binding using a standard curve for [^{35}S]GTPγS.

The [^{3}H]methylspiperone displacement curve for dopamine was best described by a two site binding curve (F test, $p<0.05$ vs. one site binding curve, GraphPad Prism 3.0), with $pK_H = 6.24 \pm 0.41$ and $pK_L = 4.39 \pm 0.09$, $n = 3$.

The receptor expression level was found to be 6.18 ± 2.60 pmol/mg, n = 3. The expression level of G protein, activated by maximal stimulation with dopamine, was found to be 6.22 ± 3.11 pmol/mg, n = 3. The receptor : G protein stoichiometry in the CHO-D_{2S} membrane preparation was thus found to be 1 : 1. Allowing for the error on the expression level determinations, the stoichiometry could be between 2.8 : 1 and 1 : 2.6.

Using an Excel model of the extended ternary complex model it was shown that biphasic binding data would be predicted for an agonist when the levels of G protein are just limiting with respect to the receptor. Within the range of receptor to G protein stoichiometries determined for this dopamine D_{2S} membrane preparation, the biphasic displacement curves obtained experimentally for dopamine would therefore be predicted by the extended ternary complex model.

In conclusion, the results of this study support the extended ternary complex model.

Samama P. *et al.* (1993) *J. Biol. Chem.*, **268**: 4625 - 4636

185P THERMODYNAMIC ANALYSIS OF LIGANDS AT HUMAN CCK_{2S} RECEPTORS EXPRESSED IN NIH3T3 CELLS

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The pioneering study of Weiland *et al.* (1979) was the first to demonstrate a relationship between the pharmacological properties (agonist/antagonist) of ligands and their thermodynamic binding parameters (enthalpy [H] and entropy [S]). This phenomenon has since been confirmed for most but not all receptors and ion channels on which these studies have been conducted (eg 5-HT_{1A}; Dalpiaz *et al.*, 1996). We have had a long interest in trying to use radioligand binding assays to predict intrinsic activity at human receptors. We therefore investigated whether H_3 receptor agonists (1=proxyfan; 2=chloroproxyfan; 3=bromoproxyfan; 4=iodoproxyfan; 5=R-α-methylhistamine; 6=imetit; 7=immepip) and antagonists (8=thioperamide; 9 = JB96132; 10=clobenpropit), as defined by a 'functional' bioassay of the guinea-pig ileum, can be discriminated using receptor binding thermodynamics.

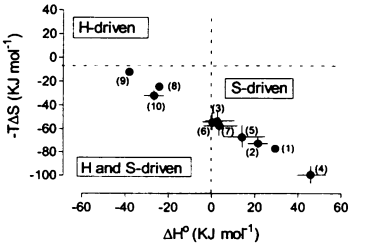
Guinea-pig cortex membranes were prepared as described previously (Harper *et al.*, 1999) and resuspended in 20mM Hepes-NaOH buffer (pH 7.4 containing 3mM metyrapone). For saturation (S) and competition (C) assays, the membranes (1.6mg/ tube) were incubated (2.75h; 21,30°C; 24h; 4, 12°C) with [^{3}H]-10 as the radioligand (S, 0.04-30nM; C, 0.2nM). Non-specific binding was defined with 8 (1µM).

At all temperatures, at the membrane concentration used for (C) and kinetic (K) assays (1.6mg/tube) ~10% of added [^{3}H]-10 was bound. There was a linear relationship between specific binding of [^{3}H]-10 and membrane concentration up to 10mg ml^{-1} (4mg/tube). K assays indicated that the specific binding of [^{3}H]-10 reached equilibrium after ~3, 25, 30 and 80min at 30, 21, 12 and 4°C, respectively. The binding was fully dissociated by 8 (1µM) and the $t_{1/2}$ values (4°C~80min; 12°oC~20min; 21°C~14min; 30°C~3min) indicated that the incubation time for the C assays was sufficient for equilibrium to have been reached. S assays demonstrated that the affinity (pK_D) of [^{3}H]-10 increased with decreasing temperature (Table; multivariate analysis of variance MANOVA, $p<0.001$) and that Hill slope parameter (n_H) and B_{max} (B) values were not temperature-dependent. Data from C assays indicated that there was a significant relationship between assay temperature and affinity values (K_A or $1/K_I$) for ligands 1,2,4,5,8,9 and [^{3}H]-10 (MANOVA, $p<0.05$). Van't Hoff plots were plotted for each ligand ($(\ln 1/K_I)$ v $1/T$). Linear regression of the data and the use of the integrated van't Hoff equation ($\ln K_A = -\Delta H°/RT + \Delta S°/R$; R=8.31 $JKmol^{-1}$; T=298K) allowed the ΔH° and ΔS° of each ligand to be calculated (slope=-ΔH°/R; y-intercept =+ΔS°/R). The slopes of the van't Hoff plots were –ve for 1,2,4 and 5 and +ve for 8,9 and [^{3}H]-10. An extrathermodynamic plot (Figure) indicated that the underlying molecular mechanism of binding differed between the antagonists (H and S-driven=8,9,10) and agonists (S-driven= 1,2,3,4,5,6,7).

Table pK_D, n_H, B and $t_{1/2}$ values for [^{3}H]-10 (n=4± s.e.mean)

Temp. (°C)	pK_D	B (fmol mg^{-1})	n_H
4	10.73 ± 0.14	3.78 ± 0.86	0.98 ± 0.02
12	10.54 ± 0.06	3.84 ± 0.45	0.94 ± 0.02
21	10.44 ± 0.05	3.73 ± 0.62	1.01 ± 0.03
30	10.30 ± 0.05	4.06 ± 0.60	0.97 ± 0.02

Figure An extrathermodynamic plot for 1,2,3,4,5,6,7,8 9 and [^{3}H]-10



The data obtained in this study suggest that like 5-HT_3 (Borea et al., 1996) and adenosine A_1 (Borea et al., 1992) receptors, H_3 receptor agonists and antagonists can be thermodynamically distinguished.

Borea PA, Varani K, Guerra L et al. (1992) Mol Neuropharmacol, 2, 273-281.
Borea PA, Dalpiaz A, Gessi S *et al.* (1996) Eur J Pharmacol, 298, 329-334
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Harper EA, Shankley NP & Black JW (1999) Br J Pharmacol, 128, 881-890.
Weiland GA, Minneman KP & Molinoff PB (1979) Nature, 281, 114-117.

186P THERMODYNAMIC ANALYSIS OF LIGANDS AT HUMAN CCK_{2S} RECEPTORS EXPRESSED IN NIH3T3 CELLS

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Several studies have shown a relationship between the pharmacological properties (agonist/antagonist) of ligands and their thermodynamic binding parameters (enthalpy (H) and entropy (S)) (eg. Borea *et al.*, 1996). In this study, we aimed to determine if thermodynamic analysis could be used to discriminate between selected CCK_2 receptor ligands, with different intrinsic activity (α) measured in the lumen-perfused mouse stomach assay (Roberts *et al.*, 1996, Shankley *et al.*, 1997; α=1.0, 1=CCK-8S, 2=pentagastrin; α=0.5, 3=PD134,308, 4=compound 4, Kalindjian *et al.*, 2001; α=0, 5=*R*-L-365,250, 6=*S*-L-365,260, 7=YM022, 8=JB95008, 9=[^{3}H]-JB93182).

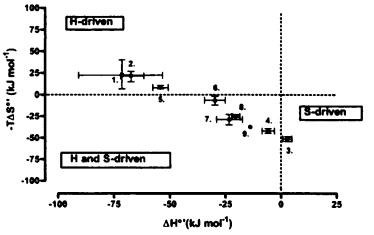
Membranes from NIH3T3 cells expressing the human CCK_{2S} receptor were prepared as described previously (Harper *et al.*, 2000). For saturation, kinetic and competition assays, the membranes were homogenised in buffer at the appropriate temperature (37, 30, 21, 12 or 4°C) to give a final concentration equivalent to $7.5x10^5$ cells ml^{-1}. For competition assays, the membranes (400µl) were incubated with 9 (50µl;10nM) for 2.5 (37, 30, 21°C) or 24h (12, 4°C).

At all temperatures, there was a linear relationship between specific binding of 9 and membrane concentration ($1.75x10^4$–$2.5x10^6$ cells ml^{-1}). At a membrane concentration of $7.5x10^5$ cells ml^{-1}, <10% of the added 9 was bound and specific binding was >50%. Kinetic experiments indicated that at all temperatures, the specific binding of 9 reached equilibrium within 5min and that the binding was reversible ($t_{1/2}$ values<15min, table). Saturation studies indicated that the affinity (pK_D) of 9 increased with decreasing temperature (Table; multivariate analysis of variance MANOVA, $p<0.05$) and that Hill slope parameter (n_H) and B_{max} (B) values showed no significant temperature-dependence. There was a significant relationship between assay temperature and affinity values (K_A or $1/K_I$), obtained from competition experiments for 1, 2, 5, 6, 7 and 8 (MANOVA, $p<0.001$). Van't Hoff plots were plotted for each ligand ($\ln K_A$) v 1/T). Use of the integrated van't Hoff equation ($\ln K_A = -\Delta H°/RT + \Delta S°/R$; R=8.31 JKmol^{-1}; T=298K) and linear regression of the data allowed the $\Delta H°$ and $\Delta S°$ of each ligand to be calculated (slope=$-\Delta H°/R$; y-intercept=$+\Delta S°/R$). An extrathermodynamic plot (Figure) indicated that the underlying molecular mechanism of binding differed between the ligands (H-driven =1, 2, 5; H and S-driven =6, 7, 8, 9; S-driven =3, 4). The agonists (1, 2 and 3, 4) were clustered at the two extremes of the extrathermodynamic plot and therefore, there appeared to be no relationship between mouse stomach α values and the underlying mechanism of ligand binding.

Table pK_D, n_H, B (fmol 3 x 10^5 cells and $t_{1/2}$ values for 9 (*p<0.05, t-test) (n=3± s.e.mean)

Temp. (°C)	pK_D	B	n_H	$t_{1/2}$ (min)
4	9.27 ± 0.08	44.66 ± 3.00	1.00 ± 0.01	12.47 ± 1.90
12	9.19 ± 0.10	42.76 ± 3.89	*0.90 ± 0.01	5.64 ± 0.22
21	9.12 ± 0.10	55.19 ± 5.19	0.95 ± 0.06	1.68 ± 0.35
30	9.00 ± 0.10	54.97 ± 6.76	1.01 ± 0.09	0.78 ± 0.17
37	9.02 ± 0.08	38.38 ± 7.53	1.03 ± 0.04	0.58 ± 0.03

Figure An extrathermodynamic plot for 1,2,3,4,5,6,7,8 and 9



It is possible that unlike, for instance, the adenosine A_1 and 5-HT_3 receptors, thermodynamic analysis cannot be used to discriminate agonists and antagonists at the CCK_{2S} receptor. However, it has been shown that receptor speciation can affect agonist potency order at the CCK_2 receptor (eg Beinborn *et al.*, 1998). Thus it is possible that the ligand α values for the mouse CCK_2 receptor are not the same as for the human CCK_{2S} receptor. To investigate this possibility, the measurement of ligand α values in the NIH3T3 cell system is in progress.

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Kalindjian, S.B., Dunstone, D.J., Low, C.M.R. *et al.* (2001) J. Med. Chem., **44**, 1125-1133.

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Shankley, N.P., Roberts, S.P., Watt, G.F. *et al.* (1997) *ASPET.*, **39**, 65.

187P G-PROTEIN ACTIVATION BY h5-HT_{1B} RECEPTORS EXPRESSED IN CHO CELLS: SCINTILLATION PROXIMITY ASSAYS REVEAL CONSTITUTIVE $G\alpha_{i3}$ ACTIVATION.

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Serotonin 5-HT_{1B} receptors couple to G-proteins of the Gi/o family. However, few studies have investigated the activation profiles of the G-protein subtypes involved. We now report the characterisation, using an innovative immuno-detection method, of $G\alpha_{i3}$ G-protein subunit activation by h5-HT_{1B} receptors stably expressed in Chinese hamster ovary cells.

Procedures were as described by De Lapp *et al.* (1999). CHO-h5-HT_{1B} cell membranes were incubated with agonists and/or antagonists and [^{35}S]GTPγS (0.2 nM) for 1h at 22°C. Reaction was stopped by addition of detergent (NP40 0.3% v/v final) and anti-$G\alpha_{i1/3}$ monoclonal antibodies were added (0.1µg of IgG per well). Because CHO cells do not express $G\alpha_{i1}$ (Gettys *et al.*, 1993), the assay detects essentially activation of $G\alpha_{i3}$. After incubating for 1h, antibody/$G\alpha_{i3}$ complexes were captured using scintillation proximity assay (SPA) beads coated with anti-mouse 2nd antibody. Radioactivity was counted following overnight incubation. Non-specific binding was defined with 10µM GTPγS. Results are expressed as mean ± s.e.m. of (n) determinations performed in duplicate.

Table 1: Stimulation of $G\alpha_{i3}$ at h5-HT_{1B} receptors.

Ligand	pEC_{50}	E_{max} (%) [b]	n
5-HT	8.97 ± 0.20	181 ± 16	4
Alniditan	8.84 ± 0.23	171 ± 10	5
BMS181,101	8.67 ± 0.33	188 ± 2	3
S18127	7.17 ± 0.55	124 ± 4	3
Methiothepin	8.04 ± 0.13 [a]	58 ± 8	3
SB224,289	7.68 ± 0.04 [a]	56 ± 1	3

[a] pIC_{50} for inverse agonists. [b] Basal binding = 100%.

The agonists, 5-HT, alniditan and BMS181,101, stimulated [^{35}S]GTPγS binding, whereas methiothepin and SB224,289 behaved as inverse agonists (Table 1). The selective 5-HT_{1B} receptor ligand, S18127, only modestly stimulated $G\alpha_{i3}$ and reversed both 5-HT (10nM)–stimulated and methiothepin (100nM)-inhibited [^{35}S]GTPγS binding [pK_b values: 7.87 ± 0.35 (4) and 6.67 ± 0.11 (3), respectively]. S18127 (1µM) also produced parallel dextral shifts of the 5-HT and methiothepin isotherms [pK_b values: 7.98 ± 0.15 (4) and 6.67 ± 0.18 (3), respectively]. pEC_{50}, pIC_{50} and pK_b values determined here generally resembled those derived by classical [^{35}S]GTPγS binding methods (Audinot *et al.*, 2001; Newman-Tancredi *et al.*, 2000), which do not distinguish between G-protein subtypes.

In conclusion, the present data indicate that h5-HT_{1B} receptors mediate agonist-induced activation of $G\alpha_{i3}$ proteins in CHO cells. Further, the inhibitory actions of inverse agonists, and their reversal by the selective ligand, S18127, reveals constitutive activation of $G\alpha_{i3}$ by h5-HT_{1B} receptors.

Audinot A., Newman-Tancredi A., Cussac D., *et al.* (2001) *Neuropsychopharmacology* **25,** 410-422.

De Lapp N., McKinzie J.H., Sawyer B.D. *et al.* (1999) *J. Pharmacol. Expt. Ther.* **289**, 946-955.

Gettys T.W., Fields T.A. & Raymond J.R. (1993) *Anal. Biochem.* **220**, 82-91.

Newman-Tancredi A., Audinot V., Moreira C. *et al.* (2000) *Mol. Pharmacol.* **58**, 1042-1049.

188P STIMULATION OF G-PROTEIN $G\alpha_{i3}$ SUBUNITS BY h5-HT_{1A} RECEPTORS EXPRESSED IN CHO CELLS: AGONISTS DISPLAY BELL-SHAPED CONCENTRATION-RESPONSE CURVES

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Serotonin 5-HT_{1A} receptors couple to multiple G-protein subtypes of the Gi/o family. However, few studies have investigated their respective activation patterns. Here, using an innovative immuno-detection method, we characterise the activation of $G\alpha_{i3}$ G-protein subunits by h5-HT_{1A} receptors stably expressed in Chinese hamster ovary (CHO) cells.

Procedures were as described by De Lapp *et al.* (1999) and Newman-Tancredi *et al.* (this meeting). CHO-h5-HT_{1A} cell membranes were incubated with agonists and/or antagonists and [^{35}S]GTPγS (0.2 nM) for 1h at 22°C. Reaction was stopped by addition of detergent (NP40 0.3 % v/v final) and anti-$G\alpha_{i1/3}$ monoclonal antibodies were added (0.1µg of IgG per point). Because CHO cells do not express $G\alpha_{i1}$ (Raymond *et al.*, 1993), the assay detects activation of $G\alpha_{i3}$. After 1h incubation, scintillation proximity assay (SPA) beads, coated with anti-mouse 2nd antibody, were added and incubated with gentle agitation overnight before radioactivity counting.

Table 1: Stimulation of $G\alpha_{i3}$ at h5-HT_{1A} receptors.

Ligand	-Log [drug] at peak (M)	E_{max} (%) [b]	n
5-HT	8.35 ± 0.13	245 ± 21	5
(+)8-OH-DPAT	8.46 ± 0.08	283 ± 15	4
S14506	8.54 ± 0.08	226 ± 20	5
Buspirone	7.32 ± 0.03	234 ± 2	3
Methiothepin	7.84 ± 0.18 [a]	80 ± 2	3
Spiperone	7.27 ± 0.03 [a]	27 ± 4	3

[a] pIC_{50} for inverse agonists. [b] Basal binding = 100%.

Non-specific binding was defined with 10µM GTPγS. Results are expressed as the mean ± s.e.m. of (n) determinations.

The agonists, 5-HT, (+)8-OH-DPAT, S14506 and buspirone, yielded bell-shaped [^{35}S]GTPγS binding isotherms, with peak stimulation at drug concentrations shown in Table 1. Isotherms gradually returned to basal values upon increasing drug concentrations to 1µM. In contrast, the inverse agonists, spiperone and methiothepin, sigmoidally inhibited [^{35}S]GTPγS binding, demonstrating constitutive activation of $G\alpha_{i3}$. The 5-HT_{1A} receptor antagonist, WAY100,635 (3 nM), shifted the spiperone isotherm and the bell-shaped curves of 5-HT and (+)8-OH-DPAT in parallel to the right with pK_b values [9.58 ± 0.27 (3), 9.78 ± 0.18 (3) and 9.78 ± 0.09 (3), respectively] which resembled its pK_i (9.91) at h5-HT_{1A} receptors (Newman-Tancredi *et al.*, 2001). WAY100,635 itself modestly stimulated [^{35}S]GTPγS binding with sigmoidal concentration-response curves [E_{max} = 135 ± 5 %, pEC_{50} = 8.52 ± 0.06 (3)], indicating mild agonist properties for activation of $G\alpha_{i3}$.

The present data show that h5-HT_{1A} receptors activate $G\alpha_{i3}$ subunits in CHO cells. However, as the concentration of high-efficacy agonists increases, stimulation of this G-protein is progressively attenuated. This paradoxical activation pattern may reflect changes in receptor conformation and/or agonist-directed trafficking of receptor signalling.

De Lapp N., McKinzie J.H., Sawyer B.D. *et al.* (1999) *J. Pharmacol. Expt. Ther.* **289**, 946-955.

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Raymond J.R., Olsen C.L. & Gettys T.W. (1993) *Biochem.* **32**, 11064-11073.

189P DEXAMETHASONE INDUCES SERINE PHOSPHORYLATION OF ANNEXIN 1: INVOLVEMENT OF PKC AND PI3-KINASE

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Annexin 1 (ANXA1) belongs to a family of Ca^{2+} and phospholipid binding proteins. It is an important component of the signalling mechanisms used by glucocorticoids (GCs), particularly in the neuroendocrine and host defence systems where it appears to act as a paracrine agent following GC-induced exportation from specific cells. The mechanism by which GCs export ANXA1 from cells is unclear but our recent studies on rat pituitary tissue, where ANXA1 is expressed predominantly in the non-secretory folliculostellate (FS) cells, suggest a role for protein kinase C (PKC) dependent phosphorylation (John et al, 2001). In order to investigate this further, we are examining the effects of dexamethasone ± kinase inhibitors on the expression, cellular disposition and phosphorylation status of ANXA1 in a human FS cell line (PDFS) which expresses ANXA1 in abundance. ANXA1 in the cytoplasm (ANXA1) and bound to the cell surface (ANXA1) was detected by western blot and fluorescent activated cell (FAC) analyses, using antisera which distinguish between Ser-27-phosphorylated (Ser-27P) and non-phosphorylated species of the protein. The pattern of results obtained with the two methods was similar. Here we show data from FAC analysis (mean fluorescence intensity) expressed as a percentage of the corresponding control (100%), corrected for non-specific fluorescence.

Dexamethasone (1 µM) induced (a) a prompt but transient increase in cytoplasmic Ser-27P-ANXA1 (150.2 ± 0.5% of control, t=30min., P<0.001, n=6), (b) translocation of Ser-P-ANXA1 to the cell surface (cell surface ANXA1 = 300.6 ± 2.7%, t=30min., P<0.001, n = 6) and (c) subsequent *de novo* ANXA1 synthesis (total cellular ANXA1 = 190.1 ± 0.9%, t=2h, P<0.01, n=3) to replenish the depleted stores of the protein.

The dexamethasone-induced phosphorylation and exportation of ANXA1 were inhibited by the GC receptor (GR) antagonist, mifepristone (1µM, Ser-27P-ANXA1 = 60.5 ± 1.2 % of control, P<0.05, n=3), but not by the translation inhibitor, cycloheximide (5µM, P<0.05, n=3). The phosphorylation and exportation of ANXA1 was also blocked by the non-selective PKC inhibitor (peptide 19-31, 5 µM, 61.2 ± 2.1 %, P<0.001, n=6), the PI3 kinase inhibitor (wortmannin, 5nM, 58.1 ± 1.5%, P<0.05, n=3) and the Ca^{2+} chelator BAPTA (3 µM, 68.2 ± 1.1%, P<0.05, n=3). By contrast, inhibition of PKCδ (rottlerin 10µM), MAPK (PD9805, 20µM) or MEK kinase (UO126, 100nM) did not affect the capacity of dexamethasone to induce serine phosphorylation and exportation of ANXA1 while pre-treatment of the cells with verapamil (50µM) potentiated the response to the steroid (183.2 ± 1.1 %, P< 0.001, n= 3).

The results suggest that dexamethasone acts via GR to induce Ser-27 phosphorylation of ANXA1 and that its actions are independent of protein synthesis but dependent on PKC, PI3 kinase and Ca^{2+}.

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We are grateful to the Wellcome Trust and MRC for generous support.

190P LPS ACTS VIA IL-6 AND IL-1 TO MODULATE THE EXPRESSION AND PHOSPHORYLATION OF ANNEXIN 1 IN CELL LINES DERIVED FROM THE PUTATIVE MICROGLIA OF THE NEUROENDOCRINE SYSTEM.

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Annexin 1 (ANXA1), a Ca^{2+} and phospholipid binding protein, is strongly implicated in the interplay between the immune and neuroendocrine systems. Within the neuroendocrine system ANXA1 is expressed in particular abundance by the non-secretory pituitary folliculo-stellate (FS) cells (Traverso *et al.* 1999), which show some of characteristics of resident microglia in the brain and may play a role in neuroendocrine-immune communication.

We have previously reported that IL-6 up-regulates ANXA1 expression in the liver *in vivo* and in A549 cells (lung epithelial) *in vitro* through the trans-activation of c/EBP β (Solito *et al.* 1998; deCoupade *et al.* 2001) and suggested that ANXA1 may serve as as new acute phase protein. We have also shown that endotoxin (LPS) increases ANXA1 expression in the rat anterior pituitary gland and hypothalamus (Buckingham, 1996). In the present study we used RT-PCR, western blot analysis and fluorescence activated cell (FACs) analysis to examine the effects of LPS on ANXA1 expression and phosphorylation status in a FS cell line (murine TtT/GF). As the profiles of data obtained with western blot and FACs analyses were similar we show results from the latter only. All data are expressed as a percentage of the corresponding control (n=3). RT-PCR and FACs analysis showed that TtT/GF cells express CD14 and the Toll receptors 2 and 4 (Tlrs, a family of receptors, which recognise pathogens).

LPS (100ng/ml) induced *de novo* ANXA1 expression (mRNA =140.3 ± 1.4%, P<0.005; protein = 137.5±1.5%, P<0.005) and caused ser-phosphorylation of the protein (115 ± 0.4, P<0.003) which was maximal at 2h. Inclusion of a specific antibody directed against IL-6 suppressed the LPS-induced up-regulation of *de novo* ANXA1 synthesis (70.14 ± 6.5%, P<0.001) but did not affect the capacity of LPS to induce serine phosphorylation of ANXA1. In contrast, a specific antibody directed against interleukin 1 (IL-1) inhibited the serine phosphorylation of the of ANXA1 (34.3 ± 8.6%, P<0.001) induced by LPS without affecting the increase in *de novo* ANXA1 synthesis. Interestingly, a similar inhibition of serine phosphorylation was achieved with an antibody directed against the toll receptor 4 (43.4 ± 2.0%, P<0.005) and by the PI3-kinase inhibitor wortmannin (250nM, 78.6± 0.8%, P<0.001).

In conclusion, the results suggest that LPS induces *de novo* ANXA1 expression and ser-phosphorylation of the protein by two distinct mechanisms. The former is dependent upon IL-6 while the latter is mediated Tlr4 or possibly the IL-1 receptor (which shares a high C-terminal homology with Tlr4) and PI3-kinase. These findings thus support a role for ANXA1 as a mediator of immune-neuroendocrine communication.

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This work was supported by Wellcome Trust.

191P A DIRECT INHIBITORY ACTION OF PROSTAGLANDINS UPON ACTH SECRETION AT THE LATE STAGES OF THE SECRETORY PATHWAY OF AtT-20 CELLS

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The mouse AtT-20/D16-16 anterior pituitary tumour cell line is used as a model system for the study of the late stages of the adrenocorticotrophin (ACTH) secretory pathway (Guild, 1991). A recent study has indicated an inhibitory action for cyclo-oxygenase products upon these late-stages of the secretory pathway in AtT-20 cells (Guild, 2001). This study, therefore, investigated the effects of prostaglandins upon ACTH secretion in secretory studies using both intact and electrically-permeabilised cells as previously described (Guild, 2001).

In permeabilised cells basal ACTH secretion was 73 $\pm$6 pg /10^5 cells/30 min. Calcium (10^{-9} -10^{-4} M), GTP-γ-S (10^{-6} -10^{-4} M) and mastoparan (10^{-6} M and 10^{-5} M) all stimulated ACTH secretion from permeabilised AtT-20 cells in a concentration-dependent manner to a maximum of 410$\pm$35 pg /10^5 cells/30 min for calcium (10^{-5}M), 255 $\pm$ 25 pg /10^5 cells/30 min for GTP-γ-S (10^{-4}M) and 305 $\pm$ 31 pg /10^5 cells/30 min for mastoparan (10^{-5}M). Co-incubation with prostaglandins E_1 and E_2 (10^{-5} M) but not $PGF_{2\alpha}$ significantly inhibited calcium (10^{-5}M)-evoked ACTH secretion by 50% to 205 $\pm$ 14 pg /10^5 cells/30 min and GTP-γ-S (10^{-4}M)-evoked secretion by 50% to 125 $\pm$ 11pg /10^5 cells/30 min but mastoparan (10^{-5}M)-evoked secretion by only 30% to 198 $\pm$ 13 pg /10^5 cells/30 min.

The effects of prostaglandins E_1 and E_2 upon the calcium-independent agents GTP-γ-S (10^{-4} M)- and mastoparan-evoked secretion were concentration-dependent. PGE_1 significantly inhibited GTP-γ-S (10^{-4} M)-evoked secretion at concentrations of 10^{-6} M and above but only inhibited mastoparan (10^{-5} M)-evoked secretion at the highest concentration of PGE_1 investigated.i.e.10^{-5} M. PGE_2 however, was more potent than PGE_1 and significantly inhibited GTP-γ-S (10^{-4} M)-evoked secretion at 10^{-8} M and above and inhibited mastoparan (10^{-5} M)-evoked secretion at concentrations above 10^{-6}M. Mastoparan-evoked secretion thus proved less sensitive to inhibition by prostaglandins. The inhibitory effects of PGE_1 and PGE_2 upon calcium-, GTP-γ-S- and mastoparan-stimulated ACTH secretion from permeabilised cells were pertussis toxin (PTX) sensitive and were not observed in cells treated with 1μg ml^{-1} PTX for 16 hr as previously described (Erlich et al.,1998).

In intact cells basal secretion of ACTH was 510 + 45 pg/well/3h which was largely unaffected by co-incubation with PGE_1, PGE_2 and $PGF_{2\alpha}$ (10^{-9}-10^{-5} M). However, PGE_2 (10^{-9} - 10^{-5} M) but not PGE_1 or $PGF_{2\alpha}$ significantly inhibited CRF-41 (10^{-7}M)-evoked secretion in a concentration-dependent manner from 1582 $\pm$ 104 pg/well/3h in the absence of PGE_2 to 1312 $\pm$ 68 pg/well/3h in the presence of PGE_2 (10^{-5}M).

The present study finds that prostaglandins of the E series exert an inhibitory action, via a pertussis toxin-sensitive G-protein, at the late stages of the ACTH secretory pathway distal to the Ge/calcium (Erlich et al., 1998) point of control.

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192P REGULATION OF C-JUN N-TERMINAL KINASE AND P38 IN HUMAN VASCULAR SMOOTH MUSCLE CELLS BY G-PROTEIN COUPLED P2Y RECEPTORS AND PDGF RECEPTORS

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Regulation of intimal smooth muscle proliferation is a key event leading to the development of vascular diseases such as atherosclerosis and restenosis following angioplasty. We have previously shown that PDGF acts as a mitogen in saphenous vein (SV) explants (White *et al* 2000), also shown by other groups (Yang *et al* 1998). ATP and UTP has been shown to be mitogenic in rat vascular smooth muscle cells (VSMCs) (Harper *et al*, 1998). However, we have reported a novel UTP anti-proliferative effect in cultured human VSMCs (White *et al* 2000). This study investigates the role of two mitogen activated protein kinases (MAPK), c-jun N-terminal kinase (JNK) and p38, in the nucleotide signalling network leading to control of proliferation in human VSMCs.

Primary cultures (p3-p7) of human SV VSMCs were grown in 6 well plates and serum starved at 80% confluence for 48 hours. Cells were stimulated for 30 min at 37°C with agonists following 30 min pre-incubation with inhibitors where indicated. Samples were prepared for western blot in ice-cold lysis buffer and separated by electrophoresis. Blots were probed with anti-phospho specific JNK or p38 antibodies. Data from blots was collected by directly imaging chemi-illuminesence using ECL reagent. One way ANOVA was used to statistically analyse data.

Anisomycin was used as a positive control for the activation of JNK and p38. JNK phosphorylation was increased in a dose dependant manner by anisomycin (EC_{50}=5.18±2.55ng/ml, n=3, P<0.05). PDGF also increased JNK phosphorylation in a dose dependant manner (EC_{50}=326.7±5.9pM, n=4, P<0.01). 1nM PDGF significantly increased the phosphorylation of JNK (1412±512% of basal P<0.001 n=4). UTP alone had no significant influence on JNK phosphorylation. However, in the presence of PDGF, 300μM UTP was able to significantly attenuate JNK activity induced by PDGF (42.1±12.7% of the PDGF maximal response, P<0.05, n=4). ATP did not influence JNK in the presence or absence of PDGF. Phosphorylation of p38 was not activated by PDGF despite positive activation by anisomycin. ATP and UTP gave small but inconsistent increases in phosphorylation of p38.

PD98059 and U0126 are inhibitors of extracellular receptor kinase 1/2 (ERK1/2), which is another MAPK. Cells were pre-incubated with 30μM PD98059 or 10μM U0126 prior to stimulation. The JNK phosphorylation induced by PDGF was significantly reduced by PD98059 and U0126 to 37.4±18.1% (P<0.05) and 14.5±1.2% of the PDGF maximal response (P<0.01) respectively. The decrease of the PDGF response by these inhibitors was not attenuated any further by UTP.

These results show that JNK activation by PDGF is dependent on ERK1/2 pathway and also implicates the JNK pathway in the anti-proliferative action of UTP in human VSMCs. This also implicates the coupling of P2Y receptors activated by UTP but not ATP to JNK, which may play a role in modulating proliferation. P38 does not appear to be involved in proliferation induced by PDGF.

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193P SPHINGOSYLPHOSPHORYLCHOLINE INHIBITS ACTIVATION OF HUMAN PLATELETS

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Lysosphingolipids such as sphingosine-1-phosphate are stored in human platelets from which they can be released upon activation (Yatomi *et al.* 2000). Since sphingosine-1-phosphate was reported to induce platelet activation (Gueguen *et al.* 1999), we have investigated the effects of the related lysosphingolipid sphingosylphosphorylcholine (SPPC) on human platelet function.

Platelets were isolated from citrate-anticoagulated blood of healthy donors, who did not take any medication known to interfere with platelet function. Platelets were washed with a citrate-buffer and resuspended in a HEPES-buffer at $2x10^8$ platelets ml^{-1}. Fibrinogen (0.5 mg ml^{-1}) was added just prior to experiments. Platelet activation was induced by adenosine diphosphate (ADP), the thrombin receptor activating peptide TRAP-6 (SFLLRN), collagen, and the thromboxane A_2 mimetic U-46619 (9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$) as platelet agonists. SPPC [0.1-20 μM] or vehicle were added 2 minutes prior to stimulation (5 min for flow cytometry). Platelet aggregation response was quantified by turbidimetry. Agonist-induced expression of activation-dependent platelet surface markers P-selectin, glycoprotein (GP) 53, GP Ib, the activated GP IIb/IIIa receptor and fibrinogen binding to the cell surface were used to characterize platelet activation by flow cytometry. Agonist-induced changes of intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) were analysed fluorometrically in Fura-2-AM-loaded washed platelets. Data are means ± SEM of 4-6 experiments.

SPPC alone caused only very small elevations of $[Ca^{2+}]i$ (<25 nM) and neither significantly changed surface receptor expression nor induced platelet aggregation.

Pre-incubation with SPPC concentration-dependently and almost completely inhibited platelet aggregation in response to different agonists [pEC_{50} against 5 μM ADP 5.80±0.25, against 20 μM ADP 5.41±0.29, against 5 μM TRAP-6 5.78± 0.29, against 20 μM TRAP-6 4.77±0.58, against 20 μg ml^{-1} collagen 6.19±0.30, against 50 μg ml^{-1} collagen 5.53±0.31, against 1 μM U-46619 5.27 ± 0.29. Agonist-induced surface expression of P-selectin, GP 53, the activated GP IIb/IIIa receptor, internalisation of GP Ib, and fibrinogen binding were also inhibited by SPPC in a concentration dependent manner with an almost complete inhibition at 20 μM SPPC. ADP (20 μM) and TRAP-6 (5 and 20μM) elevated $[Ca^{2+}]i$ by 239±27, 359±50 and 796±71 nM, respectively, and this was nearly completely inhibited by addition of SPPC with a pEC_{50} of 6.01±0.12, 6.15±0.13 and 5.80±0.14, respectively.

We conclude that exogenous SPPC concentration-dependently inhibits activation and aggregation of washed human platelets in response to different agonists.

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194P STUDIES INTO THE MECHANISM OF INHIBITION OF PLATELET ACTIVATION BY SPHINGOSYLPHOSPHORYLCHOLINE

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We have found that the lysosphingolipid sphingosylphosphorylcholine (SPPC) inhibits several parameters of the activation of human platelets including elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$), expression of activation-associated surface markers and aggregation (Altmann *et al.*, 2001). The present experiments were designed to obtain further insight into the underlying mechanisms.

Platelets were isolated from citrate-anticoagulated blood of healthy donors, who did not take any medication known to interfere with platelet function. Platelets were washed with a citrate-buffer and resuspended in a HEPES-buffer at $2x10^8$ platelets ml^{-1}. Fibrinogen (0.5 mg ml^{-1}) was added just prior to experiments. Platelet activation and aggregation was induced by adenosine diphosphate (ADP), the thrombin receptor activating peptide TRAP-6 (SFLLRN), the Ca^{2+}-ATPase inhibitor thapsigargin, and the ionophore calcimycin (A 23187). SPPC [5-20μM], prostaglandin E_1 (PGE_1) or vehicle were added 2 or 5 minutes prior to stimulation. In some experiments, cells were pre-incubated with of H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesufonamide) for 2 or 5 minutes to inhibit protein kinase A (PKA). Platelet aggregation response was quantified by turbidimetry. Expression of activation-dependent platelet surface marker P-selectin and fibrinogen binding to the cell as well as phosphorylation of the intracellular vasodilator-stimulated phosphoprotein (VASP) were analysed by flow cytometry. Changes of $[Ca^{2+}]i$ were analysed fluorometrically in Fura-2-AM-loaded platelets. Data are means ± SEM of 4 experiments.

SPPC (10 and 20 μM) inhibited 0.5 μM thapsigargin induced $[Ca^{2+}]i$ elevation (1070±152 nM) by 68±1% and 71±4% ($p<0.01$ vs. vehicle in t-test), but did not significantly affect $[Ca^{2+}]i$ increases by 0.5 μM calcimycin (716±94 nM). Nevertheless, 20 μM SPPC inhibited platelet aggregation by both thapsigargin and calcimycin 84±8 % and 57±6 %, respectively ($p<0.01$ each vs. vehicle in t-test).

VASP phosphorylation, an indicator of PKA activation, was induced by SPPC but to a smaller extent than by the known adenylyl cyclase-stimulating agonist PGE_1. PGE_1 (1 μM) also inhibited $[Ca^{2+}]i$ elevation, surface expression of P-selectin and fibrinogen binding and aggregation induced by 10 μM TRAP-6; the PKA inhibitor H89 (20 μM) almost completely antagonised the PGE_1 effects on $[Ca^{2+}]i$ and partly inhibited those on the other parameters. H89 also partly antagonised the inhibition of TRAP-6-induced $[Ca^{2+}]i$ elevation by SPPC (10 μM SPPC 22±5 vs. 56±10% of control; 20 μM SPPC 5±1 vs. 31±7% of control; $p<0.05$ each in t-test). In contrast, H89 did not significantly antagonise the inhibition of TRAP-6 induced P-selectin expression, fibrinogen binding and platelet aggregation.

We conclude that SPPC inhibits $[Ca^{2+}]i$ elevation and $[Ca^{2+}]i$ elevation-associated aggregation in human platelets. While the inhibition of $[Ca^{2+}]i$ elevation may involve PKA activation, this cannot fully explain the inhibition of platelet activation by SPPC.

Altmann, C. *et al.* (2001) *This meeting*

195P HIGH DENSITY LIPOPROTEIN REDUCES RENAL DYSFUNCTION AND INJURY MEDIATED BY ISCHAEMIA/REPERFUSION OF THE RAT KIDNEY *IN VIVO*

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High density lipoproteins (HDLs) have been shown to reduce organ injury and mortality in animal models of endotoxic and haemorrhagic shock (Levine *et al.*, 1993, Cockerill *et al.*, 2001). The aim of this study was to investigate whether HDL could reduce renal dysfunction and injury in a rat model of renal I/R.

Forty male Wistar rats (220-330 g) were anaesthetised using sodium thiopentone (120 mg kg^{-1} i.p.). After performing a midline laparotomy, rats were administered either saline (2 ml kg^{-1}, i.v.) or human HDL reconstituted in saline (80 mg kg^{-1}, i.v.) and maintained under anaesthesia for 30 minutes. Rats were then divided into the following groups; (i) **'Sham-sal'**, in which rats were maintained under anaesthesia for the duration of the experiment (N=12), (ii) **'Sham+HDL'**, which were identical to the Sham-sal group except for the administration of HDL (N=4), (iii) **'I/R-only'**, in which rats underwent bilateral clamping of the renal pedicles for 45 min followed by reperfusion for 6 h (N=12), (iv) **'I/R+HDL'**, in which rats underwent I/R but were administered HDL (N=12). On completion of experiments, renal function and injury were assessed by measurement of serum levels of urea (sUr), creatinine (sCr) and urinary concentrations of *N*-acetyl-β-D-glucosaminidase (uNAG). I/R injury was determined by measurement of serum levels of aspartate aminotransferase (sAST) and γ-glutamyl transferase (sγGT).

Table 1	sUr (mM)	sCr (μM)	uNAG (iuL^{-1})	SAST ($iu\ L^{-1}$)	sγGT ($iu\ L^{-1}$)
Sham-sal	6±0.4 +	43±4 +	11±2 +	144±7 +	0.3±0.1 +
Sham+ HDL	7±0.4 +	39±1 +	11±1 +	177±8 +	1±0.4 +
I/R-only	24±1 *	227±11 *	47±4 *	2218±235 *	6±1 *
I/R+ HDL	19±1 *+	173±6 *+	13±3 +	843±110 *+	3±0.5 *+

Table 1: Effect of I/R and HDL on biochemical indicators of renal dysfunction and injury. *$P<0.05$ *vs.* Sham-sal, +$P<0.05$ *vs.* I/R only. Data are expressed as mean±s.e.mean, analysed using one-way ANOVA/Bonferroni's.

Compared to the Sham group, renal I/R produced significant increases in serum and urinary indicators of renal dysfunction and injury (**Table 1**). Administration of HDL 30 minutes prior to renal I/R significantly reduced I/R-mediated increases in sUr, sCr, uNAG, sAST and sγGT, indicating a reduction of renal dysfunction, renal and I/R injury (**Table 1**).

Thus, administration of HDL significantly reduced the renal dysfunction and injury associated with I/R of the rat kidney. The mechanism(s) involved warrant further investigation.

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Levine, D.M., Parker, T.S., Donnelly, T.M. *et al.* (1993) *Proc Natl Acad Sci USA*, **90**, 12040-12044.

196P THE NOVEL INOS INHIBITOR GW274150 REDUCES RENAL DYSFUNCTION MEDIATED BY ISCHAEMIA/REPERFUSION OF THE RAT AND MOUSE KIDNEY *IN VIVO*

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Generation of nitric oxide (NO) by inducible NO synthase (iNOS) contributes to the development of renal ischaemia/ reperfusion (I/R) injury (Lieberthal, 1998). The aim of this study was to investigate the effect of GW274150, a potent, long acting and highly selective inhibitor of iNOS Alderton *et al.*, 1999), in rat and mouse models of renal I/R.

Thirty-six male Wistar rats (220-320 g) were anaesthetised using sodium thiopentone (120 mg kg^{-1} i.p.). After performing a midline laparotomy, rats were administered either saline (2 ml kg^{-1}, i.v.) or GW274150 (5 mg kg^{-1}, i.v.). Rats were then divided into 4 groups; (i) '**Sham-saline**', which were maintained under anaesthesia for the duration of the experiment (N=12), (ii) '**Sham+GW**', which were identical to the Sham-saline group except for the administration of GW274150 (N=4), (iii) '**I/R- only**', which underwent bilateral clamping of the renal pedicles for 45 min followed by reperfusion for 6 h (N=12), (iv) '**I/R+GW**', which underwent I/R, but were administered GW274150 (N=12). In a separate study, 15 male wild-type mice and 5 iNOS knockout mice (C57BL/6, 15-25 g) were anaesthetised using chloramide hydrochloride (125 mg kg^{-1}). After performing a midline laparotomy, mice were divided into 4 groups; (i) '**Sham-sal**', which were maintained under anaesthesia for 30 min (N=5), (ii) '**I/R- only**', which underwent bilateral clamping of the renal pedicles for 30 min (N=5), (iii) '**I/R+GW**', which underwent I/R, but were administered an i.v. bolus of GW274150 (5 mg kg^{-1} i.p.) 30 min before ischaemia (N=5), (iv) '**I/R+iNOS-/-**', as described for I/R-only but using iNOS knockout mice (N=5). Abdominal incisions were then sutured and mice were returned to their cages, allowed to recover from anaesthesia and left for 24 h. Renal function in rats and mice was assessed by measurement of serum urea (sUr) and creatinine (sCr).

Rat I/R	sUr (mM)	sCr (μM)	Mouse I/R	sUr (mM)	sCr (μM)
Sham-sal	8±1 +	34±2 +	**Sham-sal**	2±0.2 +	6±0.4 +
Sham+ GW	6±1 +	44±5 +	**I/R-only**	17±0.2 *	50±15 *
I/R-only	28±1 *	206±11 *	**I/R+GW**	10±1 * +	7±0.9 +
I/R+GW	19±1 * +	172±5 * +	**I/R+ iNOS-/-**	11±0.3 * +	16±3 * +

Table 1: Effect of GW274150 on renal dysfunction. *P<0.05 *vs.* Sham-sal, ^{+}P <0.05 *vs.* I/R-only. Data expressed as mean±s.e.mean, analysed using ANOVA/Bonferroni's.

These results suggest that enhanced formation of NO contributes to the I/R-mediated renal dysfunction in the rat and mouse, which is significantly reduced by GW274150. Therefore, GW274150 may be beneficial in conditions involving nitrosative stress of the kidney.

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197P RELATIVE CONTRIBUTIONS OF POLY(ADP-RIBOSE)POLYMERASE (PARP) ISOFORMS IN THE PATHOGENESIS OF MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

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Although PARP inhibition has been well documented to limit cardiac ischemia-reperfusion injury (Thiemermann et al,1997; Pieper et al., 2000), no information has been reported so far regarding the relative contributions of various PARP isoforms in mediating myocardial infarction. The purpose of this study was to determine whether myocardial infarction caused by ischemia-reperfusion was reduced in PARP-1 deficient mice and whether other PARP isoforms could be involved in the pathogenesis of this cardiac damage.

Male PARP-$1^{-/-}$ mice and littermate wild-type (WT) control PARP-$1^{+/+}$ mice weighing 25-27 g were submitted to 45-minute left coronary artery occlusion followed by 24-hour reperfusion and received before the induction of myocardial ischemia either the PARP inhibitor, 3-aminobenzamide (3-AB)(30 mg/kg ip + 30 mg/kg/24 hr sc via an osmotic minipump), or its vehicle. For the surgical procedure, animals were anaesthetized by a mixture of pentobarbital (40 mg/kg ip) and xylazine (40 mg/kg ip), intubated and ventilated with room air. Infarct size was determined after triphenyltetrazolium chloride (1 %) staining by computerized morphometric analysis and expressed as percentage of the area at risk. In another set of experiments, cardiac myeloperoxidase (MPO) and plasma lactate dehydrogenase (LDH) activities were determined and used as indices of neutrophil accumulation and cardiac cellular damage, respectively. PARP-1 and PARP-2 protein expression was determined in heart extracts of WT mice by Western blot. Results are expressed as mean ± s.e.m.

The elevations of cardiac MPO and plasma LDH activities found 24 hr after reperfusion injury in WT PARP-$1^{+/+}$ mice, were partially inhibited in PARP-$1^{-/-}$ mice from 2.9±1.1 to 1.4±0.87 mU/mg of tissue (-52%, $p<0.05$), and from 0.46±0.06 to 0.33±0.02 arbitrary units (-28%, $p<0.05$), respectively. After 24 hour of reperfusion, WT PARP-$1^{+/+}$ mice presented a major cardiac infarct (70 ± 3% of area at risk, n=7), which was significantly reduced by 27% ($p<0.05$) in PARP-$1^{-/-}$ mice (n=8). By comparison, chronic infusion of 3-AB in WT PARP-$1^{+/+}$ mice was more effective than genetic disruption of PARP-1 alone in limiting myocardial infarction, since the infarct size was reduced by 59% ($p<0.05$) in WT PARP-$1^{+/+}$ mice treated with 3-AB (n=7). Interestingly, in PARP-$1^{-/-}$ mice, 3-AB treatment also reduced myocardial infarction (-43%, $p<0.05$, n=6), giving a residual infarct of similar size (30 ± 7% of area at risk) to that found in WT PARP-$1^{+/+}$ mice infused with 3-AB (29 ± 7% of area at risk). In the heart of WT mice, the concentration of PARP-2 protein was 4-6 times higher than that of PARP-1.

These results suggest that although PARP-1 activation plays a significant role in cardiac ischemia-reperfusion injury in the mouse, PARP-2 or other 3-AB-sensitive PARP isoforms are also likely to contribute to the severity of the infarct.

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Thiemermann, C., Bowes, J., Myint, F.P. et al., (1997). Proc. Natl. Acad. Sci. U.S.A., 94,697-683.

198P ANNEXIN 1 PEPTIDE PROTECTS AGAINST ISCHAEMIA REPERFUSION IN THE MOUSE MESENTERY

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A recent study has described a functional interaction between the formyl peptide receptor (FPR) and peptides derived from the N-terminus of the anti-inflammatory protein annexin 1 (ANXA1) (Walther *et al.*, 2000). In this study we have assessed for the first time whether peptide Ac2-26 modulates leukocyte-endothelium interaction in a model of ischaemia reperfusion (IR) in the mouse mesentery and whether this effect was indeed mediated by the FPR.

Male C57/BL6 wild type mice (WT, 12-15 g) or FPR knock-out mice (FPR-KO) were anaesthetised with Diazepam™ and Hypnorm™, and the left jugular vein and right carotid artery cannulated. Cautery incisions were made along the abdominal region and the superior mesenteric artery was clamped of 30 min. Saline (100 µl), peptide Ac2-26 (20-100 µg mouse^{-1}) or the FPR antagonist N-t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc 2; Sigma) (10 µg mouse^{-1}) were injected at the beginning of the reperfusion period. After 45 min, the mesentery was exteriorised, superfused with 37°C warm bicarbonate-buffered solution, gassed with 5% CO_2/95% N_2. One to three randomly selected post-capillary venules (diameter 20-40 µm; length ≥ 100 µm) were observed for each mouse. For each vessel, leukocyte velocity (in µms^{-1}), adhesion (no. per 100 µm) and emigration (no. per 50x100 µm^2) were measured. Data (mean ± s.e.mean) were analysed by ANOVA and post-hoc Bonferroni test taking a P value <0.05 as significant.

In saline-treated WT mice IR produced a >50% decrease in cell velocity (sham, 68 ± 16 µms^{-1}, IR, 30 ± 5 µms^{-1}), whereas cell adhesion (from 1.4 ± 0.4 cells, to 4.0 ± 0.5 cells) and emigration (from 1 ± 0.2 to 3 ± 0.1) increased significantly compared to sham mice (n=6; P<0.05). Treatment of mice with 100 µg (33 µmol) peptide Ac2-26 inhibited IR-induced leukocyte-endothelium interaction back to sham values (adhesion, 1 ± 0.3 cells, emigration 1 ± 0.2 cells, n=6, P<0.05), but had no effect on rolling velocity (25 ± 11 µms^{-1}). The peptide was not active at the lower dose of 20 µg. The FPR antagonist Boc2 was inactive when given alone to IR-treated WT mice, but it prevented the inhibitory effects of peptide Ac2-26 (n=5, P<0.05 vs. peptide alone). In FPR-KO mice, results for saline-treated I/R mice were similar to WT mice for rolling velocity (44 ± 8 µms^{-1}) and cell adhesion (4.5 ± 0.3 cells), whereas there was a higher degree of cell emigration (6 ± 0.5, n=6, P<0.05 vs. WT). Treatment of FPR-KO mice with 100 µg peptide Ac2-26 resulted in a lower degree of inhibition on IR-induced cell adhesion and emigration, with values of 2.5 ± 0.3 and 4.0 ± 0.5 cells, respectively (n=4, P<0.05 vs. saline-treated group; P<0.05 vs. peptide group in WT mice).

In conclusion, this study indicates that the ANXA1-FPR pathway may exert a tonic inhibitory control on leukocyte-endothelium interaction during experimental IR. Also, it seems that FPR is not the sole target responsible for the anti-migrating properties of peptide Ac2-26.

Walther A *et al.* (2000) Mol Cell **5**, 831-840.

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199P TETRAHYDROBIOPTERIN ENHANCES PULMONARY ARTERIAL RELAXATION AFTER BIRTH.

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Relaxation to acetylcholine (ACh) in porcine pulmonary arteries, varies with age. It is lowest before birth, rising to a maximum at 3- 14 days after birth. 3 day hypoxic animals (animals placed in a hypobaric chamber between birth and 3 days of age) show little or no relaxation to ACh. The mechanisms of these changes are unclear.

Tetrahydrobiopterin (BH4) is one of the essential cofactors required for nitric oxide synthase (NOS) activity. Nitric oxide (NO) is a potent endothelium derived relaxing factor. Sub optimal levels of BH4 impair NO production and turn NOS into a generator of harmful superoxide anions. The rationale behind this study was to test the hypothesis that increasing BH4 levels via the use of the BH4 precursor, sepiapterin (SEP) and quenching superoxide anions using the superoxide dismutase (SOD) mimetic Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MNTPY) would increase endothelium dependent relaxation of porcine pulmonary arteries.

Intrapulmonary arterial rings, obtained from foetal (1-2 weeks preterm), newborn, 12-24 hour, 3 day, 3 day hypoxic, 14 day and adult Large White pigs, were dissected and mounted in organ chambers for the measurement of isometric force. Tissues were maintained in modified Krebs solution and were incubated for 30 mins with SEP (30uM), MNTPY (10uM), SEP + MNTPY or neither (control). Tissues were precontracted with the thromboxane analogue U46619 (to EC80), after which cumulative concentration response curves were constructed to ACh and sodium nitroprusside (SNP). Finally arterial rings were relaxed fully with 10uM papaverine. Results are shown in Figure 1.

Figure 1 Percentage maximal relaxation to ACh (% of pre contraction) (mean ± SEM).

	Foetal (n=4)	New born (n=3)	24hr (n=4)	3 day (n=5)	14day (n=5)	Adult (n=4)	3 hyp (n=3)
Control	7.30 ±2.5	8.40 ± 3.3	8.70 ± 1.2	18.7 ± 5.1	22.9 ± 2.9	19.6 ± 2.0	4.80 ± 2.0
SEP	10.5 ± 2.2	8.40 ± 1.6	*15.9 ± 4.0	*29.4 ± 5.4	*31.7 ± 4.1	21.7 ± 5.1	4.80 ± 2.3
Mntpy	6.40 ± 3.5	10.1 ± 3.6	*12.6 ± 2.6	*33.9 ± 4.2	*31.6 ± 2.9	18.9 ± 4.5	2.90 ± 1.9
SEP + Mntpy	5.50 ± 3.2	16.7 ± 0.6	*16.2 ± 3.0	*33.5 ± 5.6	*43.2 ± 4.1	21.2 ± 6.9	*9.40 ± 3.4

* indicates p<0.05 drug treatment compared to control value.

There were no significant differences in response to SNP between drug treated and control for any age groups, suggesting any effects seen were endothelium dependent.

Western blotting indicated that expression of GTP-CH1 (the rate limiting enzyme for BH4 generation) was lower in the groups in which supplementary BH4 produced effects.

These results suggest that BH4 may be rate limiting for NO mediated relaxation 12-24 hours after birth. The finding that SOD also increases relaxation may indicate over production of superoxide anions. These may derive from BH4 deficient NOS or from other sources. Further experiments will be required to test this directly.

200P REGIONAL HAEMODYNAMIC RESPONSES TO INFUSION OF HUMAN UROTENSIN II (hUII) IN CONSCIOUS SPRAGUE-DAWLEY RATS

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Recently, we described dose-dependent depressor effects of incremental i.v. bolus doses of hUII in conscious rats, associated with rapid-onset, pronounced, but short-lived mesenteric vasodilatation, and slower-onset, prolonged hindquarters vasodilatation (Gardiner *et al.*, 2001). We have now studied the effects of i.v. infusion (300 pmol kg^{-1} h^{-1}), or a high bolus dose (3 nmol kg^{-1}), of hUII in conscious, naïve rats, since pilot experiments indicated pre-exposure might influence subsequent responses.

Male Sprague-Dawley rats (400-450g) were anaesthetised (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.) and had pulsed Doppler flow probes and intravascular catheters implanted in a two-stage procedure separated by at least 10 days. Starting 24 h after the last procedure (catheterisation), measurements of mean arterial blood pressure (MAP), heart rate (HR), and renal (R), mesenteric (M) and hindquarters (H) vascular conductances (VC) were made in conscious animals.

Infusion of hUII caused slowly developing pressor, tachycardic, and hindquarters vasodilator effects, but no mesenteric vasodilatation (Table 1). Following the high bolus dose of hUII in naïve rats, the changes in MVC and HVC were qualitatively similar to those reported previously (Gardiner *et al.*, 2001), but they were substantially less, and there was no fall in MAP (Table 1). These results indicate that the mode of administration of hUII, and/or prior exposure to this peptide, may influence the haemodynamic effects seen. The mechanisms involved remain to be determined.

Table 1. Cardiovascular variables before and during infusion (300 pmol kg^{-1} h^{-1}, n = 5) or following bolus injection (3 nmol kg^{-1}, n = 4) of hUII in conscious Sprague-Dawley rats. Values are mean ± s.e. mean. Units for VC are (kHz $mmHg^{-1}$) 10^3. * P < 0.05 Friedman's test

Infusion	**Before**	**10 min**	**60 min**	**120 min**
HR (beats min^{-1})	332 ± 14	349 ± 29	356 ± 18*	421 ± 11*
MAP (mm Hg)	102 ± 1	105 ± 3	110 ± 3	117 ± 4*
RVC (units)	86 ± 7	83 ± 7	77 ± 4*	87 ± 8
MVC (units)	75 ± 2	71 ± 3	66 ± 5	61 ± 6
HVC (units)	39 ± 3	38 ± 3	41 ± 2	52 ± 4*
Bolus	**Before**	**1 min**	**5 min**	**10 min**
HR (beats min^{-1})	337 ± 6	373 ± 7*	449 ± 11*	416 ± 17*
MAP (mm Hg)	96 ± 3	109 ± 3*	90 ± 6	95 ± 4
RVC (units)	75 ± 11	64 ± 11	81 ± 17	72 ± 9
MVC (units)	126 ± 11	144 ± 12*	153 ± 19*	130 ± 12
HVC (units)	36 ± 2	32 ± 3	59 ± 8*	53 ± 4*

Gardiner, S.M. *et al.* (2001) Br. J. Pharmacol., **132**, 1625-1629

201P REGIONAL HAEMODYNAMIC RESPONSES TO PHENTOLAMINE IN CONSCIOUS, SPRAGUE-DAWLEY RATS

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An important involvement of β-adrenoceptors in the hypotensive responsiveness to α-adrenoceptor antagonism has previously been noted (e.g., Gardiner & Bennett, 1988), but the detailed haemodynamic events underlying this putative interaction have not been delineated. The present experiments examined the haemodynamic sequelae of administering the non-selective α-adrenoceptor antagonist, phentolamine, to conscious rats, in the absence or presence of propranolol.

Male Sprague-Dawley rats (350-450 g) were instrumented with intravascular catheters (for measurement of heart rate (HR), mean arterial blood pressure (BP), and drug administration) and pulsed Doppler flow probes (to monitor coeliac, superior mesenteric, and hindquarters flows). All surgery was carried out under fentanyl and meditomidine anaesthesia (300 μg.kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg.kg^{-1} of each s.c.) Resting haemodynamic values were recorded for 30 min. Animals were randomised into 4 groups and given phentolamine (1 mg.kg^{-1} i.v. bolus, 1 mg.$kg.h^{-1}$ infusion; n=9), phentolamine plus propranolol (1 mmg.kg^{-1} i.v. bolus, 0.5 mg.$kg.h^{-1}$ infusion; n=8), saline (0.4 ml.kg^{-1} i.v. bolus, 0.4 ml.$kg.h^{-1}$ infusion; n=8), or propranolol (1 mg.kg^{-1} i.v. bolus, 0.5 mg.$kg.h^{-1}$ infusion; n=8, data not shown).

Table 1 summarises some of the results. Baseline values for the phentolamine, phentolamine and propranolol, and saline groups, respectively, were: HR (beats min^{-1}) 351±8, 345±5, 344±9; BP (mm.Hg) 106±1, 101±1, 104±1; vascular conductance (VC) ([kHz mm Hg^{-1})]10^3), coeliac (CVC) 110±10, 124±8, 111±10, mesenteric (MVC) 92±6, 95±8, 94±6, and hindquarters (HVC) 37±2, 45±3, 39±4.

The administration of phentolamine alone caused substantial and sustained hypotension, which was probably due to the dramatic increases in HVC. These effects were accompanied by a marked increase in HR, with surprisingly little change in CVC and MVC. In the presence of propranolol, the hypotensive, tachycardic, and hyperaemic hindquarters vasodilator effects of phentolamine were strikingly reduced, and were accompanied by a significant decrease in CVC. In a separate group of animals, propranolol alone had little haemodynamic effect (data not shown).

Table 1: Cardiovascular changes (Δ) after phentolamine (Phent), phentolamine and propranolol (Phent+Prop), or saline. HR; BP; CVC, MVC, HVC (% min). Values (mean ± s. e. mean) are areas under or over the curve (0-30min); $^{*}P<0.05$ vs. saline, $^{\#}P<0.05$ vs. phentolamine (Kruskal-Wallis Test).

	Phent	Phent+Prop	Saline
ΔHR (beats)	3542 ± 483*	-766 ± 202$^{*\#}$	235 ± 83
ΔBP (mm Hg min)	-868 ± 161*	-358 ± 69$^{*\#}$	-31 ± 36
ΔCVC (% min)	-337 ± 105	-685 ± 113$^{*\#}$	-95 ± 32
ΔMVC (% min)	-316 ± 35*	-274 ± 82*	84 ± 24
ΔHVC (% min)	4870 ± 705*	894 ± 72$^{*\#}$	153 ± 43

These findings are consistent with the hypotensive effects of phentolamine, in conscious rats, being due to β-adrenoceptor-mediated vasodilatation. The clinical corollary of these findings may be of interest.

Gardiner, S.M. & Bennett, T. (1988). Am. J. Physiol., **255**, H813-H824.

202P β_2-ADRENOCEPTOR INVOLVEMENT IN THE HINDQUARTERS VASODILATOR EFFECTS OF WIN 55212-2, HU 210 AND ANANDAMIDE IN CONSCIOUS SPRAGUE-DAWLEY RATS

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In conscious rats, the synthetic cannabinoids, HU 210 and WIN 55212-2, and the endocannabinoid, anandamide, cause vasodilatation only in the hindquarters vascular bed (Gardiner *et al.*, 2001). Since β_2-adrenoceptor agonist-induced vasodilatation also occurs predominantly in the hindquarters (Gardiner *et al*, 1996), we have now examined the effects of β_2-adrenoceptor antagonism, with ICI 118551, on the regional haemodynamic responses to HU 210, WIN 55212-2 and anandamide.

Under anaesthesia (fentanyl and meditomidine, 300 $\mu g\ kg^{-1}$ of each i.p. reversed with nalbuphine and atipamezole, 1 $mg\ kg^{-1}$ of each s.c.), male, Sprague-Dawley rats (400-450g) had pulsed Doppler flow probes and, at least 14 days later, intravascular catheters, implanted. On the day following catheterisation, measurements of mean arterial blood pressure (MAP), heart rate (HR) and renal (R), mesenteric (M) and hindquarters (H) vascular conductances (VC) were made. In separate groups of animals, WIN 55212-2 (150 $\mu g\ kg^{-1}$; n = 7), or anandamide (3 $mg\ kg^{-1}$; n = 11) were administered before and 90 min after administration of ICI 118551 (0.2 $mg\ kg^{-1}$; 0.1 $mg\ kg^{-1}\ h^{-1}$). Since HU 210 has a long duration of action, different groups of animals received HU 210 (100 $\mu g\ kg^{-1}$) in the absence (n = 6) or presence (n = 8) of ICI 118551.

Due to the fact that the cardiovascular effects of the different cannabinoids were complex, and differed in time course, the data are presented as the mean changes ($\Delta \pm$ s.e. mean) at the time of maximum hindquarters vasodilatation.

Following administration of WIN 55212-2 in the absence of ICI 118551, the increase in HVC (maximum Δ +49 ± 16 % at 120 s) was associated with an increase in MAP (+16 ± 5 mm Hg) and decreases in RVC and MVC (-22 ± 5 %, -33 ± 5 %, respectively). In the presence of ICI 118551, the increase in HVC was abolished (-6 ± 4 %) but the other cardiovascular effects of WIN 55212-2 were unchanged. HU 210 alone caused an increase in HVC (maximum Δ +83 ± 11 % at 20 min), a decrease in MAP (-11 ± 5 mm Hg), no change in RVC, and a decrease in MVC (-15 ± 3 %). In the presence of ICI 118551, the increase in HVC following HU 210 (+17 ± 7 %) was significantly ($P < 0.05$, Mann-Whitney test) reduced, and the associated falls in MAP and MVC were abolished. Anandamide caused an increase in HVC (maximum Δ +47 ± 10 % at 60 s), a fall in MVC (-12 ± 3 %), but no change in MAP or RVC. Pre-treatment with ICI 118551 abolished the increase in HVC following anandamide (+2 ± 7 %); the fall in MVC was unaffected (-13 ± 6 %), but there was a fall in RVC (-15 ± 4 %) and a rise in MAP (+14 ± 3 mm Hg).

These results are consistent with the hindquarters vasodilator effects of WIN 55212-2, HU 210, and anandamide being due to adrenomedullary adrenaline release, although the mechanisms involved must be different because the actions of the synthetic cannabinoids are inhibited by CB_1-receptor antagonism, whereas those of anandamide are not (Gardiner *et al.*, 2001).

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203P LACK OF FUNCTIONAL β_3- OR PUTATIVE β_4-ADRENOCEPTORS IN RAT ISOLATED AORTA

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Evidence for the presence of atypical β-adrenoceptors in blood vessels is largely based on relaxant responses to selective β_3-adrenoceptor agonists, such as BRL 37344 and SR 58611A and to non-conventional partial agonists, such as CGP 12177A and cyanopindolol (Oriowo, 1994; Trochu *et al*, 1999; Brawley *et al.*, 2000). We previously reported that relaxations to CGP 12177A and BRL 37344 were not blocked by the selective β_3-adrenoceptor antagonist, SR 59230A (Brawley *et al.*, 2000; Brahmadevara *et al.*, 2001). The present study further characterises responses to selective β_3-adrenoceptor agonists and to non-conventional partial agonists in rat isolated aorta.

Male Wistar rats (200 - 250 g) were stunned and killed by cervical dislocation before removal of thoracic aortae. Ring preparations were suspended in Krebs physiological saline, gassed with 95/5 % O_2/CO_2, at 37 °C for isometric recording. Rings were pre-constricted with phenylephrine (PE, 0.6 μM) or $PGF_{2\alpha}$ (3 μM) before carrying out cumulative concentration-response curves to relaxants. In antagonist studies, tissues were incubated with antagonist for 30 min before constriction. Values are mean±s.e.mean. Statistical analysis was carried out using ANOVA followed by post tests.

CGP 12177A produced concentration-dependent full relaxation of PE-constricted rat aorta. However this relaxant effect also occurred with other β-adrenoceptor antagonists, irrespective of reported partial agonist activity [pEC_{50}s: CGP 12177A, 4.40±0.02 (8); cyanopindolol, 5.40±0.02 (5); pindolol, 5.30±0.07 (3); alprenolol, 5.08±0.05 (5); bupranolol, 5.50±0.03 (5); ICI 118551, 4.57±0.08 (5); CGP 20712A, 4.35±0.03 (5); propranolol, 4.83±0.03 (6); SR 59230A, 5.47±0.03 (6)]. The relaxant effect of CGP 12177A was not affected ($P>0.05$) by propranolol (1 μM), SR 59230A (1 μM) or CGP 20712A (10 μM). In $PGF_{2\alpha}$-constricted aortae, CGP 12177A (200 μM), alprenolol (30 μM), bupranolol (100 μM) and cyanopindolol (10 μM) had no relaxant effect. SR 58611A produced concentration-dependent relaxation in PE-constricted rat aorta (pEC_{50}, 4.94±0.01 (8)) which was unaffected ($P>0.05$) by propranolol (1 μM) or SR 59230A (1 μM). SR 58611A also produced relaxation in $PGF_{2\alpha}$-constricted aortae (pEC_{50}, 4.71±0.01 (6)) and this was unaffected ($P>0.05$) by propranolol (1 μM), alprenolol (30 μM) or bupranolol (30 μM). CL 316243 (3 nM - 100 μM), a potent and selective β_3-adrenoceptor agonist, had no relaxant effect in either PE- or $PGF_{2\alpha}$-constricted rings.

In conclusion, relaxant effects obtained in rat aorta with some selective β_3-adrenoceptor agonists and with non-conventional partial agonists are not related to stimulation of β_3- or putative β_4-adrenoceptors.

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204P (-)-PINDOLOL ACTS THROUGH TWO STATES OF THE β_1-ADRENOCEPTOR IN HUMAN ATRIUM AND CHO CELLS.

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(-)-Pindolol and (-)-CGP12177 ((-)-4-(3-tertiary butylamino-2-hydroxypropoxy) benzimidazol-2-one) are β-adrenoceptor (βAR) blocking agents that exert agonist effects resistant to blockade by (-)-propranolol (Kaumann, 1989). The cardiostimulant effects of (-)-CGP12177 persist in β_2AR knockout mice but are abolished in β_1AR/β_2AR double knockout mice, indicating mediation through a (-)-propranolol-resistant state of the β_1AR (Kaumann et al., 2001). We investigated whether (-)-pindolol exerts agonist effects through a (-)-propranolol-resistant state of the β_1AR in human atrium and CHO cells expressing 98 fmol.mg^{-1} protein β_1AR (Arg389 variant). Trabeculae of human right atrium were set up to contract at 1 Hz at 37°C as described (Kaumann, 1996), and exposed to 20 μM 3-isobutyl-1-methylxanthine (IBMX) and 200 nM (-)-propranolol. A concentration-effect curve to (-)-pindolol was obtained up to 6 μM and followed by a curve for (-)-CGP12177 in the presence of 6 μM (-)-pindolol. On a second trabeculum from the same atrium, a curve for (-)-CGP12177 was determined in the absence of (-)-pindolol. To determine the intrinsic activity (IA) of the partial agonists, the experiments were terminated with 600 μM (-)-isoprenaline. Cyclic AMP was determined in CHO cells exposed to 1 mM IBMX. (-)-Isoprenaline (100 nM) produced a ~100-fold increase in the levels of cyclic AMP in CHO cells (-$logEC_{50}$=8.08±0.10, n=8). (-)-CGP12177 was a partial agonist in both atria (-$logEC_{50}$=7.59±0.07; IA=0.68±0.05, n=7) and CHO cells (-$logEC_{50}$=7.72±0.07, IA=0.07±0.01).(-)-Pindolol produced positive inotropic effects and enhanced cyclic AMP levels in CHO cells. (-)-Propranolol (200 nM) failed to block the effects of (-)-pindolol but antagonised the effects of (-)-isoprenaline with a pK_B of 9.0 in CHO cells. (-)-Pindolol (6 μM) caused surmountable antagonism of the effects of (-)-CGP12177 in atria and CHO cells. (-)-Pindolol (6 μM) was ~200 times more effective in antagonising the effects of (-)-isoprenaline than those of (-)-CGP12177 in CHO cells.

Table 1. Pindolol data (mean ± s.e.m., n=5-7 per group)

	Atrium	CHO cells
-$LogEC_{50}$, M	6.53±0.08	6.54±0.17
Intrinsic activity	0.33±0.05	0.018±0.004
pK_B vs (-)-CGP12177	6.45±0.09	6.25±0.11
pK_B vs catecholamine	9.11±0.05*	8.61±0.22

*vs (-)-noradrenaline, from Kaumann & Lobnig (1986)

The results from both atrium and CHO are consistent with the existence of two β_1AR states for (-)-pindolol. The low-affinity state mediates the agonist effects. The high-affinity state mediates blockade of the effects of catecholamines.

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Kaumann, A.J. et al. (2001) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **363**, 87-93.

205P EFFECTS OF ACE-INHIBITORS AND β-BLOCKERS ON NORADRENALINE TRANSPORTER-DENSITY AND -ACTIVITY (UPTAKE$_1$) IN THE HUMAN HEART

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In chronic heart failure (CHF) elevated plasma noradrenaline (NA) levels and a disparity between the neuronal release and the effective re-uptake of NA lead to an increased concentration of NA in the presynaptic cleft, that might be involved in downregulation of the myocardial β-adrenoceptors (Böhm *et al.*, 1995).

Recently, evidence has accumulated that ACE-inhibitors and/ or long-term β-blocker therapy re-modulate the cardiac adrenergic nervous system in the failing human heart. Thus, it could be demonstrated that the release of [^{125}I]-metaiodobenzylguanidine ([^{125}I]-MIBG), an analog of noradrenaline, was decreased and [^{125}I]-MIBG re-uptake into sympathetic nerve endings was increased in patients with CHF due to long-term ACE-inhibitor and/or metoprolol treatment (Lotze *et al.*, 2001). The aim of the present study was to find out whether this holds also true for noradrenaline as physiological substrate of the NA transporter (NAT) in dependence of therapy and age.

For this purpose we determined NAT-density in crude membranes isolated from right atrial (RA) appendages by [^{3}H]-nisoxetine binding as recently described (Leineweber *et al.*, 2000) at 6 concentrations ranging from 0.31-10 nM. Non-specific binding was determined in the presence of 1 μM desipramine (uptake$_1$ antagonist). Furthermore, we determined the NAT-activity as described (Leineweber et al., 2000) by incorporation of [^{3}H]-NA within a range of 1.56-25 nM in the presence of 40 μM corticosterone (uptake$_2$ inhibitor) during a 15 min incubation at 37°C in RA slices (0.25 mm) obtained from 5 different groups of patients: group A, children (n=9; mean age 9±3 yrs); group B, patients not treated with ACE-inhibitors and/or β-blockers (n=16; mean age 62±3 yrs); group C, patients treated with ACE-inhibitors (n=14; mean age 63±4 yrs); group D, patients treated with metoprolol (n=14; mean age 63±4 yrs), and group E, patients treated with ACE-inhibitors and metoprolol (n=17; mean age 60±7 yrs). All patients were undergoing coronary artery bypass grafting and were in NYHA-class II-III; children were undergoing open-heart surgery because of acyanotic congenital heart disease. Data are given in table 1.

Group	A	B	C	D	E
NAT-density	169±13 (5)	105±7[a] (11)	145±16[b] (9)	158±14[b] (8)	195±16[b] (9)
NAT-activity	274±44 (4)	128±15[a] (5)	147±14 (5)	217±14[b] (6)	194±13[b] (8)

Table 1: NAT-density (fmol/mg protein); NAT-activity (pmol NA/mg tissue/15 min); Data are means±S.E.M.; [a] $p<0.05$ vs. group A; [b] $p<0.05$ vs. group B

These data show that with aging and/ or CHF cardiac NAT-density and -activity are decreased, and that they can be restored by long-term treatment with ACE-inhibitors and β-blockers.

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206P PREJUCTIONAL AND POSTJUNCTIONAL INHIBITORY ACTIONS OF THE SELECTIVE AT1-ANTAGONISTS EPROSARTAN AND CANDESARTAN IN THE RABBIT ISOLATED MESENTERIC ARTERY

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Angiotensin II (Ang II) has a well known facilitatory effect on sympathetic neurotransmission (Reid, 1992).

We investigated the effects of the AT_1-receptor antagonists eprosartan and candesartan and the AT_2-receptor antagonist PD123319 on angiotensin II-induced facilitation of noradrenergic neurotransmission in the isolated rabbit mesenteric artery under isometric conditions in a wire myograph setup. The sympatho-inhibitory (presynaptic) potency of the AT_1-blockers was compared to their potency concerning inhibition of the vasoconstrictor (postsynaptic) effect of Ang II. New Zealand White Rabbits of either sex weighing 2000-3400 g killed using pentobarbital 30 mg/kg i.v.

To investigate blockade of the pre-synaptic AT_1 and AT_2-receptors, we studied the effects of Ang II (0.5 nM) on electrical field stimulation (EFS)-induced contractions in the presence or absence of eprosartan (0.01-0.1 nM), candesartan (0.01-0.1 nM) or PD 123319 (10 nM). To investigate blockade of the post-synaptic AT_1-receptors, we studied the effects of either eprosartan (0.1-10 nM) or candesartan (0.001-0.1 nM) on cumulative concentration-response curves (CRC) of Ang II. In addition, the effect of Ang II on postsynaptic α-adrenoceptor mediated responses was studied using noradrenaline. The isolated mesenteric artery preparations were incubated with vehicle or either the AT_1 or AT_2-antagonists for 15 minutes before applying EFS or constructing the CRC's to Ang II or NA. In stimulation experiments, Ang II was added 2 minutes prior to EFS. Values are presented as mean ± SEM. Groups consisted of 7-12 animals. Student's t-test was used to compare means.

EFS (1, 2 and 4 Hz) caused a frequency-dependent increase of contractile force. At stimulation frequencies of 1, 2 and 4 Hz, a sub-pressor concentration of Ang II (0.5 nM) increased the stimulation-induced (S-I) vasoconstrictor responses by a factor 2.8 ± 0.5, 2.4 ± 0.4, and 1.6 ± 0.1, respectively ($p<0.05$ compared to control for all frequencies).

The enhancement could be antagonised by eprosartan (pIC_{50} 9.7 ± 0.30) and candesartan (pIC_{50} 9.7 ± 0.26) ($p > 0.05$). The AT_2-antagonist PD123319 (10 nM) did not influence the Ang II induced facilitation of SI-contractions ($p > 0.05$ at all frequencies). Contractile responses to exogenous noradrenaline were unaltered in the presence of Ang II 0.5 nM (pEC_{50}-values were 8.26 ± 0.06 and 8.20 ± 0.07 in the presence and absence of Ang II, respectively ($p>0.05$). Ang II (0.5 nM – 0.5 μM) caused a concentration-dependent increase in contractile force, which could be antagonised in a non-competitive fashion by eprosartan (pD_2' 8.8 ± 0.19) and candesartan (pD_2' 11.3 ± 0.23)($p < 0.05$).

We conclude that the facilitating effect of Ang II on noradrenergic neurotransmission is mediated by pre-synaptically located AT_1-receptors, and not by AT_2-receptors. Regarding sympatho-inhibition, eprosartan and candesartan are equipotent. Regarding inhibition of the vasoconstrictor effect of Ang II, candesartan is more potent than eprosartan. For eprosartan, sympatho-inhibition was achieved at concentrations which also block AT_1-receptors on vascular smooth muscle. In contrast, for candesartan, the presynaptic inhibitory concentrations were considerably higher than those required for postsynaptic inhibition.

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207P ATTENUATION OF ANGIOTENSIN II AUGMENTED NORADRENALINE RELEASE BY SEVERAL SELECTIVE AT_1-RECEPTOR ANTAGONISTS IN THE RABBIT ISOLATED THORACIC AORTA

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Angiotensin II (Ang II) is known to enhance stimulation-induced (S-I) sympathetic outflow (Hughes & Roth, 1971). Release of transmitter from sympathetic nerves can be measured after treatment with labelled noradrenaline (Su & Bevan, 1970).

In the present study we evaluated the facilitating effect of Ang II on sympathetic neurotransmission in order to quantitatively compare the sympatho-inhibitory potencies of the selective AT_1-receptor antagonists losartan, irbesartan and telmisartan.

Isolated rabbit thoracic aortic rings (NZW, either sex, 2-2.8 kg) were pre-treated with 0.1 μM [^{3}H]-noradrenaline and mounted in an organ bath set-up filled with physiological salt solution (carbogenated, kept at 37°C). Desipramine (0.6 μM), corticosterone (40 μM) and yohimbine (1 μM) were added to the medium. Three consecutive periods of electrical field stimulation (EFS, S_1 - S_3: 2 Hz, 3 ms, 150 mA, 2 min.) were applied in order to evoke sympathetic transmission. Tritium overflow was assessed by sampling and calculated into fractional release (*FR*) of tritium. Tritium overflow evoked by S_2 served as an internal control for that evoked by S_3. Pharmacological interventions were performed before S_3. The effects of the different compounds were represented by the ratio FR_3/FR_2. ANOVA followed by Dunnett's test was used for multiple comparisons with control. Linear regression was performed and analysis of covariance was used to determine differences between regression lines.

Ang II (0.01 *n*M - 0.1 μM) caused a concentration-dependent enhancement of EFS-evoked sympathetic neurotransmission (control v.s. concentrations 0.1 *n*M - 0.1 μM: $p<0.05$). The maximal augmentation was observed at a concentration Ang II of 1 *n*M (FR_3/FR_2 2.03 ± 0.11 v.s. control 0.99 ± 0.03). Interestingly, a further increase of Ang II to 0.1 μM produced less than maximal facilitation

The augmentation of EFS-evoked sympathetic transmission by Ang II could be reduced by AT_1-receptor blockade. Losartan (0.1 *n*M – 0.1 μM), telmisartan (0.01 *n*M – 10 *n*M) and irbesartan (0.1 *n*M – 0.1 μM) all could concentration-dependently attenuate the enhancement of EFS-evoked sympathetic outflow by Ang II (1 *n*M). The concentrations that reduced the enhancement by 50% (IC_{50}, expressed as –log M ± SEM) were losartan 9.05 ± 0.16, telmisartan 10.28 ± 0.20 and irbesartan 9.20 ± 0.23 ($p<0.05$ between regression lines of telmisartan and irbesartan and that of telmisartan and losartan) respectively. Accordingly, the order of potency with respect to sympatho-inhibition proved telmisartan > irbesartan = losartan.

It is concluded that the facilitating effect of Ang II on the sequalae of neuronal stimulation is mediated by pre-synaptically located AT_1-receptors. Facilitation can be concentration-dependently attenuated by blockade of AT_1-receptors. The order of potency with respect to sympatho-inhibition is telmisartan > irbesartan = losartan. These differences might be explained by differences in affinity for the presynaptic AT_1-receptor.

Hughes J. Roth RH. *Br J Pharmacol* 1971; 41: 239-55

Su C. Bevan JA. *J Pharmacol Exp Ther* 1970; 172: 62-8

208P TOXIN IV-5 ACCOUNTS FOR THE RELAXING ACTIVITY OF *TITYUS SERRULATUS* VENOM IN THE RABBIT CORPUS CAVERNOSUM

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Tityus serrulatus venom (TSV) relaxes rabbit (Teixeira *et al.*, 1998) and human (Teixeira *et al.*, 2001) corpus cavernosum (CC) through activation of nitrergic fibres causing the release of nitric oxide (NO). We aimed to identify and characterise the effects of the toxin responsible for NO release in rabbit CC.

Extracted proteins from TSV were loaded onto a preparative RP-HPLC. Eleven pooled fractions were tested and only fraction VII relaxed the CC. Fraction VII was rechromatographed and samples collected and pooled according to UV spectrum. An active peptide was isolated and its molecular weight (MW) was obtained by ESI/MS using a Quattro-LC Z-spray triple quadrupole mass spectrometer. Male New Zealand rabbits (2-3 kg) were anaesthetised with sodium pentobarbitone (40 mg/kg, i.v.) and the penis excised. Strips of CC were transferred to 10-ml organ baths containing Krebs solution (95%O_2 - 5%CO_2, 37°C, pH 7.4) and connected to isometric transducers under tension of 10 mN. Changes in isometric force were recorded using a MacLab data acquisition system. The preparations were allowed to equilibrate for a 60 min period and phenylephrine (PE, 10 μM) was added in order to increase the basal tone. Electrical field stimulation (EFS) was performed using a Grass S48 stimulator delivering single square-wave pulses (10 s train duration; 20 V) at varying frequencies (2-16 Hz).

The isolated peptide (MW=7427.66 ± 0.15 Da) was identified as toxin IV-5 (tx IV-5; Possani *et al.*, 1991). The NO synthesis inhibitor L-NAME (100 μM) markedly reduced ($p<0.01$) the relaxations evoked by EFS (2-16 Hz; n=9) and subsequent addition of L-arginine (L-Arg, 1 mM; n=9) partially restored ($p<0.01$) these responses. L-NAME also significantly inhibited ($p<0.01$; n=4) the relaxations elicited by tx IV-5 (30 nM; 52 ± 4% in the absence and 8 ± 3% in the presence of L-NAME). Prior addition of L-Arg prevented this inhibition (n=4). The soluble guanylyl cyclase inhibitor ODQ (10 μM) significantly reduced ($p<0.01$) the relaxations elicited by EFS (n=6). In addition, the relaxations induced by tx IV-5 were virtually abolished by ODQ (98 ± 1% inhibition). The Na^+ channel blockers tetrodotoxin (TTX, 100 nM; n=10) and saxitoxin (STX, 100 nM; n=7) abolished EFS-evoked relaxations ($p<0.01$). Similarly, TTX and STX (n=3, each) virtually abolished the relaxations induced by tx IV-5 (98 ± 1% and 95 ± 3% inhibition for TTX and STX, respectively). Interestingly, these blockers promptly reversed the established relaxations (n=3, each).

Our results indicate that toxin IV-5 exerts nitrergic actions and is responsible for the relaxing activity of TSV in rabbit CC.

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Teixeira CE, Faro R, Moreno RA *et al.* (2001) *Urology*, 57:816-820.

We thank FAPESP for their financial support.

209P VASOPRESSIN-INDUCED PRE-SYNAPTIC FACILITATION OF SYMPATHETIC NEUROTRANSMISSION IN THE PITHED RAT.

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Several studies have shown that arginine vasopressin (AVP) potentiates the sympathetic nervous transmission in isolated vessels. The objective of the present study was to investigate this enhancement in the pithed rat model and to characterize the receptor subtypes involves by means of the selective antagonists, SR 49059 (V_1), SR 121463B (V_2), respectively and the V_2 agonist desmopressin.

Male Wistar rats weighing 270–310 g were used. In the experiments with the V_1-antagonist, SR 49059 10 mg/kg (p.o.), was given at least 60 min. before the measurements. The animals were anaesthetized using hexobarbital 100 mg/kg i.p., pithed and subsequently artificially respirated. Blood pressure was monitored via a carotid arterial catheter. In the pithed rat the thoraco-lumbal (T5-L4) sympathetic nervous system (SNS) was electrically stimulated with 4 Hz. 15 minutes after the pithing procedure, either saline or the V_2-antagonist, SR 121463B 3 mg/kg were administered intravenously. Another 15 minutes later, spinal cord stimulation was applied in the presence or absence of the subpressor dose of AVP (1 pmol/kg/min), i.v.. In addition, the effect of AVP on post-synaptic α-adrenoceptor mediated responses was studied using exogenously administered noradrenaline (NA). Therefore dose-response curves (DRC) to AVP (i.v.) and (NA) (i.v.) were constructed. Data are presented as means S.E.M., n=6-10 per group. Student's t-test was used for statistical analysis.

The pithed rat is a high-renin model, in which endogenously generated angiotensin II facilitates neurally mediated increments in vascular resistance. Without the administration of the AT_1-antagonist irbesartan (30 mg/kg), the facilitating effect of AVP was not visible, but after the administration of the AT_1-antagonist irbesartan, which decreased the diastolic blood pressure (DBP) to approximately 20 mmHg, the facilitating effect of AVP was apparent. The stimulation induced rise in DBP was 63.7 ± 4.5 mmHg, at a stimulation frequency of 4 Hz. This increase was facilitated in the presence of AVP (1 pmol/kg/min) to 78.6 ± 4.2 mmHg ($p<0.05$). The V_1 antagonist SR 49059 (10 mg/kg) completely inhibited this AVP-induced facilitation. Whereas the V_2 antagonist SR 121463B (3 mg/kg) or the V_2-agonist desmopressin (100 pmol/kg/min) had no influence. The dose response curve of exogenously administered NA was not influenced by AVP, even in the presence of the AT_1-antagonist irbesartan.

The facilitating effect of angiotensin II on the SNS in the pithed rat model masks the facilitating effect of AVP on this structure. This effect of vasopressin on sympathetic neurotransmission is completely dependent on the stimulation of presynaptically located V_1 receptors. This might be of importance in diseases like chronic heart failure, which are characterized by both high vasopressin plasma levels and an activated sympathetic nervous system.

210P THE SECOND PHASE OF ATP MEDIATED RELAXATION IS MEDIATED THROUGH P2Y11 RECEPTORS

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ATP is released by a range of vascular cells in response to stress. ATP induces vasodilatation in some vascular beds via the release of NO and PGI_2 from the endothelium, by way of $P2Y_1$ receptor activation (see Ralevic and Burnstock, 1998). Recently, we have shown that ATP can also stimulate vasodilatation is a sustained manner, by a NO and PGI_2-independent pathway (Stanford et al., 2001). This pathway resembles, in some respects, endothelial-derived hyperpolarising factor (Gitlin et al., 2001a). Furthermore and by contrast, to the initial transient NO-mediated vasodilatation induced by ATP, the sustained response does not appear to be mediated by $P2Y_1$ receptors (Gitlin et al., 2001b). Here we examine the effects of $P2Y_{11}$ ligands on vascular responses in order to further classify the effects of ATP.

Male Wistar rats (250-300g) were anaesthetised with sodium pentobarbitone (100 $mg.kg^{-1}$; *i.p.*) and sacrificed by cervical dislocation. The mesenteric artery was cannulated, the mesentery excised and perfused with Kreb's buffer (37°C; 95% O_2, 5% CO_2; $10ml^{-1}$). Perfusion pressure, measured by an arterial cannula, was raised to approx. 120 mmHg by methoxamine ($1x10^{-6}M$ – $1.2\ x10^{-5}M$). The effects of 1-3µl bolus doses of 2-meSATP, which activate $P2Y_1$ and $P2Y_{11}$ receptors or AR-C67085MX, which selectively activates $P2Y_{11}$ preceptors were recorded. All data are shown as mean±s.e.mean.

2-MeSATP ($3x10^{-11}$ to 10^{-9} moles) evoked a transient single phase dilator response. At doses of 10^{-8} to $3x10^{-7}$ moles this was accompanied by a second, sustained phase (Fig A). By contrast, AR-C67085MX evoked single phase vasodilator responses which resembled the second phase induced by 2-MeSATP (Fig B).

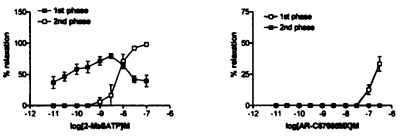

Figure 1 *shows the effect of A) 2-MeSATP (n=4) and B) AR-C67085MX (kindly supplied by Astra Zeneca; n=4) on first (filled) and second (clear) phase relaxation in the isolated rat mesenteric artery.*

Similarly to ATP (Stanford et al., 2001), 2-MeSATP induced biphasic vasodilator responses. Furthermore, AR-C67085MX (Communi et al., 1999) induced a vasodilator response that was sustained in nature. These observations, together with our previous work, suggest that the first and second phase of dilator response induced by ATP are mediated by separate receptors. We would suggest that the first transient phase of dilation is mediated by $P2Y_1$, whilst the second sustained phase is mediated by $P2Y_{11}$ receptor activation.

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211P CHARACTERIZATION OF THE INHIBITION MECHANISM OF CATECHOL *O*-METHYLTRANSFERASE WITH TWO NOVEL INHIBITORS, BIA 3-202 AND BIA 3-335

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BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone) and BIA 3-335 (1-[3,4-dihydroxy-5-nitrophenyl]-piperazine-1-propanone dihydrochloride) are novel peripheral catechol-O-methyltransferase (COMT) inhibitors. The aim of the present study was to characterize the type of interaction of BIA 3-335 and BIA 3-202 with recombinant rat soluble COMT (rS-COMT) at the functional and structural levels.

The rS-COMT was produced and purified as previously described (Bonifácio *et al.*, 2001). Enzyme activity was evaluated by measuring the formation of metanephrine, the *O*-methylated product of adrenaline, in the presence of a saturating concentration of S-adenosyl-L-methionine (SAM, 500 µM) (Bonifácio *et al.*, 2000). Apparent K_i values were determined from non-linear regression analysis of steady-state rate values obtained for different concentrations of the enzyme and inhibitors and adrenaline (1 mM). True K_i were obtained by dividing the apparent K_i by $(1+S/K_m)$ (Williams & Morrison, 1979). Results are arithmetic means with s.e.mean. Statistical differences were determined by ANOVA followed by the Newman-Keuls test.

The steady state between the enzyme and the inhibitors was evaluated with 460 nM rS-COMT in the absence or in the presence of 300 nM of either inhibitor and by starting the reactions with the enzyme or the substrate after a preincubation of 20 min. For both inhibitors, the inhibition of metanephrine formation was independent of preincubation time. The increase in adrenaline concentration led to a linear increase in IC_{50} values for both inhibitors, indicating a competitive type of inhibition. On the other hand, when SAM concentrations were varied, a linear increase in IC_{50} values was observed with the inverse of SAM concentration, which is indicative of uncompetitive type of inhibition. The tight-binding nature of BIA 3-202 and BIA 3-335 was evidenced by the asymptotic curves obtained when velocity was plotted against enzyme concentration at different inhibitor concentrations. BIA 3-202 was slightly more potent than BIA 3-335 ($K_i = 1.4 \pm 0.4$ and 6.0 ± 1.6 nM, respectively). Modeling studies based on the 3D-structure of the complex BIA 3-335 +r-S-COMT+SAM (Rodrigues *et al.*, 2001) were performed to predict the mode of binding of BIA 3-202. Both inhibitors interact with the substrate binding site having the same type of interactions between the nitrocatechol part and the enzyme active site.

It is concluded that BIA 3-202 and BIA 3-335 are potent COMT inhibitors of the fast tight-binding type interacting with the enzyme in a competitive manner.

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212P SUBCELLULAR EXPRESSION OF CHOLINE ACETYLTRANSFERASE IN AND RELEASE OF ACETYLCHOLINE FROM THE HUMAN ISOLATED PLACENTA

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Non-neuronal acetylcholine is ubiquitously synthesized in non- neuronal cells (Grando & Horton, 1997; Wessler *et al.*, 1998). However the knowledge about the subcellular distribution of choline acetyltransferase (ChAT) and the release of acetylcholine from non-neuronal cells is very scanty. The isolated human placenta was used as model to investigate ChAT expression by immunogold electron microscopy. Villus was homogenized and centrifuged to measure ChAT activity in the P1 (mitochondria and lysosome) and P2 (plasma membrane, microsomal fraction) pellet. Release of acetylcholine from isolated villus strips was measured by HPLC combined with bioreactors and electro-chemical detection.

ChAT activity was 0.35 ± 0.03 and 0.12 ± 0.01 (n=8) µmol h^{-1} g $protein^{-1}$ in the soluble and particulate fraction of homogenized villus, respectively. P1 and P2 pellets showed ChAT activities of 0.060 ± 0.007 and 0.09 ± 0.01 (n=15) µmol h^{-1} g $protein^{-1}$, respectively. Anti-ChAT immunogold electron microscopy was performed with a polyclonal rabbit anti-ChAT antibody. These experiments confirmed the wide subcellular expression. Immunogold particles conjugated to anti-ChAT antibodies were found within endosomes, cytoskeletal filaments, nucleus, plasma membrane and microvilli. In control experiments which were carried out in the absence of the specific primary antibody the investigated specimens did not show immunogold particles.

Villus strips incubated in organ baths showed a constant release of acetylcholine which could be measured in the absence of a cholinesterase inhibitor. This baseline release of acetylcholine amounted to 1.20 ± 0.05 (n=25) nmol g^{-1} wet weigth 10 min^{-1}. Release of acetylcholine was significantly reduced by substrate inhibitors of organic cation transporters (amiloride, cimetidine, verapamil). Noradrenaline, a substrate of organic cation transporters (subtype 1, 2 and 3), caused a concentration- dependent inhibition of acetylcholine release. A concentration of 0.1 µM noradrenaline was ineffective and 10 µM noradrenaline reduced acetylcholine release by 30 ± 5.8% (n= 6).

The human placenta is a useful model to characterize the non-neuronal cholinergic system. The experiments demonstrate a wide subcellular expression of ChAT in organelles associated with cell functions like proliferation, cytoskeletal activity, absorption and secretion. At least in the human placenta non-neuronal acetylcholine is released via organic cation transporters. Noradrenaline acts as a substrate inhibitor affecting the release of non-neuronal acetylcholine. This opens a new view about adrenergic-cholinergic interactions which can occur independently of receptors.

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213P LEVETIRACETAM AND PHENYTOIN SHOW GREATER EFFICACY AT REDUCING NICOTINE-KINDLED SEIZURES THAN PTZ-KINDLED SEIZURES IN MICE

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Most models of chemical kindling are closely linked to GABA-ergic mechanisms. We have recently demonstrated that daily administration of nicotine induced kindling in mice (Bastlund *et al.*, this meeting). In the following experiments we compared the effect of three different antiepileptic drugs on seizures induced in animals kindled by nicotine, or by pentylenetetrazole (PTZ). Each drug (phenytoin, tiagabine and levetiracetam) had different mechanisms of action.

NMRI mice (22 g at the start of the experiment) were kindled by repeated injection of either nicotine bitartrate (7 mg/kg, IP) every weekday for two weeks or by repeated injection of PTZ (37 mg/kg, IP) Mondays, Wednesdays and Fridays for 19 days. After this time, mice that did not show convulsions in response to their drug were discarded. Mice were then placed in groups of 12-15 for further studies. On the following 6 days each group of mice was tested daily as follows; Vehicle, Dose 1, Vehicle, Dose 2, Dose 3, Vehicle. Thirty minutes after these SC injections, the mice were injected with the convulsant drug with which they were kindled and placed in individual cages. The incidence of clonic convulsions was noted over the next 15 min. Results were expressed as the number of mice per group experiencing clonic convulsions, and an ED_{50} for suppression of kindled seizures was calculated by log-probit analysis. The ED_{50}s for each drug on PTZ and nicotine-induced seizures were compared by the method of Litchfield and Wilcoxon (1948).

The results in table 1 show that in the kindled animals, tiagabine suppressed of both PTZ and nicotine induced seizures, whilst levetiracetam was markedly more potent at reducing the nicotine induced seizures than PTZ ($P < 0.05$). Finally, phenytoin was ineffective at reducing PTZ-induced seizures (at doses up to 40 mg/kg), whilst it significantly suppressed nicotine-induced seizures ($P < 0.05$)

These experiments demonstrate that whilst the process and symptoms of kindling induced by nicotine and PTZ may appear similar, the pharmacology of the kindled state is quite different. These experiments indicate that PTZ kindling may be a model of "phenytoin resistant" seizures; whilst exploring the reasons for levetiracetam's greater efficacy in the nicotine kindled model may help elucidate this compound's, as yet unidentified, mechanism of action.

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Litchfield, J.T. & Wilcoxon, F. (1948) J. *Pharm. Exp. Ther.*, 96, 99-113

Table 1: ED_{50} ± s.d.(mg/kg) for inhibition of seizures in mice kindled with PTZ or nicotine (* $P < 0.05$ ED_{50} on PTZ cf nicotine)

Drug	ED_{50} (mg/kg) - Nicotine kindled seizures	ED_{50} (mg/kg) - PTZ kindled seizures
Tiagabine	0.39 ± 0.04	0.38 ± 0.04
Levetiracetam	1.41 ± 0.27 *	13.6 ± 4.6
Phenytoin	9.67 ± 1.70 *	> 40

214P PHYSIOLOGICAL AND BEHAVIOURAL MONITORING IN RATS IN RESPONSE TO PHARMACOLOGICAL AGENTS AND LABORATORY STRESSORS WITH BATTERY FREE TELEMETRY.

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Bioradiotelemetry offers a sensitive and reliable means to monitor physiological parameters in laboratory animals. A limiting feature of many bioradiotelemetric systems is the necessity for battery powered transmitters limiting the length of a study to the lifetime of the implanted battery. In the present investigation we describe the use of a new commercially available telemetry and data acquisition system to concurrently record heart rate, body temperature and activity of freely behaving rats with battery-free transmitters. The system uses PDT 4000HR E-Mitters (Mini Mitter Co., Inc., USA) to acquire data. E-Mitters obtain power from a radiofrequency field produced by an ER-4000 energizer/ receiver so that implanted transponders can collect data on heart rate, body temperature and gross motor activity for the lifetime of the animal. ER-4000 energizers/receivers are designed to be placed below the implanted animals cage. Data output from receivers is managed by Vital View™ an integrated Windows PC based data acquisition system,

Adult male Sprague Dawley rats (250-300g) were used in all studies. E-Mitters were implanted intra-abdominally under chloral hydrate (375 mg/kg, IP) anaesthesia. Animals were allowed 14 days to recover following surgery prior to any experimental testing. In all tests there were 4-5 animals per group. All data represent peak effects and are expressed as mean ± s.e mean change from pre-drug baseline. Data were analysed by repeated measures ANOVA followed by Fisher's LSD post hoc test.

Home cage 24 hour rhythms in heart rate, body temperature and activity ranged between 300-340 beats per minute (bpm), 36–37 °C and 0–50 counts/hour during day time (light) and 360-400 bpm, 37.5–38 °C and 150-300 counts/hour during the night (dark), respectively.

Administration of the non-selective dopamine agonist apomorphine (3 mg/kg, SC) induced tachycardia [peak change of +51±19 bpm, 120 min post challenge (PC)], hypothermia (peak change of –2.3±0.4°C 60 min PC) and hyperactivity (peak change of +48±17 counts 50 min PC). The α_2 adrenoceptor agonist clonidine (0.4 mg/kg, IP) induced bradycardia (peak change of –127±7 bpm 175 min PC) and hypothermia (peak change of –3.6±0.7°C 190 min PC). Exposure of the animals to air puff (5 puffs each minute for 5 minutes) induced a tachycardia (peak change of +79±13 bpm, 10 min PC), hyperthermia (peak change of +0.6±0.2 °C 25 min, PC) and hyperactivity (peak change of +11±1 counts 10 min, PC). Paired housing (implanted animal housed with an unfamiliar rat of similar sex and weight for 2 hours) also induced tachycardia (peak change of +161 ± 7 bpm, 5 min PC), hyperthermia (peak change of +2° ± 0.1 °C, 35 min PC) and hyperactivity (peak change of +81 ± 8 counts, 5 min PC). Effects reported are significant changes from baseline ($P<0.01$).

In conclusion, battery free PDT-4000 HR E-Mitters with Vital View™ data acquisition offer a valid and reliable means to monitor heart rate, body temperature, motor activity following pharmacological and environmental challenge to freely behaving laboratory rats.

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215P POTENTIATION OF MUSCIMOL-INDUCED LONG-TERM DEPRESSION BY BENZODIAZEPINES BUT NOT ZOLPIDEM

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Long-term depression (LTD) is a long lasting decreases in synaptic activity that follows some type of electrical stimulation in the hippocampus (Akhondzadeh & Stone, 1995). There is growing evidence that LTD does, in fact, occur in the nervous system and is believed to be involved in learning and memory processes (Akhondzadeh & Stone, 1996a). We have recently reported a new protocol for inducing LTD through activation of $GABA_A$ receptors in the hippocampal slices (Akhondzadeh & Stone, 1996a,b, 1999). This type of LTD is reversed by bicuculline and potentiated by neurosteroids. It was also shown that glutamate receptor activity or extracellular calcium are not involved in the induction of this type of LTD (Akhondzadeh & Stone, 1996b). In this study we used the CA1 region of rat hippocampal slices to compare the effects of diazepam and chlordiazepoxide, classic benzodiazepines, which binds equipotently to the BZ1and BZ2 sites and of non-benzodiazepine zolpidem, an imidazopyridine which binds preferentially to the BZ1 sites of $GABA_A$ receptors on the $GABA_A$ -induced long-term depression, a possible cellular mechanism for their different memory impairment profile.

Hippocampal slices 450 µm thick were prepared from male Wistar rats (170-210 g) and were superfused at 30°C with ACSF (in mM : KH_2PO_4 2.2, KCl 2, $NaHCO_3$ 25, NaCl 115, $CaCl_2$ 2.5, Glucose 10, $MgCl_2$ 1.2 saturated with 95% O_2/ 5% CO_2). Extracellular recordings of orthodromic potentials were made in the CA1 pyramidal cell layer following stimulation of Schaffer collateral fibres in the stratum radiatum (0.01 Hz).

Under normal experimental conditions, when Schaffer collateral/ commissural fibers were stimulated at a frequency of 0.01 Hz, the addition of $GABA_A$ agonist muscimol at concentration of 10 µM for 10 minutes induced a stable LTD of population potentials in which spike sized was reduced by 97.6%±0.1 (mean±s.e.m., $P<0.001$, n=4, Bonferroni post hoc test). The LTD induced by muscimol was concentration and time dependent as described previously (Akhondzadeh & Stone, 1995).

There was no sign of recovery for at least 120 minutes. Diazepam and chlordiazepoxide were superfused over the slices at concentrations of 10 and 20 µM. Neither compound had any effect itself on population potentials. However, both agents potentiated the ability of muscimol to induce LTD. A concentration of 2 µM of muscimol, which was not able to induce LTD was applied for 10 minutes in the presence of 10 µM diazepam or 20 µM chlordiazepoxide. These combinations of $GABA_A$ agonist and benzodiazepines proved able to induce LTD. Both agents either potentiate the inhibitory effect of muscimol on population spike or the ability of muscimol to induce LTD. As for muscimol alone, increasing stimulation frequency to 1 Hz for 10 seconds was sufficient to reverse the LTD and restore potentials to their control size (98% ± 2.2 of control, non-significant., n=4). In another set of experiment, zolpidem at concentrations of 10 and 20 µM failed to potentiate muscimol-induced long-term depression.

The results suggest that the potentiation of muscimol-induced LTD by diazepam or cholordiazepoxide and the lack of this effect by zolpidem may be an interesting explanation for their different cognition impairment profile.

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216P REGIONAL BRAIN CORTICOSTERONE CONCENTRATIONS: LACK OF CORRELATION WITH BLOOD LEVELS

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Many neuronal effects of the glucocorticoid hormone corticosterone have been reported (Joels, 1997), but few studies have compared the in vitro effects with the concentrations of corticosterone in the brain *in vivo*. Normally only blood hormone concentrations are measured. We now report regional brain corticosterone concentrations under different environmental conditions and the comparison with blood levels.

Male TO strain mice were used, housed 10 per cage, under normal light phase (lights on 08.00-20.00h). Mice were moved in their home cages into a preparation room 2h prior to the experimental procedures (all made 12.00h-13.00h) and after treatment were removed individually into a separate laboratory for killing. In the first experiment, animals were either given a single intraperitoneal injection of saline (Sal), handled as if intraperitoneal injections were to be made, or left undisturbed (No Treat.). In the second study, intraperitoneal injections of tween vehicle (0.05% Tween 80 in distilled water, Tw) or corticosterone 2 or 10 mg/kg in tween vehicle were given. Samples were taken 5 min and 30 minute after the treatments

Brain regions, hippocampus (Hipp), striatum (dorsal + ventral), and cerebral cortex (Cortex), were homogenised and extracted into 100% ethanol. Trunk blood was taken for total (Total) and free (Free) corticosterone assays. All concentrations were measured by radioimmunoassay (ICN). Statistical analysis was one-way analysis of variance and Newman-Keuls post hoc test; n=6 per treatment group.

Handling increased blood concentrations of corticosterone but did not increase concentrations in any brain region, compared with no treatment. Injections of saline (Table 1) significantly increased corticosterone concentrations in blood and hippocampus, with smaller increases in striatum and cerebral cortex. In contrast, systemic administration of corticosterone (Table 2) increased blood concentrations, and increased, then decreased striatal levels, but did not increase hippocampal or cerebrocortical concentrations compared with vehicle injection.

The brain corticosterone measured would include both that bound to receptors and that free in the cytosol. The results indicate that different changes can occur in regional brain corticosterone concentrations and that these do not parallel the blood concentrations.

Table 1. Corticosterone concentrations after injection of isotonic saline, mean± s.e.m.. *P<0.05 compared with home cage values.

Treatment	Hipp ng/g	Striatum ng/g	Cortex ng/g	Total nM	Free nM
No Treat.	9.1±0.3	6.3±0.2	3.7±0.2	261±5	50±1
Sal 5 min	18.5±1.4*	8.8±0.8*	5.4±0.5*	466±14*	89±3*
Sal 30 min.	17.3±1.3*	8.2±0.1*	5.5±0.4*	661±30*	127±6*

Table 2. Corticosterone concentrations mean ± s.e.m. after injection of corticosterone 10 mg/kg (Ct 10). A similar pattern was seen with 2 mg/kg corticosterone *P<0.01 compared with Tween vehicle.

Treatment	Hipp ng/g	Striatum ng/g	Cortex ng/g	Total nM	Free nM
Tw 5 min	6.5±0.2	4.5±0.2	3.9±0.4	105±8	21±1.4
Tw 30 min	6.6±0.3	3.6±0.7	4.3±0.4	506±37	97±7
Ct 5 min	6.6±0.5	15.1±3.0*	4.8±0.6	739±14*	140±3*
Ct 30 min	2.1±0.3*	2.2±2.7	2.2±0.3	1029±9*	196±2*

Joels M (1997) *Front. Neuroendocrin.* 18, 2-48

217P CHARACTERIZATION OF BRADYKININ-INDUCED PROSTAGLANDIN E_2 RELEASE FROM ADULT RAT TRIGEMINAL NEURONES

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The inflammatory mediator, bradykinin (BK) is a known activator of nociceptive sensory neurones that has been shown to stimulate meningeal primary afferents and increase the firing rate of second order neurones in the trigeminal nucleus caudalis (Ebesberger *et al.*, 1997). These actions suggest a potential role for BK in the pathogenesis of migraine headache. Also implicated in migraine is the arachidonic acid derivative, prostaglandin E_2 (PGE_2). In the present study we have investigated the effects of BK on the release of PGE_2 from cultured adult rat trigeminal neurones.

Primary cultures were derived as previously described (Carruthers *et al.*, 2001). Neurones were maintained in culture for 4-6 days in the continuous presence of nerve growth factor (50 ng ml^{-1}) and cytosine-β-D-arabinofuranoside (20 μM). The release of immunoreactive PGE_2 was determined using an enzyme immunometric assay kit (Cayman Chemicals, Ann Arbor, USA) and quantified in pg ml^{-1}. To account for differences in neuronal numbers between wells, PGE_2 release was measured 30 min before and 30 min after exposure to drugs. Data are presented as the mean±s.e.mean from 3-8 independent experiments, unless otherwise stated. Statistical comparison between treatments was by one-way analysis of variance followed by the Tukey test.

Stimulation of the cultures with BK (1 μM) resulted in an increase in PGE_2 levels from 30 ± 2 pg ml^{-1} to 68 ± 7 pg ml^{-1}. This release of PGE_2 was not observed in control wells (35 ± 1 pg ml^{-1} & 13 ± 2 pg ml^{-1}, respectively) or in those treated with KCl (30 mM; 39 ± 7 pg ml^{-1} & 20 ± 7 pg ml^{-1}) or capsaicin (10 nM; 39 ± 7 pg ml^{-1} & 6 ± 3 pg ml^{-1}). The effects of BK (1 μM) were abolished by the B_2 receptor selective antagonist, HOE140 (1 μM; PGE_2 levels before and after treatment were 53 ± 14 pg ml^{-1} & 9 ± 1 pg ml^{-1}, respectively), but not by the B_1 receptor-selective antagonist, [Leu^8]desArg^9BK (1 μM; PGE_2 levels before and after treatment were 21 ± 11 pg ml^{-1} and 80 ± 28 pg ml^{-1}, respectively). In the presence of the non-selective cyclooxygenase inhibitor, piroxicam (10 nM-1 μM), the BK-induced PGE_2 release was concentration-dependently inhibited, and was abolished in the presence of 1 μM piroxicam. Likewise, the inclusion of the phospholipase A_2 inhibitor, $AACOCF_3$ (30 μM) also abolished BK-induced PGE_2 release (PGE_2 levels before and after stimulation were 22 ± 3 pg ml^{-1} and 6 ± 2 pg ml^{-1}, respectively). However, the inclusion of the nitric oxide synthase inhibitor, L-NAME (10 μM) did not significantly modify the observed PGE_2 release. PGE_2 levels before and after stimulation were 19 ± 7 pg ml^{-1} and 44 ± 8 pg ml^{-1} in the presence of BK (1 μM) and L-NAME, compared to 14 ± 4 pg ml^{-1} and 51 ± 13 pg ml^{-1} following stimulation with BK (1 μM) alone.

In conclusion, these data suggest that bradykinin, acting via B_2 receptors can cause PGE_2 release from cultured trigeminal neurones that is dependent on the activation of cyclooxygenase and phospholipase A_2, but not the secondary release of nitric oxide.

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218P THE EFFECT OF A SELECTIVE CANNABINOID CB_1 RECEPTOR AGONIST ON MECHANICAL EVOKED RESPONSES OF SPINAL NEURONS IN ANAESTHETISED RATS.

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The cannabinoid CB_1 receptor is present in the DRG and dorsal horn of the spinal cord, non-selective cannabinoid agonists are antinociceptive following spinal administration (Drew *et al.*, 200). Arachidonyl-2-chloroethylamide (ACEA), a derivative of anandamide, has been described as a selective CB_1 receptor agonist (Hillard *et al.*, 1999). Here, effects of spinal ACEA on mechanical punctate-evoked responses of spinal neurones was studied in halothane anaesthetised rats.

Extracellular recordings of convergent dorsal horn neurones were made in anaesthetised (1% halothane in 66% N_2O / 33% O_2) male Sprague Dawley rats (Chapman *et al.*, 1994). Neuronal responses to mechanical punctate stimulation (Von Frey hairs: 8, 12, 21, 45 and 80g for 10secs) of the receptive field were recorded. Responses were quantified as neuronal firing rate (Hz) during a 10sec stimulus duration. Behavioural studies have demonstrated that 15g stimulation evokes reflex withdrawal in awake rats suggesting that stimulation with >15g is nociceptive. Control responses were determined and the effects of spinal administration of ACEA (0.5-500ng/50µl) on evoked responses of spinal neurones was studied (n=5 rats). Data are presented as mean maximal effects and standard error of the mean; statistical analysis was performed using repeated measures ANOVA and Dunnett's *post hoc* test, compared to controls. The mean depth of recorded neurones was 712 ± 51µm. Mechanical stimulation of peripheral receptive fields with von Frey filaments evoked an incremental increase in spinal neuronal firing.

ACEA (0.5-500ng/50µl) significantly reduced the 8g evoked response of spinal neurones to 28 ± 12, 35 ± 14, 38 ± 21, and 18 ± 11% of control ($p<0.0001$) and 12g evoked response of neurones to 47 ± 10, 33 ± 15, 40 ± 20, and 38 ± 21% of control ($p<0.0001$).

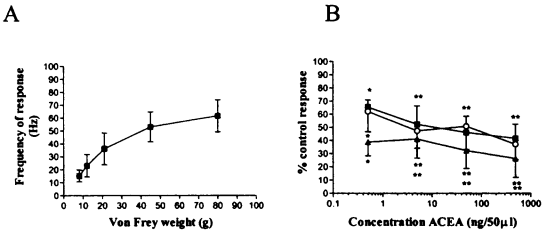

Fig 1. **A.** Control mechanical punctate-evoked responses of spinal neurones. **B.** Effect of ACEA on mechanical punctate-evoked (filled triangles: 21g, open circles: 45g, filled squares: 80,) responses of neurones. *$p<0.05$, **$p<0.01$.

Spinal ACEA also significantly reduced the 21, 45 and 80g evoked responses of spinal neurones (Fig 1B).

Our data demonstrate that spinal administration of a selective CB_1 receptor agonist can inhibit spinal responses to peripheral mechanical stimulation, which correspond to both innocuous and noxious stimulation.

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219P CANNABINOID INHIBITION OF CAPSAICIN-EVOKED SUBSTANCE P RELEASE FROM THE MOUSE SPINAL CORD, IS REVERSED BY NALOXONE

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Substance P (SP) is an excitatory neuropeptide transmitter, released by primary afferent nociceptors after stimulation. Cannabinoids acting on type 1 cannabinoid receptors (CB_1) in the spinal cord, can reduce SP release from nociceptor terminals stimulated by capsaicin, (Lever & Malcangio. In Press). This effect is likely to contribute to the antinociceptive action of intrathecally applied cannabinoids in pain models. Cannabinoid signalling may act directly to inhibit pre-synaptic neurosecretion of SP, or indirectly, by activating other inhibitory systems. In this study we investigated the mechanism of cannabinoid inhibition of capsaicin-evoked SP release from the mouse spinal cord *in vitro*.

Male CD1 mice (20-30g) were decapitated and the spinal cords removed. 16mm sections of the lumbar enlargement region were mounted in chambers and superfused (1ml/min) with oxygenated Krebs' solution, (Malcangio & Bowery 1993). Superfusate samples were collected in 8 min fractions. Four fractions were collected before cord stimulation to determine basal SP release levels. During collection of the fifth fraction, sections were stimulated by superfusion of a capsaicin solution (300nM) for 3 min. Three subsequent fractions were collected to assess release recovery. SP content in superfusates was measured by radioimmunoassay, (sensitivity 0.5 fmol/tube). Statistical analysis was performed by ANOVA followed by the Dunnett's test.

In control cord sections, superfusion of capsaicin significantly increased mean basal superfusate levels of SP (fmol/ml), from 3.65 ± 1.5 (mean ± s.e. mean, n=6) to 21.90 ± 3.9, ($p<0.05$). CB_1 agonist WIN55,212-2 (WIN-2) (100nM) was superfused 1 min prior to capsaicin stimulation, then co-superfused with capsaicin for 3 min. WIN-2 inhibited capsaicin evoked release of SP, (mean basal SP: 3.71 ± 0.5; capsaicin-evoked SP release: 3.95 ± 1.0, n=5). Co-superfusion of the CB_1 antagonist SR141716A (5µM) with WIN-2, reversed this effect, n=3. Superfusion of Naloxone (100nM) 8mins before & during capsaicin stimulation, also reversed WIN-2 inhibition of capsaicin-evoked SP release. Capsaicin evoked a mean SP release of 19.00 ± 4.3, which was significant above mean basal SP levels: 5.97 ± 0.6, n=6, ($p<0.05$). Naloxone alone, had no effect on either basal or evoked SP release.

These data indicate that cannabinoid (WIN-2) inhibition of SP release from central nociceptor terminals after capsaicin stimulation, is dependent on the functioning of naloxone sensitive opioid receptors in the spinal cord. This study supports existing evidence for the synergistic action of cannabinoid and opioid signalling systems in spinal cord antinociception.

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220P REGIONALLY SELECTIVE ALTERATIONS IN AMPA AND NMDA RECEPTOR SUBUNIT mRNA EXPRESSION IN RAT BRAIN FOLLOWING REPEATED ADMINISTRATION OF Δ^9-TETRAHYDROCANNABINOL

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In humans, the neural mechanisms underlying sustained cannabis use and accompanying deficits in short-term memory (Miller & Branconnier, 1983) and executive function (Solowiji, 1995) are incompletely understood. The neural substrates implicated in these effects (nucleus accumbens, prefrontal cortex and hippocampus) display dense glutamatergic innervation, and glutamate is known to mediate forms of synaptic plasticity. Furthermore, cannabinoids decrease presynaptic glutamate release (Shen et al, 1996). Therefore we hypothesise that some of the long-term effects of cannabinoids may be mediated by regional alterations in glutamatergic transmission. The aim of this study was to investigate whether repeated cannabinoid exposure produces regional changes in expression of mRNA encoding ionotropic glutamate receptor subunits in the rat, at doses comparable to human use (Agurell et al, 1986).

Sixteen male Long-Evans hooded rats (250-300g) were administered 0.01 mg / kg Δ^9-tetrahydrocannabinol (THC) or vehicle (0.9% NaCl; 0.1% Tween 80) i.p. (n = 8 / group) once per week for 4 weeks and again on week 7. Brain tissue was processed for *in situ* hybridisation using radiolabelled oligonucleotide probes complementary to NR1 and NR2a NMDA receptor subunit mRNA, and GluR1 and GluR2 AMPA receptor subunit mRNA. Regional mRNA expression was quantified using computer – based densitometry.

Two-way ANOVA of relative optical density values revealed a significant effect of drug treatment on GluR1 expression in the prefrontal cortex ($p = 0.03$), GluR2 expression in the hippocampus ($p = 0.01$) and NR2a mRNA expression mRNA expression in the hippocampus ($p = 0.002$) and striatum ($p = 0.001$). Post – hoc analysis revealed that, in the THC-treated group, GluR1 mRNA expression in the prelimbic area of the prefrontal cortex was significantly reduced compared to control (vehicle 0.165±0.018; THC 0.126±0.017), while NR2a mRNA was significantly reduced in the CA3 hippocampal field (vehicle 0.281±0.012; THC 0.248±0.006) but increased in the shell (vehicle 0.014±0.002; THC 0.023±0.003) and core (vehicle 0.030±0.003; THC 0.042±0.004) of the nucleus accumbens in THC-treated compared to control groups. Drug treatment did not significantly alter NR1 mRNA expression in any of the regions analysed.

These results suggest that repeated cannabinoid exposure may alter ionotropic glutamate receptor stoichiometry in areas involved in short-term memory (hippocampus), executive function (prefrontal cortex) and drug dependence (nucleus accumbens). Altered receptor responses to glutamate may therefore contribute to some of the behavioural and cognitive deficits following long-term cannabis use.

AE is supported by an AJ Clark Studentship.

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221P THE EFFECT OF INTRA-PERIAQUEDUCTAL GREY ADMINISTRATION OF THE CANNABINOID AGONIST HU210 IN A RAT MODEL OF AVERSION

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The midbrain periaqueductal grey (PAG) mediates behavioural responses to aversive stimuli (Bandler *et al.*, 2000). Chemical stimulation of the rat PAG with the excitatory amino acid D,L-homocysteic acid (DLH) induces aversive responses such as explosive running and jumping behaviour or immobility (Beckett *et al.*, 1992). The PAG contains CB_1 receptors (Tsou *et al.*, 1998) which may mediate some of the aversive effects observed following cannabis consumption in humans. This study investigated the effects of intra-PAG administration of the CB agonist HU210 (6aR-*trans*-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1 hydroxy-6,6-dimethyl-6H dibenzo [b,d]pyran -9-methanol) on DLH-induced aversive behaviour in rats.

Male Sprague-Dawley rats (250-300 g) were cannulated in the dorsal PAG (AP: –6.7 ML: + 1.7, DV: -4.1 mm relative to bregma and skull surface, 20° angle) under isoflurane/N_2O anaesthesia. Rats received an intra-PAG micro-injection of HU210 (0.1, 1 or 5 µg/250 nl) or vehicle (250 nl 60% DMSO in aCSF) 6-7 days post-surgery. Using Ethovision, behaviour was tracked 10 min post-HU210 and 5 min after an intra-PAG injection of DLH (5nmol in aCSF). Post-DLH data were split into two 2.5 min time-bins. Distance moved, velocity, grooming, rearing and jumping behaviour were measured. Data were analysed by one way ANOVA followed by Fisher's PLSD post hoc test.

DLH administration induced explosive running and jumping behaviour in 71% of rats. In these rats, HU210 (5 µg/250 nl) significantly reduced the maximum velocity during the first 2.5 min min 2.5 min post-DLH compared with vehicle controls (Table 1a). HU210 had no significant effect on the distance moved during the first 2.5 min post-DLH (Table 1a). The distance moved 2.5-5 min post-DLH was significantly less in HU210 (0.1, 1 and 5 µg/250 nl) treated animals compared with vehicle controls (Table 1b). The maximum velocity of movement during this period was also significantly reduced by HU210 compared with controls. HU210 did not significantly affect grooming, rearing or jumping behaviour post-DLH compared with controls.

(a)	Vehicle	HU210 (0.1)	HU210 (1)	HU210 (5)
Distance (cm)	2448 ± 223	3512 ± 699	2895 ± 820	1868 ± 767
Max Velocity (cm/s)	169 ± 10	168 ± 9	136 ± 17	122 ± 18*
(b)				
Distance (cm)	934 ± 243	220 ± 131*	217 ± 100*	203 ± 59*
Max Velocity (cm/s)	39 ± 7	11 ± 4**	11 ± 4.7**	17 ± 10*

Table 1. The effect of intra-PAG HU210 (0.1, 1 and 5 µg/250 nl) on DLH-evoked behaviour of rats (a) 0-2.5 min and (b) 2.5-5 min. Values expressed as mean ± s.e. mean (n = 6-14). *$P < 0.05$, **$P < 0.01$ compared with vehicle treated controls.

Our data demonstrate the presence of functional CB receptors in the PAG and their ability to modulate DLH-evoked aversive responses. The inhibitory effects of HU210 on the aversive behaviour may be due to cannabinoid mediated inhibitory modulation of efferent pathways involved in the behavioural response.

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