40P PULMONARY VASOCONSTRICTION BY DEXFENFLURAMINE IS NOT MODIFIED BY α_1-ADRENOCEPTOR ANTAGONISM OR PRE-TREATMENT WITH AN SSRI OR SNRI IN THE WISTAR RAT LUNG

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Intake of the 5-hydroxytryptamine (serotonin; 5HT)-releasing agent dexfenfluramine (dFEN), is a risk factor for the development of pulmonary hypertension (PH; Abenhaim *et al.*, 1996). Whilst dFEN is a substrate for the 5HT transporter and facilitates the release of 5HT (Rothman *et al.*, 1999), the causative link between dFEN-induced PH and 5HT, or any other vasoconstrictor, has not been proven. We have shown that dFEN causes vasoconstriction in the isolated blood-perfused Wistar rat lung preparation (IPL), which is unaffected by $5HT_2$ receptor blockade (Sisodiya *et al.*, 2000). In the current study, we have questioned whether pre-treatment with citalopram (CIT), a selective serotonin reuptake inhibitor (SSRI) or venlafaxine (VEN), a mixed serotonin and noradrenaline reuptake inhibitor (SNRI), could influence the dFEN-induced increase in pulmonary arterial pressure (Ppa). In addition, the response has been examined in the presence of prazosin, an α_1-adrenoceptor antagonist.

Male Wistar rats (8-12 weeks) were anaesthetised with sodium pentobarbitone (Sagatal; 60mg.kg^{-1}, intraperitoneally). Their lungs were isolated *in situ*, ventilated (air + 5% CO_2) and perfused with autologous blood at a constant flow (20 mL.min^{-1}, 39°C). Changes in Ppa, which was measured continuously (Acqknowledge v 3.0.1), reflect changes in pulmonary vascular tone. A cumulative dose response curve to dFEN (0.4-144.4 μM, ≤ 0.1 mL) was constructed, either alone (n= 5) or after pre-treatment with CIT (0.25 μM, 0.3 mL; n=6), VEN (3 μM, ≤ 0.3 mL; n=7) or prazosin (0.3 μM, ≤ 0.3 mL; n=5). Saline was given as a volume control. Statistical analysis employed ANOVA with Tukey's post-hoc test.

The doses of CIT and VEN were based on their affinities for the transporters (Owens *et al.*, 1997). 0.3 μM prazosin was chosen because it selectively abolished the increase in Ppa to phenylephrine, an α_1 agonist. Results are presented as the mean change in Ppa (ΔPpa ± SEM) from the pre-dFEN baseline.

dFEN caused a dose-dependent sustained increase in Ppa in all test groups (Table 1; ANOVA $p < 0.05$). Both CIT and VEN caused a small rise in Ppa (ΔPpa ± SEM CIT 1.4 ± 0.4; VEN 1.0 ± 0.1), but did not modulate the pulmonary pressor effects of dFEN. The pressor responses were also not altered by α_1-adrenoceptor blockade.

Table 1: Cumulative (mean ΔPpa ± SEM mmHg) to dFEN is not affected by pre-treatment with CIT, VEN or prazosin

		Pretreatment		
Cumulative dFEN (μM)	dFEN alone	CIT	VEN	Prazosin
Saline	0.1 ±0.2	0.0 ± 0.1	-0.2 ± 0.0	-0.2 ± 0.1
0.4	1.8 ±0.5	1.0 ± 0.3	1.6 ± 0.5	1.7 ± 0.2
4.4	2.8 ±0.7	2.3 ± 0.5	2.6 ± 0.7	3.2 ± 0.4
44.4	6.3 ±0.9	5.0 ± 0.8	5.8 ± 1.0	5.4 ± 0.6
144.4	10.3 ± 1.1	8.2 ± 1.0	9.5 ± 1.3	7.5 ± 0.8

In summary, these data suggest that dFEN does not require the 5HT/noradrenaline transporters or α_1-adrenoceptor stimulation to mediate its pulmonary vasoconstrictor action.

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41P TRIFLUOROMETHYLPHENYLIMIDAZOLE (TRIM) PRODUCES SELECTIVE INHIBITION OF CAPACITATIVE CALCIUM ENTRY IN SMOOTH MUSCLE

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Capacitative (store-operated) calcium entry (CCE) may play an important role in excitation-contraction coupling in several smooth muscles (Gibson et al., 1998). However, research into CCE is hindered by a paucity of drugs that selectively inhibit store-operated channels (SOCs) over other calcium entry processes. In this study, we report that trifluoromethylphenylimidazole (TRIM) produces selective inhibition of thapsigargin (Tg)-induced CCE in mouse anococcygeus, and that this effect is independent of its previously reported ability to inhibit nitric oxide synthase (Moore & Handy, 1997).

Anococcygeus muscles were dissected from male mice (LACA; 25-35 g) and set up for the recording of isometric tension responses to drugs and field stimulation (Wallace *et al.*, 1999). The Krebs solution contained phentolamine (1 μM), L-N^G-nitroarginine (L-NOARG; 50 μM) and verapamil (10 μM); in addition, muscles were pre-incubated with guanethidine (30 μM; 10 min). Verapamil was omitted when responses to high K^+ Krebs (60 mM) were to be investigated; L-NOARG was absent in studies involving field stimulation. Single smooth muscle cells were freshly dispersed from the mouse anococcygeus, loaded with FURA-2, and fluorescence ratio ($R_{340/380}$) recorded by methods described fully elsewhere (Wallace *et al.*, 1999). Results are given as mean ± s.e.m with a minimum n value of 5; analysis was by Student's t test.

Tg (100 nM) produced sustained contractions (550 ± 55 mg), which we have shown to be dependent on CCE (Wallace et al., 1999). Field stimulation (10 Hz for 10 s every 100 s) of the contracted tissue caused nitrergic relaxations which were abolished by 50 μM L-NOARG, but were unaffected by TRIM (1-333 μM). Rather, TRIM produced clear concentration-related relaxation of Tg-induced tone (pD_2, 4.38 ± 0.07; 94 ± 3% relaxation at 333 μM). High K^+ caused a similar sustained contraction (440 ± 40 mg) but in this case TRIM had little effect on tone, producing only a small relaxation (12 ± 5%) at the highest concentration used. As a comparison, papaverine was equally effective as a relaxant of tone induced by either Tg (pD_2, 5.55 ± 0.07) or high K^+ (pD_2, 5.75 ± 0.09).

In FURA-2 loaded smooth muscle cells bathed in calcium-free medium, 100 nM Tg caused a small, transient increase in fluorescence ratio; subsequent re-admission of 2.5 mM calcium caused a large, sustained increase (0.23 ± 0.02) which was significantly reduced by 200 μM TRIM (by 35 ± 12%). High K^+ did not produce the transient effect seen with Tg, but calcium again caused a sustained increase in fluorescence ratio (0.27 ± 0.02) which was, however, unaffected by TRIM.

Thus, TRIM provides a novel, selective inhibitor of CCE and should be useful in improving our understanding of this ubiquitous calcium entry pathway. It may also act as a template for the production of more potent analogues, free from the complication of nitric oxide synthase inhibition.

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42P PRELIMINARY PHARMACOLOGICAL STUDY OF THE HUMAN H_{3A} HISTAMINE RECEPTOR TRANSIENTLY EXPRESSED IN HUMAN EMBRYONIC KIDNEY (HEK) 293 CELLS.

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The cloning of the human H_{3A} receptor by Lovenberg *et al.* (1999) has revealed that it is a new member of the G-protein coupled receptor superfamily. We have transiently expressed the human recombinant H_{3A} receptor in HEK 293 cells and performed a preliminary pharmacological study using the novel radiolabelled H_3 receptor antagonist [^{3}H]-clobenpropit.

HEK 293 cells were cultured and transfected with pCI-neoH_{3A} (20 μg) using the calcium phosphate precipitation method (Chazot *et al.*, 1994). Transfected cells were harvested 24h post-transfection, cell homogenates were prepared (50-100 μg protein) and assayed immediately, using a [^{3}H]-clobenpropit (0.2 nM) binding assay essentially as described by Harper *et al.* (1999). 100 μM thioperamide was used to define non-specific binding. All data cited are mean ± SD values for 3-6 independent transfection experiments. [^{3}H]-clobenpropit specifically bound to H_{3A} receptors expressed in HEK 293 cells with a K_D of 0.96 ± 0.18 nM, and a B_{max} of 471 ± 37 fmol/mg protein. In competition studies, thioperamide and (R)-α-methyl histamine (RαMH) produced a concentration dependent inhibition of specific [^{3}H]-clobenpropit binding, with overall apparent IC_{50} values of 0.28 ± 0.08 μM and 14.79 ± 0.4 nM respectively, and pseudo Hill Coefficients (n_H) which were significantly less than unity (0.57 ± 0.07 and 0.55 ± 0.11, respectively). These data fitted best to a two component competition model. Thioperamide displayed a high affinity (IC_{50} = 0.13 ± 0.02 μM) and a low affinity (IC_{50} = 16.6 ± 1.25 μM) in the ratio 75:25 (high:low %, SD=9). RαMH displayed a high affinity (IC_{50} = 1.10 ± 0.06nM) and a low affinity (IC_{50} = 89.13 ± 3.29 nM) in the ratio 40:60 (high:low %, SD = 3). The presence of 5-guanylylimidodiphosphate (GppNHp) (0.1mM) elicited a rightward shift in the RαMH competition curve (n_H = 0.75 ± 0.07; overall apparent IC_{50} value of 31.62 ± 0.30 nM). These data again fitted best to a two component model, indicating that, in the presence of GppNHp, RαMH can still discriminate between two binding sites, (IC_{50} = 1.62 ± 0.09 nM and 60.26 ± 0.92 nM, respectively), but in the ratio 22:78 (high:low %, SD = 9). Heterogeneity of thioperamide binding has been noted previously in the mammalian brain (Harper *et al.*, 1999). The high affinity site corresponds well with the affinity of thioperamide for the recombinant human H_{3A} receptor expressed in other cell lines (Lovenberg *et al.*, 1999). The low affinity site may represent a population of non-histamine H_3 receptor sites, or a different conformational state of the H_{3A} receptor for which thioperamide can discriminate.

The sensitivity to GppNHp of the high-affinity RαMH binding component suggests that we have successfully expressed a G-protein coupled H_{3A} receptor in the HEK 293 cells. Therefore, we have a useful model system to further dissect the molecular pharmacology and signalling properties of the H_{3A} receptor.

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43P DECREASED SENSITIVITY TO PANCREATIC POLYPEPTIDE IN COLONIC MUCOSA FROM Y_2 RECEPTOR KNOCKOUT MICE

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Isolated descending colon mucosal preparations from normal (129SV) mice are sensitive to a range of neuropeptide Y (NPY) and peptide YY (PYY) analogues, including pancreatic polypeptide (PP; Cox *et al.* 2001). Pre-treatment with a combination of Y_1 (BIBO3304; Wieland *et al.* 1998) and Y_2 (BIIE0246; Doods *et al.* 1999) antagonists resulted in significant (95%) inhibition of PYY responses and, unexpectedly 50% of PP effects. We concluded that Y_1, Y_2 and Y_4 receptors are responsible for the atypical pharmacology in this tissue. The present study set out to determine how unconditional knockout of the Y_2 receptor gene altered Y receptor-mediated responses in the mouse intestinal mucosa.

Descending colon from male and female adult $Y_2^+/_+$ or $Y_2^-/_-$ mice was stripped of overlying smooth muscle and mucosae placed in Ussing chambers and voltage-clamped at 0 mV (Cox *et al.* 2001). Tissues were maintained at 37 C in oxygenated Krebs-Henseleit solution and once stable, peptide additions made to the basolateral reservoir, recording short-circuit current (I_{sc}) continuously. All preparations were pre-treated with vasoactive intestinal polypeptide (VIP, 30nM) before single additions of a Y agonist. Concentration-response curves were constructed from pooled data, and EC_{50} values (with 95% confidence limits) calculated using GraphPad Prism. Data groups were compared using Student's unpaired *t*-test with a significance level of $p \leq 0.05$.

Age-matched knockout mice were significantly lighter (25.0±0.6g, n=47, $p<0.05$) than their wild type littermates (29.2±0.7g, n=31). There were no significant differences between VIP responses in $Y_2^+/_+$ and $Y_2^-/_-$ colonic tissue. Subsequent antisecretory responses to PYY(3-36) resulted in an EC_{50} of 10.4 nM (2.1-50.8) in $Y_2^+/_+$ tissue but were absent (up to 100nM) in $Y_2^-/_-$ colon. In the latter residual PYY(3-36) responses were observed at 300nM and were abolished by BIBO3304 (300nM). The Y_1-preferred agonist, Pro^{34}PYY reduced VIP-elevated I_{sc} in both $Y_2^+/_+$ and $Y_2^-/_-$ tissue (EC_{50} values were 12.3nM (3.0-50.4) and 34.4nM (22.9-52.1) respectively). We observed a right shift in the concentration-response curve for human (h) PP in $Y_2^-/_-$ compared with $Y_2^+/_+$ colon (EC_{50} 24.9nM (7.0-89.3) and 4.1nM (1.6-10.4) respectively). BIBO3304 (300nM) inhibited hPP responses (>100nM) in both tissues.

Loss of sensitivity to PYY(3-36) in $Y_2^-/_-$ was predictable, however the reduced sensitivity to hPP was not. The decrease in hPP sensitivity and body weight in $Y_2^-/_-$ mice may be related to elevated circulating levels of PP (Sainsbury *et al.* submitted) and is consistent with the lean phenotype of PP over-expressing mice (Ueno *et al.* 1999). We suggest that loss of Y_2-mediated inhibition of PP release *in vivo*, results in elevations in PP levels with resulting Y_4 (and Y_1) receptor desensitisation in peripheral target tissues, including the colon.

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44P PANCREATIC POLYPEPTIDE RESPONSES IN COLONIC MUCOSAL AND SMOOTH MUSCLE PREPARATIONS FROM WILD TYPE AND Y_4 RECEPTOR KNOCKOUT MICE

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Y_1, Y_2 and Y_4 receptors mediate the antisecretory effects of peptide YY (PYY) and pancreatic polypeptide (PP) in mouse isolated colon mucosa (Cox *et al.* 2001). As yet the effects of Y agonists upon murine intestinal smooth muscle have not been established, however in rat colon a combination of Y_2, Y_4 (but not Y_1) receptor-mediated contractile effects have been observed (Pheng *et al.* 1999). Given that RT-PCR studies show Y_4 receptor expression in sub-epithelial, as well as epithelial layers of the rat large intestine (Goumain *et al.* 1998), our aim was to determine the effects of a Y_4-preferred agonist, PP, upon colon mucosal and smooth muscle preparations from wild type ($Y_4^+/_+$) and knockout ($Y_4^-/_-$) mice.

Mucosal tissue from descending colon was prepared and set up in Ussing chambers as described in detail previously (Cox *et al.* 2001). Changes in short-circuit current (I_{sc}) in response to PP and other Y agonists were recorded following pre-treatment with vasoactive intestinal polypeptide (30nM). Segments of ascending colon were cleared of lumenal contents, attached with thread and suspended in 10 ml organ baths containing oxygenated (95%O_2/5%CO_2) Krebs-Henseleit solution, maintained at 37°C. Tissues were stretched to a resting tension of 1 g and allowed to equilibrate (45 min) with intermittent washes. Isometric changes in basal tension were recorded for porcine (p) PP (30nM) in the presence or absence of tetrodotoxin (TTX, 200nM). The Y_1 and Y_2 antagonists, BIBO3304 (300nM) and BIIE0246 (1μM) were used as indicated. Data from the two tissues were pooled and statistical comparisons performed using Student's unpaired *t*-test. GraphPad Prism was used to calculate EC_{50} values (with 95% confidence limits).

The concentration-response curve for human (h) PP in mucosal preparations from $Y_4^+/_+$ colon was biphasic; the first phase saturating at ~ 100nM (EC_{50} 14.6nM (4.0-53.4)). Pre-treatment with BIBO3304 and BIIE0246 shifted the hPP curve to the right (EC_{50} 197.8nM (138.8-281.9)). $Y_4^-/_-$ mucosa was also sensitive to hPP, but these responses (≤100nM) were abolished by BIBO3304 ($p < 0.01$). The Y_1 antagonist also blocked Pro^{34}PYY (30nM) responses in $Y_4^-/_-$ mucosae ($p < 0.05$). Y_2-mediated PYY(3-36) effects (≤100nM) were similar in wild type and knockout mucosae.

Longitudinal muscle from $Y_4^+/_+$ and $Y_4^-/_-$ colon was stimulated by TTX and subsequent pPP increased basal tone (as observed by Pheng *et al.* 1999), in both $Y_4^+/_+$ (0.16±0.02 g , n=3) and $Y_4^-/_-$ tissue (0.19±0.05 g, n=3). BIBO3304 had no effect upon pPP responses in $Y_4^+/_+$ (0.19±0.03 g, n=3) but abolished them in $Y_4^-/_-$ colon (0.16±0.02 g, n=4, $p< 0.01$). We conclude therefore that PP can stimulate Y_1 receptors with resultant contractile and antisecretory effects in $Y_4^-/_-$ tissues. The lack of BIBO3304 sensitivity in $Y_4^+/_+$ muscle, indicates that the Y_1 receptor is not involved in wild type pPP responses, but following Y_4 deletion, Y_1 receptor expression occurs in this tissue.

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45P THE EFFECT ON CGRP-BINDING OF MUTATIONS TO THE HYDROPHILIC RESIDUES WITHIN THE FIRST TRANSMEMBRANE REGION OF HUMAN CALCITONIN RECEPTOR-LIKE RECEPTOR (CRLR).

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The 37 amino acid neuropeptide, calcitonin-gene related peptide (CGRP) is an extremely potent vasodilator. Functional CGRP receptors require the heterodimersation of a type II G-protein coupled receptor (GPCR) known as the calcitonin receptor-like receptor (CRLR) and a single-pass trans-membrane protein known as the receptor activity modifying protein 1 (RAMP1) (McLatchie *et al.*, 1998). The molecular basis of type II GPCR-ligand interactions has been studied extensively via the use of site-directed point mutations. The mutation of hydrophilic residues within the transmembrane regions of the secretin-receptor, for example, led to a marked reduction in ligand-affinity (Di Paulo *et al.*, 1998). This approach has been used to investigate the role of hydrophilic residues within the first transmembrane domain of CRLR. Histidine at position 128 (H128) and serines (S) at positions 131, 134 and 138 were mutated to alanine (A).

The genes encoding the mutant and wild-type CRLRs contained within the pcDNA3- mammalian expression vector were transiently transfected into a RAMP1-transformed HEK293 cell-line. Point mutations were created using the Stratagene Quick-change Mutagenesis method. Cells were transfected at 50-80% confluency on 10mm cell culture dishes according to the CalPhos transient transfection system (Clontech). Transfected cells were homogenised and CGRP-affinity measured via competition for ^{125}I-human CGRP by unlabelled human CGRP, as described previously (Poyner *et al.*, 1998). pIC_{50} (-log IC_{50}) values were calculated using Graphpad Prism version 3.00 and the mutant pIC_{50} values were compared to wild type using a one-way ANOVA followed by Dunnett's test.

Average pIC_{50} values ± standard errors (n 3) were 8.41 ± 0.12 for wild-type CRLR and 8.72 ± 0.18, 8.48 ± 0.23, 8.67 ± 0.60 and 8.22 ± 0.62 for the CRLR mutants, H128A, S131A, S134A and S138A respectively. None of the mutant pIC_{50} values was significantly different from that of wild type (i.e. $P>0.05$ for each mutant pIC_{50} compared with wild-type).

This study suggests that individual hydrophilic residues within the first transmembrane helix of CRLR are not crucial for CGRP-recognition or RAMP1 interaction. We are currently investigating the remaining helices.

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46P ADENOSINE RECEPTOR AGONISTS MEDIATE THE PHOSPHORYLATION OF MITOGEN ACTIVATED PROTEIN KINASE (MAPK) IN MCF-7 HUMAN BREAST CANCER CELLS.

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Adenosine is a purine nucleoside which exerts its effects via a family of G protein-coupled receptors of which 4 have been identified. Agonists and antagonists have been made for the A_1, A_{2A} and A_3 receptors but not for the A_{2B} receptor (Fredholm *et al.*, 1998). Adenosine has both cytoprotective and cytotoxic effects in a number of cells (Jacobson *et al.*, 1998). Mitogen activated protein kinase (MAPK), also known as extracellular regulated kinases 1\2 (ERK 1\2), is involved in the regulation of cell proliferation, differentiation, survival and death in response to growth factors (Wildmann *et al.*, 1999). This study investigated the effect of adenosine and other adenosine receptor agonists on the activation of MAPK in MCF-7 human breast cancer cells.

Studies were performed using MCF-7 cells which had been quiesced by serum starvation for 24 hours prior to lysis. The cells were treated with drugs made up in media and left at 37°C for different lengths of time. The treatment was stopped by washing the cells with PBS and the addition of lysis buffer. After lysis for 15 min, the cells were centrifuged and the supernatant stored at –20°C. The amount of phosphorylated MAPK was studied by Western blotting using a specific Phospho-ERK 1\2 antibody. Equal amounts of protein were loaded for each sample to allow comparisons to be made. All experiments are n=3 except for IB-MECA, n=2.

Incubating MCF-7 cells for 10 minutes with adenosine, 10^{-6}M, $3x10^{-6}$M, 10^{-5}M, $3x10^{-5}$M and 10^{-4}M, in the presence of dipyridamole (0.3μM), produced a concentration-related increase in phosphorylation of MAPK concentration dependence. A time course using adenosine (10^{-5}M) and dipyridamole (0.3μM) for 0, 5, 10, 20, 30 and 60 min showed that the phosphorylation of MAPK was time-dependent with the maximum apparent phosphorylation at 5 min. A variety of adenosine receptor agonists were incubated with the MCF-7 cells for 10 min at 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M. NECA (non-selective agonist) and CPA (A_1 agonist) caused concentration-dependent phosphoryl-ation of MAPK, whereas addition of IB-MECA (A_3 agonist) had no detectable effect on MAPK phosphorylation. Time courses using NECA and CPA at 10^{-5}M for 0, 5, 10, 20, 30 and 60 min showed that the phosphorylation of MAPK by these agonists was time-dependent with maximum apparent phosphorylation at 5 min.

This study has shown that A_1 and A_2 adenosine receptor agonists, including adenosine itself, can induce concentration- and time- dependent activation of MAPK in MCF-7 cells. This study would suggest that A_1 and A_2 receptors are found in these cells. A_3 receptors may also be present but do not appear to be coupled to the activation of MAPK. Given that MAPK is involved in cell proliferation and cell death these results suggest that adenosine may exert some of its cytoprotective and cytotoxic effects via the activation of MAPK.

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47P EVIDENCE FOR A ROLE OF PKA AND PROTEIN SYNTHESIS IN ENDOGENOUS SECRETIN RECEPTOR RESPONSIVENESS

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The G_s-coupled secretin receptor endogenously expressed in NG108-15 mouse neuroblastoma x rat glioma cells exhibits agonist-induced desensitization (Mundell *et al.*, 1997). We have previously shown that PKA and PKC can regulate heterologous desensitization of secretin receptor responsiveness (Ghadessy & Kelly, 2001). Here, we further investigated the specific role of PKA in this process.

Wild type NG108-15 cells, cultured as previously described (Mundell *et al.*, 1997), were seeded into 24 well plates. The PKA inhibitors H-89 (10 μM), H-7 (150 μM) and Rp-cAMPS (50 μM), and the protein synthesis inhibitor cycloheximide (10 μM) were added to the wells during agonist challenge. The phosphodiesterase inhibitor Ro201724 (100 μM) was also added to each well. With the exception of basal, cells were challenged with either secretin (100 nM), the adenosine receptor agonist NECA (10 μM), the IP-prostanoid receptor agonist iloprost (1 μM), or the adenylyl cyclase activator forskolin (10 μM). Whole cell cAMP accumulation was subsequently determined by a protein binding assay (Mundell *et al.*, 1997). Data are expressed as means ± s.e.mean.

Pretreatment of cells with H-89, H-7 and Rp-cAMPS resulted in an increase in secretin-stimulated cAMP accumulation after 60 min of agonist stimulation (cAMP accumulation in the presence of the PKA inhibitors was 176 ± 21%, 118 ± 4% and 194 ± 37% that of the control secretin response, respectively; n = 3-5). In contrast , cAMP accumulation stimulated by forskolin, iloprost and NECA was not affected by PKA inhibition (cAMP accumulation after 60 min of agonist stimulation in the presence of H-89 for forskolin, iloprost and NECA was 104 ± 18%, 118 ± 32% and 128 ± 15% that of the control agonist response, respectively; n=5).

Pretreatment of cells with cycloheximide led to a time-dependent increase in secretin-stimulated cAMP accumulation (cAMP accumulation after 60 and 120 min of stimulation in the presence of cycloheximide was 153 ± 5% and 323 ± 61% that of the control secretin response at the same time-point, respectively; n=7). Protein synthesis inhibition with cycloheximide also slightly increased cAMP accumulation due to iloprost and NECA, but to a smaller extent than with secretin (cAMP accumulation after 120 min of stimulation in the presence of cycloheximide was 176 ± 6% and 154 ± 25% that of the control agonist response, respectively; n=3-4). Protein synthesis inhibition did not affect forskolin stimulated cAMP accumulation (cAMP accumulation after 120 min of stimulation in the presence of cycloheximide was 128 ± 24% that of the control agonist response; n=5).

The results suggest that a novel pathway(s) involving cAMP-dependent PKA activation and protein neosynthesis may regulate secretin receptor responsiveness in NG108-15 cells. Such a potential feedback mechanism(s) may regulate secretin receptor responsiveness at the level of the receptor itself since adenylyl cyclase responses to other endogenous receptors, or directly through forskolin, were unaffected by PKA inhibition.

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48P FUNCTIONAL INTERACTION BETWEEN CASEIN KINASE Iα AND THE MUSCARINIC M_3 RECEPTOR

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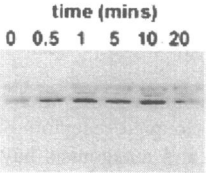
Phosphorylation of G protein-coupled receptors (GPCRs) primarily by G protein-coupled receptor kinases (GRKs) on sites within the third intracellular loop and C-terminal tail are thought to act as molecular switches and to mediate processes such as receptor desensitisation, sequestration and down-regulation (Ferguson, 2001). The mechanisms by which GRKs are able to co-localise with their receptor substrates are diverse buts includes isoprenylation of C-terminal CAAX motifs, palmitoylation of C-terminal cysteines, binding to free G-protein βγ-subunits and electrostatic interactions between GRKs and plasma membrane phospholipid (Ferguson, 2001). Recently, we have demonstrated that casein kinase Iα (CKIα) is able to phosphorylate the muscarinic M_3 receptor in intact cells (Budd et al., 2001), however unlike members of the GRK family, it is not apparent how CKIα may be localised to the appropriate intracellular compartment to allow interaction with the muscarinic M_3 receptor. Here we provide evidence of a direct interaction between CKIα and the third intracellular loop of the M_3-receptor *in vitro* and in intact cells and that this interaction can be modified by agonist activiation.

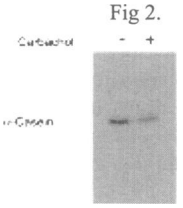
Chinese Hamster Ovary cells expressing the m3 receptor (CHOm3) were stimulated with 1mM carbachol (CCH) for 5 mins and cytosolic lysates produced. The cytosolic lysates were mixed with glutathione-S-transferase-muscarinic M_3 third intracellular loop fusion proteins. Associated CKIα was precipitated on glutathione sepharose beads and detected by western blotting using anti-CKIα antisera (fig 1). These experiments demonstrate an increase in cytosolic CKIα following M_3-receptor stimulation (fig 1).

Fig.1



CHOm3 were stimulated with 1mM carbachol for 5 mins and cells lysed in 2% CHAPS, 1M NaCl, 1% bovine serum albumin (BSA). Muscarinic M_3 receptors were then immunoprecipitated with M_3 receptor antisera. The immunoprecipitate was washed four times and was subsequently provided with exogenous α-casein in order to detect any associated kinase activity. Upon addition of agonist, the level of casein kinase activity associated with the muscarinic M_3 receptor was reduced suggesting that the tight interaction observed *in vitro* is modified by receptor phosphorylation in intact cells (fig. 2).

Fig 2.



We provide evidence in live cells that the receptor and kinase may interact directly and that this interaction can also be modified by agonist activation of the muscarinic M_3 receptor.

Budd DC et al., (2001) J. Biol. Chem. 276:4581-87.

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49P PREJUNCTIONAL RECEPTORS OF ANGIOTENSIN II AND BRADYKININ IN THE HEART OF NEWBORN RATS

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Cross-talk between α_2-autoreceptors and prejunctional receptors mediating a facilitatory effect on noradrenaline release was shown to be present in adult mouse atria (Cox *et al.*, 2000) or to be absent in adult rat tail artery (Mota *et al.*, 2000). The facilitation by angiotensin II of the transmitter release is possibly linked to protein kinase C (PKC) activation (Musgrave *et al.*, 1991). The present investigation was undertaken to further look for this kind of interaction in newborn rat heart.

Neonatal Wistar rats less than 24 hours old, with a body weight of 5.4-5.9 g were sacrificed by decapitation and the hearts dissected free. The organs were labelled with [^{3}H]-noradrenaline (0,2 μM), superfused with cocaine-containing medium and stimulated electrically for 4 min (1 Hz, 0.2 ms, 55 mV) at 120 (S_1), 160 (S_2) and 200 (S_3) min. In a first set of experiments the prejunctional effect of angiotensin II (10-300 nM) or bradykinin (3-100 nM) on tritium release was compared in the absence and in the presence of the α_2-adrenoceptor antagonist yohimbine (100 nM). The results obtained are shown in Table 1. In a second set of experiments the influence of angiotensin II and bradykinin on noradrenaline release was studied in the absence and in the presence of the PKC inhibitor chelerythrine (1 μM). The influence of chelerythrine is shown in Table 2.

Drug	*$pEC_{30\%}$*	*Maximal effect (%)*	*n*
Angiotensin II	7.37±0.20	65.08±7.68	5
Angiotensin II +yohimbine	6.59±0.15*	38.30±12.46*	5
Bradykinin	8.40±0.20	91.30±5.72	5
Bradykinin +yohimbine	7.75±0.10*	85.48±3.32	5

Table 1. Effect of angiotensin II and bradykinin on the overflow of tritium evoked by electrical stimulation. Shown are mean±SEM of *n* experiments. $EC_{30\%}$ represents the concentration of the drug that increased the evoked overflow by 30% ($pEC_{30\%}$ = negative logarithm of $EC_{30\%}$).*p<0.05 (Mann-Whitney test)

The results show that the blockade of α_2-autoreceptors of neonatal rat heart decreases the facilitatory responses mediated by angiotensin II and bradykinin receptors indicating the existence of cross-talk between them. Under these experimental conditions, angiotensin II- and bradykinin–mediated enhancements of noradrenaline release were unexpectedly increased by inhibition of PKC.

Table 2. Effect of angiotensin II and bradykinin on the overflow of tritium evoked by electrical stimulation

Drug	*$pEC_{30\%}$*	*Maximal effect (%)*	*n*
Angiotensin II	7.18±0.10	59.20±8.30	5
Angiotensin II +chelerythrine	7.79±0.20*	120.00±11.40*	5
Bradykinin	8.00±0.30	83.55±7.35	4
Bradykinin + chelerythrine	9.68±0.42*	110.82±10.40*	5

Shown are mean±SEM of *n* experiments. *p<0.05 (Mann-Whitney test)

Cox, S.L., Schelb, V., Trendelenburg., A.U., *et al.* (2000). *Br. J. Pharmacol.*, 129, 1095-1102.

Mota, A., Paiva, M.Q. Moura, D., *et al.* (2000). *Pharmacol. Res.*, 42, 383-387.

Musgrave, I.F., Foucart, S. and Majewski, H. (1991). *J. Auton. Pharmacol.*, 11, 211-220.

50P PERINUCLEAR $P2X_7$-LIKE IMMUNOREACTIVITY IN THE GUINEA-PIG VAS DEFERENS

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P2X receptors are adenosine 5'-triphosphate (ATP)-gated ion channels located in the plasma membrane. Seven subtypes have been cloned and designated $P2X_{1-7}$. The $P2X_7$ receptor is expressed on immune cells and is involved in apoptotic and immunological events. In addition to the plasma membrane, ion channels are also present in the nuclear envelope, allowing for fine control of intranuclear ion concentrations. ATP-dependent ion channel activity resembling $P2X_7$ receptor activation has been reported in the *Xenopus* oocyte nuclear envelope *in situ* (Mazzanti *et al.*, 1994). Therefore the aim of this study was to examine the cellular distribution of $P2X_7$ receptors using $P2X_7$ subunit-specific antisera.

Five male albino guinea-pigs (400-500 g) were killed by cervical dislocation. The vasa deferentia were fixed in 10% neutral buffered formalin, wax embedded and sectioned at 10 μm. Two rat $P2X_7$ subunit antisera were employed, each generated against different antigenic sequences near the carboxy terminus (Roche, USA: TWRFVSQDMADFAIL, residues 555-569; Alomone, Israel: KIRKEFPKTQGQYSGF KYPY, residues 576-595). In sections of guinea-pig vas deferens, $P2X_7$-like immunoreactivity was visualised using the avidin-biotin technique; nuclei were counterstained with haematoxylin. To establish whether these antisera possessed avidity for the guinea-pig $P2X_7$ receptor subunit we performed standard Western blotting experiments using membrane preparations from guinea-pig vas deferens nuclei and human embryonic kidney cells stably expressing rat $P2X_7$ receptors (HEK293-$P2X_7$). In sections, $P2X_7$-like immunoreactivity was predominantly seen in cell nuclei. Using the Alomone anti-serum, nuclei were stained in the longitudinal smooth muscle layers, with fewer stained in the oblique layer (estimated at 50% and 30% of total nuclei, respectively). Immunostaining was more intense in the outermost smooth muscle layer. A small proportion of nuclei were weakly stained in the lamina propria, but a large majority of the adjacent basement lamina nuclei were stained (~80%). All epithelial cell nuclei were intensely stained. Experiments using the antiserum provided by Roche gave a similar pattern of immunoreactivity. Negative controls (omission of the primary antisera or incubation with cognate peptide) displayed no specific staining. Western blotting with nuclear envelope or HEK293-$P2X_7$ membrane preparations revealed a single band of the correct molecular weight, which was lost upon pre-incubation with the antigenic peptide.

In summary, a similar pattern of immunostaining obtained using two different $P2X_7$ subunit-specific antisera raised against different parts of the $P2X_7$ subunit sequence, and the appearance of a single band in Western blots, strongly suggests antiserum specificity. Ion transport is a critical facet of nuclear envelope function. Prolonged activation of the $P2X_7$ receptor is typically associated with channel dilation and increased membrane permeablisation. It is possible, therefore, that a $P2X_7$-like protein may be expressed in some cell nuclei and may be involved in nucleocytoplasmic transport.

Mazzanti, M., Innocenti, B. and Rigatelli, M. (1994). *FASEB J.* **8**, 231-236.

51P CHARACTERISATION OF THE SITES OF ACTION OF ATP IN THE GUINEA-PIG ISOLATED VAS DEFERENS

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Adenosine 5'-triphosphate (ATP) is released as a cotransmitter with noradrenaline from sympathetic nerves in the guinea-pig vas deferens and acts at postjuctional $P2X_1$ receptors to evoke smooth muscle contraction (Sneddon *et al.*, 1996). However, a second site of action for ATP has also been identified in this tissue (Bailey & Hourani, 1994; Reilly & Hirst, 1996). The aim of this study was to further characterise this site.

Guinea-pig isolated vasa deferentia were mounted under isometric conditions in a modified Krebs solution at 37°C and bubbled with 95% O_2/5% CO_2. The P2 receptor antagonists suramin, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and pyridoxyl-5-phosphate (P-5-P) were added to tissues 30 min before agonist addition. Data were analysed statistically by one-way ANOVA with Tukey's comparison.

Contractions evoked by ATP (300 μM) were inhibited by PPADS (IC_{50} = 2.0±1.3 μM , n=5) and P-5-P (IC_{50} = 24.7±3.2 μM, n=4). PPADS was significantly more potent than P-5-P ($P<0.001$). However, neither abolished the contractions and 20-30% of the ATP response remained resistant. Suramin did not inhibit responses to ATP (300 μM) at all. Instead, the peak contraction amplitude was significantly potentiated in a concentration-dependent manner (45 ± 14% increase at 1 mM suramin, n=5). Suramin (1 mM) abolished reponses to 10 μM ATP (n=4), but significantly potentiated those to 100 μM and 1 mM ATP by 27 ± 5% (n=6) and 32 ± 5% (n=5) respectively ($P<0.01$). The potentiating effects of suramin against each concentration of ATP were not significantly different.

The nucleotidase inhibitor ARL 67156 (100 μM) potentiated contractions to ATP (100 μM) by 95 ± 9% (n=5), to ATP (300 μM) by 77 ± 9% (n=7) and to ATP (1 mM) by 49 ± 8% (n=5). When coapplied with ARL 67156, suramin (1 mM) now had no significant effect (n=5-7). Thus, when breakdown of ATP is prevented, suramin no longer potentiates contractions to ATP, but neither does it inhibit them. A supramaximal concentration of PPADS (30 μM) inhibited contractions to ATP (300 μM) to a similar extent in the absence (by 75 ± 3%, n=6) and presence (by 68 ± 4%, n=5) of ARL 67156 (100 μM).

Responses to ATP (300 μM, n=6) and α,β-meATP (1 μM, n=4) were abolished in a calcium-free solution containing 5 mM EGTA. Contractions evoked by noradrenaline (30 μM, n=5), acetylcholine (30 μM, n=4) and histamine (100 μM, n=2) were also abolished or virtually abolished.

These results show ATP evokes contractions via the $P2X_1$ receptor and a second site. The identity of this second site remains to be determined.

Bailey, S.J. & Hourani, S.M.O. (1994). *Br. J. Pharmacol.*, **112**, 219-225.

Reilly, M.J. & Hirst, G.D.S. (1996). *J. Auton. Nerv. Sys.*, **57**, 93-100.

Sneddon, P., McLaren, G.J. and Kennedy, C. (1996). *Seminars in Neurosci.*, **8**, 201-205.

52P INVESTIGATION OF α-ADRENOCEPTOR-MEDIATED RESPONSIVENESS OF AORTA FROM $\alpha_{2A/D}$-ADRENOCEPTOR KNOCK-OUT MICE

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Although $\alpha_{2A/D}$-adrenoceptors are expressed in rat aorta (Ping & Faber, 1993), their role in the contractile response remains to be clearly elucidated. Indeed, the predominant α_1-adrenoceptor component and the lack of selectivity of drugs make this study difficult. Thus, the use of engineered knockout mice would allow us to distinguish the respective participation of the adrenoceptor subtypes to the contractile response to noradrenaline (NA).

Thoracic aortas were dissected out from female wild-type (WT) and $\alpha_{2A/D}$ adrenoceptor knockout (KO) C57 Black mice (18-22 g). Endothelium denuded aortic rings (2-3 mm long) were mounted in a Mulvany wire-myograph filled with Krebs-Henseleit (37°C, 95% O_2/ 5% CO_2) solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, EDTA 0.03 and ascorbic acid 0.28. Propanolol (3 μM) was also present. Following 30-min equilibration period at the optimal passive isometric tension corresponding to 90 % of the vessel diameter at 100 mmHg, NA (10^{-9} to 10^{-5} M) was added cumulatively to the bath. Following 30-min wash-out and a further 30-min incubation time with the α-adrenoceptor alkylating agent chloroethylclonidine (CEC, 100 μM) or vehicle, the dose-response curve to NA was repeated. In a second series of experiments, following the first dose-response curve, arteries were challenged with NA (10 μM). At the plateau of contraction yohimbine (0.1 to 1 μM) or vehicle were added. Results were expressed as mean ± s.e.m. Significant differences ($P < 0.05$) were determined by one-way ANOVA plus Bonferroni test or Student's *t* test.

Aortas from knockout mice showed an increased maximal contraction in response to NA compared to wild-type mice (Table 1). CEC inhibited the maximal effect of NA-induced vasomotion on aortas of both WT and KO mice (Table 1). Yohimbine (1 μM) increased NA-induced contraction in WT but not in KO mice (Table 2).

Table 1: Maximal NA-induced contraction (mN) in absence or presence of CEC (100 μM) in WT and KO mice. *: $P < 0.05$ *vs* Vehicle; †: $P < 0.05$ *vs* WT.

	Vehicle	CEC
WT	0.93 ± 0.18 (n = 12)	$0.42 \pm 0.10^{*}$ (n = 7)
KO	$1.39 \pm 0.24^{\dagger}$ (n = 5)	$0.48 \pm 0.16^{*}$ (n = 5)

Table 2: Effect (% of initial contraction) of yohimbine (0.1 to 1 μM) on NA (10 μM)-induced contraction. *: $P < 0.05$ *vs* WT.

	Yohimbine		
	0.1 μM	0.3 μM	1 μM
WT (n = 6)	118 ± 4 %	132 ± 7 %	136 ± 10 %
KO (n = 6)	105 ± 8 %	105 ± 10 %	97 ± 10 %*

In conclusion, CEC failed to distinguish between knock-out and wild-type but an α_2-adrenoceptor-mediated relaxation component of the response to NA exists in these vessels.

Ping, P. & Faber, J.E. (1993). *Am. J. Physiol.* 265:H1501-H1509.

53P FUNCTIONAL CHARACTERISATION OF NOVEL α_2-ADRENOCEPTOR LIGANDS IN THE MOUSE *VAS DEFERENS*

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The α_2-adrenoceptor (AR) is known to be widespread in the CNS and has been implicated in a number of disease states, including depression (French, 1995). However, studies in patients have not been possible due to a lack of suitable imaging ligands. In the present study, the functional activity of a series of iodo-substituted derivatives of idazoxan, closely related to the highly selective α_2-AR antagonist, RX821002 (2-methoxy-idazoxan) has been investigated. These compounds would, potentially, lend themselves to ^{123}I-labelling for single photon emission computed tomography (SPECT).

Male mice (CBA, 20-30g) were killed by stunning and cervical dislocation. Their *vasa deferentia* were dissected out and mounted separately in 10 ml organ baths with magnesium free Kreb's solution, gassed with 5% CO_2, 95% O_2. The tissue was electrically stimulated every 10 secs (50 Hz, 2.5 sec duration) and the twitch response recorded. Cumulative dose-response curves for UK 14, 304 were obtained in the presence (added t = -5 min) and absence of the proposed antagonist, and the equilibrium dissociation constant calculated using the single dose method described by Osterlitz and Watt, (1968). The iodo-substituted compounds were also tested alone against the twitch response to determine any intrinsic activity.

The results show that all three of the novel α_2-adrenoceptor ligands antagonise UK 14, 304-induced inhibition of the twitch response. UK 14, 304 induced a dose-dependent inhibition of the twitch response with an EC_{50} = 15.10±2.72 nM (*n*=21 animals). The inhibition induced by UK 14, 304 was attenuated by the addition of the classical α_2-AR antagonist, rauwolscine and also RX821002 at 100nM (table 1). The three iodo-substituted compounds antagonised UK 14, 304-induced inhibition at 100nM and the calculated equilibrium dissociation constants are shown in table 1. 2-(4-Iodobenzyloxy)-idazoxan induced a dose-dependent inhibition of the twitch response with an EC_{50}=0.69μM. Neither 2-(3-iodobenzyloxy)-idazoxan nor 2-(3-iodopropoxy)-idazoxan had any effect on the twitch response at concentrations up to 10μM but induced a 20-40% inhibition at 0.1mM (the highest concentration tested).

Drug	**K_e ([Antagonist] = 100nM** ***Mean (nM) ± s.e.mean***
Rauwolscine	45.48 ± 20.14
RX821002	4.81 ± 1.32
2-(3-iodobenzyloxy)-idazoxan	14.62 ± 2.37
2-(4-iodobenzyloxy)-idazoxan	106.67 ± 27.18
2-(3-iodopropoxy)-idazoxan	4.06 ± 1.71

Table 1: K_e values for known α_2-AR antagonists and the novel iodo-substituted compounds in mouse vas deferens (n=4 animals).

These data show that all three iodo-substituted compounds antagonise UK 14, 304-induced inhibition in mouse *vas deferens* suggesting they are α_2-AR antagonists. However, these compounds also inhibited the twitch response at high concentrations and require further investigations to determine the mechanism underlying this effect. These results are comparable with the reported binding affinities for these compounds at α_2-AR in mouse brain (Finch *et al.*, This meeting). In conclusion, 2-(3-iodopropoxy)-idazoxan is the most potent antagonist and lacks any intrinsic activity at α_2-adrenoceptors, thus, making it the most suitable candidate for radio labelling and further characterisation.

French N. (1995) *Pharmac. Ther.* **68**, 175-208
Finch L., Tyacke R.J., Robinson E.S.J. *et al.*, This meeting
Osterlitz and Watt (1968) *Br. J. Pharmacol.*, **33**, 266-270

54P *IN VITRO* EVALUATION OF THREE POTENTIAL SPECT LIGANDS FOR THE CENTRAL α_2-ADRENOCEPTOR

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Single photon emission computed tomography (SPECT) is a powerful technique for studying receptor function *in vivo* by using the γ-emitting radioisotope iodine-123. The major drawback is the lack of suitable ligands for the receptors of interest. One such receptor is the α_2-adrenoceptor which has been linked in the pathology of a number of disease states including depression (French, 1995). Presented here are the binding affinities for three potential SPECT ligands; 2-iodopropoxy-idazoxan, 2-(3-iodobenzyloxy)-idazoxan and 2-(4-iodobenzyloxy)-idazoxan, in mouse and guinea pig whole brain membranes. These ligands are based on the classic α_2-adrenoceptor antagonist idazoxan, but are more closely related to the α_2-adrenoceptor antagonists 2-methoxy-idazoxan (RX821002) and 2-ethoxy-idazoxan (RX811059).

Guinea pig (male, Dunkin-Hartley, ~350g) and mouse (male, CBA, ~30g) brains were homogenised (10vol 50mM Tris-HCl buffer, pH 7.4 containing 320mM sucrose) and centrifuged (1000g, 10min). The precipitate was discarded and supernatant centrifuged (32,000g, 20min). The resulting P_2 membrane preparations were washed twice by centrifugation (32,000g 20min, 50mM Tris-HCl, pH 7.4) and frozen (-70°C) until use. Aliquots of thawed membrane (300μg protein) were incubated (45min, 22°C) with the selective α_2-adrenoceptor antagonist [^{3}H]RX821002 (1nM). Non specific binding was determined using rauwolscine (10μM). The test compounds were examined for their ability to compete with labelled [^{3}H]RX821002 over the range of 0.1nM - 10μM. Bound ligands were separated by filtration and determined by liquid scintillation counting. Data were analysed using the nonlinear regression analysis supplied with GraphPad Prism version 3.02 for Windows (San Diego California USA).

These data were best fit to single site model of inhibition of binding with Hill slopes close to unity. Table 1 shows the calculated equilibrium dissociation constants at the α_2-adrenoceptor in both mouse and guinea pig for the compounds tested. 2-Iodopropoxy-idazoxan and 2-(3-iodobenzyloxy)-idazoxan show the best affinity at the α_2-adrenoceptor with inhibition constants very close to those of RX821002 and RX811059. The lowest affinity of the three compounds was shown by 2-(4-iodobenzyloxy)-idazoxan, but it was the same as that of rauwolscine.

Table 1. Inhibition of [^{3}H]RX821002 binding to whole brain membranes

Compound	K_i (nM) ± S.D.	
	Mouse	Guinea Pig
2-Iodopropoxy-idazoxan	4.1 ± 0.3	2.6 ± 1.6
2-(3-Iodobenzyloxy)-idazoxan	6.8 ± 3.1	8.2 ± 1.5
2-(4-Iodobenzyloxy)-idazoxan	57 ± 31	40 ± 4
Rauwolscine	59 ± 13	38 ± 5.5
2-Methoxy-idazoxan (RX821002)	1.3 ± 0.5	1.1 ± 0.2
2-Ethoxy-idazoxan (RX811059)	3.0 ± 1.3	1.5 ± 0.4

Data represent the mean ± the S.D. for 3 separate experiments performed in triplicate

It is clear that all the iodo-substituted compounds compare very favourably with the known structurally related α_2-adrenoceptor antagonists RX811059 and RX821002, as well as the classic α_2-adrenoceptor antagonist rauwolscine. It has also been shown that all of these compounds antagonise the effects of the α_2-adrenoceptor agonist UK 14 304 in the isolated mouse *vas deferens* (Willmott *et al.*, this meeting). In summary, these compounds show good potential to be suitable for use as SPECT ligands by using iodine-123.

French, N. (1995) *Pharmacol. Ther.* 68, 175-208
Willmott, G, Robinson, ESJ, Tyacke RJ, *et al.*, this meeting

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55P OPPOSITE INFLUENCE OF α_2-AUTORECEPTOR ACTIVATION ON THE A_1- AND A_{2A}-ADENOSINE RECEPTOR: MODULATION OF NORADRENALINE RELEASE IN ISOLATED EPIDIDYMAL PORTION OF RAT VAS DEFERENS

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In the epididymal portion of the rat vas deferens, adenosine modulates noradrenaline release by activation of A_1-inhibitory and A_{2A}-facilitatory adenosine receptors (Gonçalves & Queiroz, 1993). In the present study we investigated the influence of the activation of α_2-autoreceptors on the modulation of noradrenaline release mediated by A_1- and A_{2A}-adenosine receptors in the epididymal portion of rat vas deferens.

Epididymal portions of vasa deferentia of Wistar rats (240-400 g) were incubated in 2 ml medium containing 0.1 μM ^{3}H-noradrenaline and perfused with ^{3}H-free medium at a constant rate of 1 ml min^{-1}. The medium contained (mM): NaCl 118.6, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.23, $NaHCO_3$ 25.0, glucose 10.0, ascorbic acid 0.3, dissodium EDTA 0.031, saturated with 95% O_2 and 5% CO_2 and kept at 37°C. Desipramine (400 nM) was added throughout superfusion (in some experiments 1 μM of yohimbine was also added). A total of 5 identical periods of stimulation were applied (8Hz, 1ms, 50mA, 100 pulses) every 30 min, starting at t=30 min (S_0-S_4; t=0 was the onset of perfusion). The stimulation evoked overflow of tritium was taken to reflect action-potential-evoked release of noradrenaline. Effects of drugs on tritium overflow were expressed as % from the respective control (solvent instead of drugs); showed is the mean ± s.e.m and n represents the number of tissue preparations. Results were analysed by the Student's unpaired t test; P<0.05 was taken to be statistically significant.

Adenosine (10-1000 μM) and NECA (30-300 nM; non-selective adenosine receptor agonist) decreased noradrenaline release, an effect more pronounced when α_2-receptors were blocked (1 μM yohimbine present). CGS 21680 (10-1000 nM; A_{2A}-adenosine agonist) increased noradrenaline release up to 147 ± 6 % (n =16; $P < 0.05$) but only up to 124 ± 3 % (n = 14; $P < 0.05$) in the presence of yohimbine. Either NECA (30-300 nM) or NBTI (5 μM; adenosine uptake inhibitor) in the presence of DPCPX (20 nM; A_1-receptor antagonist), increased noradrenaline release up to 147 ± 6% (n=13; P<0.01) and to 140 ± 9 % (n = 13; P<0.01), respectively. The effects of NECA and NBTI, in the presence of DPCPX (20 nM), were antagonised either by yohimbine or by ZM 24138 (20 nM; A_{2A}-receptor antagonist). However, yohimbine potentiated the facilitatory effect of isoprenaline (30-300 nM; β-adrenoceptor agonist) and of forskolin (10-1000 nM; adenylylcyclase activator) on noradrenaline release.

It is concluded that, in the epididymal portion of the rat vas deferens, α_2-autoreceptor activation reduces the A_1-receptor mediated inhibition but potentiates the A_{2A}-receptor mediated facilitation of noradrenaline release, the latter effect being distinct from that exerted by α_2-autoreceptors upon the β-adrenoceptor mediated facilitation of noradrenaline release and does not seem to occur at the adenylylcyclase level.

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Gonçalves J. & Queiroz G. (1993) Naunyn Schmiedeberg's Arch. Pharmacol., 348, 367-371.

56P AGMATINE AND IMIDAZOLINE SITE LGANDS IN THE MOUSE ISOLATED VAS DEFERENS

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Compounds capable of binding to the nonadrenergic imidazoline recognition sites can contain the imidazolidine, imidazoline, imidazole, oxazoline or the guanidino groups (Michel and Ernsberger, 1992). It has been shown previously that the mouse *vas deferens* may contain a presynaptic imidazoline binding site distinct from the presynaptic α_2-adrenoceptor (α_2-AR) (Slough & Taberner, 1999). The activation of either presynaptic imidazoline binding sites or α_2-AR leads to the inhibition of the electrically stimulated twitches. Since agmatine is known to have affinity for the imidazoline binding site, guanfacine and guanethidine are well characterised α_2-AR ligands but contain guanidino group, and the action of metformin and phenformin are not well known, we have examined the ability of the 5 compounds to inhibit the electrically-evoked twitches in isolated *vas deferens* in the presence of the α_2-AR antagonist rauwolscine.

Vasa deferentia from adult CBA/Ca mice (30-36g) were mounted singly under 0.3 g tension in a 10 ml bath between two platinum wire electrodes in Mg^{2+}-free Krebs Ringer bicarbonate buffer (pH 7.4) gassed with 95% O_2, 5% CO_2 at 37°C. Isometric responses were recorded following addition of imidazoline compounds, or following electrical stimulation (a train of 3x 0.1 msec 120 V rectangular pulses at 10 Hz, every 10 sec) in the presence of the imidazolines. The imidazoline S22954 was used as a positive control for α_2-AR agonism (Slough *et al.*, 2000). EC_{50} values are shown with 95% confidence intervals (in parentheses)

Agmatine, guanfacine, guanethidine were capable of complete inhibition of electrically evoked twitches. EC_{50} values for S22954, agmatine, guanfacine and guanethidine were 110 nM (38-440), 1.3 mM (1.1-3.1), 65 nM (27-160) and 3.6 μM (2.7-4.9). The inhibition of the twitch response by S22954, guanfacine and guanethidine was reversibly blocked by rauwolscine (0.1 μM), EC_{50} values are 0.582 μM (0.24-1.4), 0.2 μM (0.086-0.45) and 9.4 μM (6.3-14) respectively. Rauwolscine (0.1 μM) had no effect on the inhibition caused by agmatine. However, rauwolscine (1μM) caused a leftward shift of the dose-response curve and a decreased EC_{50} value to 0.19 mM (0.078-0.48; $p<0.05$, Student's t-test). Neither metformin nor phenformin inhibited the twitch response. In unstimulated tissue, guanfacine and metformin reduced the contraction evoked by 50 μM noradrenaline; S22954, guanethidine, agmatine, metformin had no effect.

We conclude that agmatine inhibited the twitches and that the effect was not mediated through α_2-AR but possibly through the imidazoline-binding site, since this effect was not blocked by rauwolscine. Guanfacine and guanethidine both act via the presynaptic α_2-AR with guanfacine mediating some effects on the postsynaptic α_1-AR. Metformin was shown to possess some affinity for the postsynaptic α_1-AR but neither biguanide was acting at the imidazoline site.

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57P EFFECT OF NITRIC OXIDE SYNTHASE/GUANYLATE CYCLASE INHIBITION ON THE RAT VAS DEFERENS CONTRACTILITY AND NORADRENALINE EFFLUX

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In the male reproductive tract, nitric oxide (NO) can act as a potent dilator of *corpus cavernosum*, contributing to penile smooth muscle relaxation. This effect can be inhibited *in vitro* by NO synthase (NOS) inhibitors (Pickard *et al.*, 1991). Contrariwise to this effect we observed in previous studies that N^G-nitro-L-arginine (L-NOARG), a NOS inhibitor, decreased the vas deferens contractility induced by phenylephrine (Phe) and noradrenaline (NE) (Pinto *et al.*, 1999).

In the present study, we investigated the influence of N^G–nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl –L-arginine (L-NMMA), two NOS inhibitors, 1H-(1,2,4)-oxadiazol-(4,3-a)quinoxalin-1-one (ODQ), a soluble guanylate cyclase (GC) inhibitor and 3-morpholinosydnonimine hydrochloride (SIN-1), a NO source, in the rat vas deferens contractility induced by Phe. We also have analysed the NE concentration in the perfusion fluid after incremental doses of Phe–0.0 (basal)-32.0μM–, in the absence and in the presence of L-NMMA or L-NMMA + SIN-1.

Male Wistar rats (250±30g) were anaesthetised with ether and then killed by exsanguination. The vas deferens were removed and placed in organ bath with Krebs-Henseleit solution at 37 °C. Contractile responses were measured using a force transducer, and dose-response curves (drc) were obtained using incremental doses of Phe (1.0-32.0μM) in the absence (control) or presence of L-NAME (100nM) or L-NMMA (100nM) or ODQ (1μM) or SIN-1 (10μM) or L-NMMA + SIN-1 (100nM + 10μM) or ODQ + SIN-1 (100μM + 10μM).

The NE efflux was evaluated by HPLC with electrochemical detection in absence or in presence of L-NMMA (100nM) or L-NMMA + SIN-1 (100nM+10μM). Phe-drc parameters (E_{max}, $EC_{50\%}$) were calculated by a non-linear regression analysis. According to the protocol the results – mean (m) ± standard error mean (se) - were compared using Student's *t* test or one-way ANOVA analysis.

Results: Effect of the tested compounds on the Phe-drc (m±se)

Tested compound(s) (100%)	Phe E_{max} %
L-NAME 100nM (1241±107mg)	81±4*
L-NMMA 100 nM (1915±165mg)	80±6*
ODQ 1 μM (1615±74mg)	89±2*
SIN-1 10 μM (1563±129mg)	108±7
L-NMMA 100nM+SIN-1 10μM(893±90mg)	97±2
ODQ 100μM+SIN-1 10μM(1066±146mg)	75±3*

(n=8;*P *vs* Control -100%- <0.05)

No changes were observed in $EC_{50\%}$. A dose-dependent increase of NE efflux was observed in the perfusion fluid after Phe. (n=8, P<0.05 *vs* basal). Such increase was not observed in the presence of L-NMMA (n=8). No differences were observed in the NE efflux between absence and presence of L-NMMA+SIN-1 (n=6).

The results suggest that in rat vas deferens the decrease of Phe-induced contractility by NOS inhibition may be related to a decrease of NE efflux in such preparation. This decrease seems to be dependent to the inhibition of NO/cyclic guanosine monophosphate (cGMP) signalling pathway.

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58P BIA 3-202, A FAST AND COMPETITIVE TIGHT-BINDING CATECHOL-*O*-METHYLTRANSFERASE INHIBITOR

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BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone) is a new reversible tight-binding catechol-O-methyltransferase (COMT) inhibitor (Vieira-Coelho *et al*, 2000). The present study was aimed at evaluating the time dependent kinetic inhibitory profile of BIA 3-202 and the type of interaction of BIA 3-202 with the substrate and co-substrate binding sites in rat liver soluble COMT (S-COMT).

S-COMT was isolated from saline perfused livers, obtained from pentobarbitone (60 mg kg^{-1}) anaesthetised male Wistar rats (240-260 g; Harlan, U.K.). Tissues were homogenised in 5 mM phosphate buffer, pH 7.8, and centrifuged at 15,000 g for 20 min at 4°C. The high-speed supernatants (100,000 g for 60 min at 4°C) were used as the soluble fraction of liver COMT. COMT activity was evaluated by the ability to methylate the substrate adrenaline to metanephrine in the presence of a saturating concentration (500 μM) of the methyl donor, S-adenosyl-L-methionine (SAMe) as described by Borges *et al* (1997). Results are arithmetic means with s.e. mean, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

To determine whether BIA 3-202 act as fast or slow inhibitor, progress curves were obtained either by starting the reaction with the enzyme or, alternatively, by adding the substrate (adrenaline) to the enzyme (S-COMT) preincubated for 15, 30 or 60 min with BIA 3-202 (30 nM). The inhibitory effect of BIA 3-202 was not changed by preincubation times. In fact, no differences were observed in the metanephrine formation rates (in nmol mg $protein^{-1}$ min^{-1}) obtained with no preincubation and with 60 min preincubation with 30 nM of BIA 3-202 (1.30±0.02 and 1.35±0.03, respectively). This indicates an almost immediate interaction with the enzyme after mixing. Also, in experiments initiated with the enzyme (no preincubation), linearity of progress curves was observed independently of BIA 3-202 concentrations (3, 30 and 100 nM). This is indicative that the steady-state velocities were reached upon mixing. The metanephrine formation rate (in nmol mg $protein^{-1}$ min^{-1}) in control conditions (1.92±0.03) was significantly ($P<0.05$) decreased to 1.76±0.01, 1.30±0.02 and 0.56±0.03 in the presence 3, 30 and 100 nM of BIA 3-202, respectively. For substrate competition studies, IC_{50} values for BIA 3-202 were determined at different adrenaline and SAMe concentrations. An increase in the concentration of adrenaline (100, 200 and 1000 μM) resulted in a linear increase in IC_{50} values for BIA 3-202 (49.0±4.2, 59.8±4.0 and 67.0±2.4 nM, respectively), giving a straight line when plotting IC_{50} values against adrenaline concentrations. In contrast, when increasing concentrations of SAMe were used (10, 20 and 60 μM) a linear decrease in IC_{50} values (143.6±3.1, 115.4±0.7, 90.4±2.4 nM) was obtained.

In conclusion, BIA 3-202 behaves as a fast tight-binding COMT inhibitor that acts in a competitive manner to the substrate binding-site and uncompetitively to the methyl donor binding-site of rat liver S-COMT.

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59P ATYPICAL β-ADRENOCEPTOR-MEDIATED VASODILATATION IN RAT ISOLATED SMALL MESENTERIC ARTERIES

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Recent reports have described the presence of an atypical β-adrenoceptor in rat carotid artery (Oriowo, 1994) and thoracic aorta (Oriowo, 1995; Brawley *et al*., 2000), though there have been no reports of atypical β-adrenoceptors in resistance arteries. Atypical β-adrenoceptors are characterised by low affinity for classical β-adrenoceptor antagonists such as a propranolol (Oriowo, 1994) and activation by atypical β-adrenoceptor agonists, such as CGP 12177A (Mohell & Dicker, 1989). The aim of this study was investigate whether atypical β-adrenoceptors are present in small arteries of the rat mesentery.

Small mesenteric arteries (mean diameter 279.06 ± 18.18 μm) of virgin female Wistar rats (200 – 300 g), killed by CO_2 inhalation, were mounted on a wire myograph (Mulvany & Halpern, 1977). Arteries were preconstricted with phenylephrine (PE)(EC_{80}) and the integrity of the endothelium was assessed with acetylcholine (ACh) (1 μM and 10 μM). The arteries were then preconstricted with PE and cumulative concentration response curves to isoprenaline (ISO) (1 nM-100 μM) and CGP 12177A (100 nM-300 μM) were conducted, in the presence and absence of the β_1-/β_2-adrenoceptor antagonist propranolol (0.3 μM). Values are given as a mean ± S.E.M. and differences compared by students t test.

10 μM ACh produced a relaxation of 90 ± 2% (n=5) of PE induced tone. ISO produced a concentration-dependent relaxation of PE-induced tone. Propranolol shifted the ISO curve to the right with no reduction in the maximum relaxation (-logEC_{50}: control 7.28 ± 0.071, n=5; propranolol 5.75 ± 0.013, n=3, $P<0.0001$). Estimation of the magnitude of the shift from the EC_{50} values gave a 34-fold shift with a pK_b value of 8. CGP 12177A produced a concentration-dependent relaxation which was unaffected by propranolol (-logEC_{50}: control, 5.26 ± 0.43, n=5; propranolol 5.23 ± 0.084, n=4, $P>0.05$; % maximum response: control 103 ± 20, n=5; propranolol 100 ± 5, n=4, $P>0.05$).

We conclude that classical and atypical β-adrenoceptors co-exist in the rat mesenteric small arteries where they mediate vasodilatation. The role of atypical β-adrenoceptors in the small arteries in the control of peripheral vascular resistance is not yet known.

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60P AGONIST-DEPENDENT DIFFERENCES IN THE AFFINITY OF ICI 118551 AND CGP 12177 FOR ANTAGONISM OF β_2-AGONIST-STIMULATED GENE TRANSCRIPTION IN CHO-K1 CELLS EXPRESSING THE HUMAN β_2-ADRENOCEPTOR.

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ICI 118551 is a selective β_2-adrenoceptor inverse agonist with an affinity for the β_2-adrenoceptor in the nanomolar range (Hopkinson et al., 2000). CGP 12177 is also a high affinity compound with antagonist properties at the β_2-adrenoceptor (Bylund et al., 1994). We have previously reported that this latter compound is an order of magnitude more potent as an antagonist of isoprenaline-stimulated ^{3}H-cyclic AMP accumulation than of isoprenaline-stimulated cyclic AMP response element (CRE) - mediated gene transcription (Baker et al., 2001). In this study we have evaluated the effect of these two antagonists against isoprenaline and two clinically used β_2-adrenoceptor agonists (salbutamol and terbutaline) on CRE-mediated gene transcription in CHO-K1 cells.

CHO-K1 cells expressing the human β_2-adrenoceptor at 300fmol/mg protein and a secreted placental alkaline phosphate (SPAP) reporter gene under the trancriptional control of six CREs (McDonnell et al., 1998) were used in the present study.

Isoprenaline (EC_{50} 7.83 $\pm$ 2.39 nM; n=17) stimulated CRE-dependent SPAP secretion to yield a maximum response of 6.49 $\pm$ 0.18 fold over basal. Similar responses were seen with salbutamol (EC_{50} 2.77 $\pm$ 0.43nM n=14, E_{MAX} 101.40 $\pm$ 2.00% of 10μM isoprenaline response) and terbutaline (EC_{50} 5.25 $\pm$ 0.85nM n=14, E_{MAX} 93.95 $\pm$ 3.21%). As previously reported (Baker et al., 2001), CGP 12177 displayed partial agonist properties on this response (EC_{50} 0.18 $\pm$ 0.91 nM n=5, E_{MAX} 60.40 $\pm$ 3.01%).

ICI 118551 produced parallel shifts of the agonist concentration-response curves yielding apparent K_D values of 1.01 $\pm$ 0.43 nM (n=5), 0.14 $\pm$ 0.02 nM (n=7) and 0.13 $\pm$ 0.01 nM (n=7) for isoprenaline, salbutamol and terbutaline respectively. The values obtained with salbutamol and terbutaline were significantly different from those obtained with isoprenaline ($p < 0.05$; one-way Anova and Newman-Keuls multiple comparisons test).

CGP 12177 antagonized the responses to isoprenaline, salbutamol and terbutaline in a manner consistent with its partial agonist actions. Once again the dissociation constant for CGP 12177 obtained with isoprenaline as agonist (1.22 $\pm$ 0.29 nM; n= 7) was an order of magnitide greater than those obtained with salbutamol (0.097 $\pm$ 0.013 nM; n=7) or terbutaline (0.094 $\pm$ 0.020 nM n=8) as agonists ($p < 0.001$; one-way Anova, Newman-Keuls multiple comparisons).

The data from this study confirm the partial agonist actions of CGP 12177 in this CRE-mediated gene transcription system. The significant differences in K_D values for ICI 118551 and CGP 12177 obtained with the different agonists used suggests that different affinity states of the β_2-adrenoceptor may exist.

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61P EFFECTS OF $GABA_A$-RECEPTOR ACTIVATION ON ELECTRICALLY-EVOKED RESPONSES OF DORSAL HORN NEURONES IN CONTROL, SPINAL NERVE LIGATED AND SHAM OPERATED RATS *IN VIVO*.

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Changes in $GABA_A$-receptor binding have been reported following peripheral inflammation or neurectomy (Castro-Lopes *et al.*, 1995). The effects of intrathecal muscimol (MUS; $GABA_A$ receptor agonist) on electrically-evoked spinal neuronal responses were examined in anaesthetised neuropathic, sham and control rats.

Spinal nerve ligated (SNL, n=5) male Sprague-Dawley rats (120-140g) were prepared under halothane anaesthesia as previously described by Chapman *et al.* (1998). Sham-operated rats (n=5) and rats receiving no surgical intervention (n=7) were also studied. Development of allodynia was assessed with von Frey hair (10g) stimulation to measure the mechanical sensitivity of the ipsi- and contra-lateral hindpaws. Extracellular single-unit recordings of convergent dorsal horn neurones (L4-L6), ipsilateral to SNL or sham procedure were made in anaesthetised (1% halothane in 2:1 N_2O:O_2) rats two weeks post-surgery. Responses of neurones to receptive field electrical stimulation (16 stimuli @ 0.5Hz, x3 C-fibre threshold) were recorded every 10mins and classified as: Aβ 0-20ms; Aδ 20-90ms; C 90-300ms, post-discharge (PD) 300-800ms post-stimulus. Following stable control recordings, MUS, 0.1μg/50μl-30μg/50μl in saline (≡17.5μM-5.3mM), was applied onto the spinal cord and the effect of each dose was followed for 50mins. Statistical significance was assessed using one-way ANOVA with a Tukey's or Dunnett's post-hoc test as appropriate (* P<0.05; *** P<0.001).

SNL, but not sham, rats developed ipsilateral mechanical allodynia.

	Aβ	Aδ	C	PD
Control	103±22	82±25	423±70	256±85
Sham	143±9	70±17	489±86	468±78
SNL	123±13	128±20	490±79	360±86

Table 1. Mean evoked responses of neurones in control, sham and SNL rats. Values are mean number of action potentials±S.E.M.

	Aβ	Aδ	C	PD
Control	87±25	24±10***	40±13***	23±12
Sham	91±12	40±11*	61±18	39±23*
SNL	85±12	54±20	56±19*	47±26

Table 2. Effect of 3μg/50μl MUS (approximate EC_{50} value) on evoked responses of neurones in control, sham and SNL rats. Values are percentage of control±S.E.M. (* P<0.05; *** P<0.001).

Depth (846±32μm) and control evoked responses of spinal neurones were similar for the three groups (Table 1). MUS significantly reduced evoked-firing in control, sham and SNL rats in a dose-dependent manner. The effects of MUS (3μg/50μl) on evoked responses are shown in Table 2. There was no difference in effect of MUS between the three groups. The inhibitory effects of MUS on neuronal firing are in agreement with previous behavioural studies in neuropathic rats (Hwang & Yaksh, 1997). Data suggest that there is no change in the functional role of spinal $GABA_A$ receptors following tight ligation of L5 and L6 spinal nerves.

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62P THE SENSITIVITY OF SENSORY NEURONES TO P2 RECEPTOR AGONISTS DIFFERS IN INTACT AND DISSOCIATED RAT DORSAL ROOT GANGLIA

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In adult rat dorsal root ganglia (DRG) *in vivo,* Aα/β neurones have thick, myelinated axons, with a fast conduction velocity (CV), Aδ cells have thin, myelinated axons with intermediate CV and C cells have unmyelinated, slowly-conducting axons (Perl, 1992). We have shown that ATP activates an inward current in many neurones of dissociated neonatal rat DRG (Robertson *et al.*, 1996: Rae *et al.*, 1998). The aims of this study were a) to characterise the electrophysiological properties of neurones in intact DRG from neonatal rats *in vitro* and b) to compare the actions of ATP, α,β-meATP and capsaicin on neurones in intact and dissociated ganglia.

Neonatal (3-7 d) rat L4/L5 DRG, with attached sciatic nerve, were pinned to a Sylgard base and superfused with a HEPES-based buffer at room temperature. Glass microelectrodes filled with 3 M KCl (40-70 MΩ) were used to record neuronal transmembrane potential. Drugs were applied in the superfusate or by pressure ejection. Somal action potentials were initiated by stimulation of the sciatic nerve via a suction electrode or by injection of depolarising current through the recording electrode. DRG were dissociated by incubation with type 1 collagenase and dispase (both 2.5 $mg.ml^{-1}$) for 45-60 min at 37C, followed by trituration. Intracellular [Ca^{2+}] was monitored using the Ca^{2+}-sensitive dye calcium green-1.

The axonal CV of 62 cells in intact DRG was determined. 26 cells had a CV >10 m s^{-1} (Aα/β), in 11 the CV was 2-10 m s^{-1} (Aδ) and in 25 cells CV was < 2 m s^{-1} (C). The action potential duration of C cells (2.3 ± 0.2 ms) was greater ($P<0.05$) than that of Aδ (1.6 ± 0.1 ms) and Aα/β (1.2 ± 0.1 ms) cells, but the values for Aδ and Aα/β cells were not significantly different.

Capsaicin (1 μM) depolarised 12% of C cells (3/25), but no Aα/β (n=21) or Aδ (n=7) cells. ATP (1mM) had no effect in all cells tested (7 Aα/β, 4 Aδ, 8 C) and no cells responded to α,β-meATP (30 μM) (4 C, 3 Aδ). However, KCl (50 mM) evoked long lasting depolarization in every cell tested (20 C, 10 Aδ, 26 Aα/β). In contrast, following ganglia dissociation, an increase in intracellular [Ca^{2+}] was evoked by capsaicin (10 μM) in 62%, by ATP (100 μM) in 69%, and by α,β-meATP 30 μM) in 62% of all cells tested.

Thus, this study has shown that the electrophysiological properties of neurones of the neonatal rat DRG vary with cell type. Also, the responsiveness of cells to ATP, α,β-meATP and capsaicin increases substantially on ganglia dissociation, as reported by Stebbing *et al.,* (1998) in adult rat DRG.

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63P UNILATERAL CHRONIC ARTHRITIS INDUCED IN THE MOUSE KNEE JOINT USING FREUND'S COMPLETE ADJUVANT

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We have been using a unilateral model of joint inflammation (injecting Freund's Complete Adjuvant (FCA) intra-articularly (i.art.) into the rat knee joint - Donaldson et al., 1993). The model allows the role of joint sensory nerves to be studied in chronic inflammation. We have now developed an equivalent murine model in order to study, behaviourally and electrophysiologically, the influence of drugs on chronic joint inflammation in normal and transgenic animals.

Male C57BL6 and DBA-1 mice (8 weeks old) were used to establish a unilateral arthritis using FCA (5 μl of 1 mg ml^{-1} heat killed *Mycobacterium tuberculosis* in paraffin oil, Sigma) introduced into the left knee joint by intra-articular injection under transient anaesthesia (3% halothane in oxygen). Chronic inflammation was induced by repeat injections, once per week for 4 weeks. The animals were monitored over the course of the experiment for changes in body weight and knee joint diameter. Joint swelling, gait, and hyperalgesia were each measured subjectively (scale 0-3, where 3 is maximum effect) and a pooled "arthritic score" calculated. Data are expressed as mean ± SEM and were analysed statistically using Wilcoxon matched pairs and Mann-Whitney tests. Knee joints were collected after killing the animal (Schedule 1), fixed in formalin and decalcified for histology. Tissues were stained with haematoxylin/eosin and toluidine blue then assessed for joint damage.

In C57BL6 and DBA-1 mice, one week after the final injection of FCA there was a significant increase in the diameter of the injected joint (compared to the uninjected joint; Figure 1, $P<0.05$, Wilcoxon) or to joints injected with vehicle ($P<0.05$, Mann-Whitney). In C57BL6 mice inflammation was reduced to normal levels after treatment with prednisolone for 7 days (Figure 1A). Histology showed marked arthritic changes in injected joints, including cell infiltration, cartilage destruction and pannus formation.

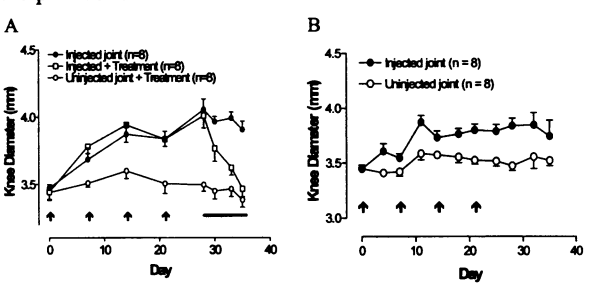

Figure 1 – Knee diameter of (A) C57BL6 and (B) DBA-1 mice. FCA (5 μl, i.art.) was injected at arrows (↑). (A) Treatment began on day 28 (marker bar) with prednisolone (1 mg kg^{-1}, daily, i.p.).

In summary, we have induced a chronic unilateral arthritis in mice using repeated injections of FCA. There is marked swelling and hyperalgesia confined to the injected joint, which coincides with extensive joint destruction. The chronic inflammation can be reversed by prednisolone.

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64P FAILURE OF CANNABINOID INHIBITION OF HIND LIMB WITHDRAWAL REFLEXES IN PENTOBARBITONE-ANAESTHETIZED RABBITS

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In decerebrated, spinalized rabbits the synthetic cannabinoid HU 210 is a very potent inhibitor of hind limb withdrawal reflexes, giving > 50% inhibition at a dose of 10 nmol kg^{-1} i.v. (Clarke *et al.* 2001). As part of a study of the effects of analgesic agents on trigeminal and spinal reflexes, we have now investigated the effects of HU 210 against a spinal flexion withdrawal reflex (FWR) and the trigeminally-mediated jaw-depressor reflex (JDR) in pentobarbitone-anaesthetized, spinalized rabbits.

Seven young adult rabbits of mixed strains and either sex, weight range 1.7 – 2.1 kg, were anaesthetized with sodium pentobarbitone (mean dose 46 mg kg^{-1} i.v.) and maintained on a continuous i.v. infusion of the same agent (mean rate 18 mg kg^{-1} h^{-1}). Electrical stimuli were applied though stainless steel needle electrodes and reflexes recorded as e.m.g. signals through percutaneous copper wire electrodes. The JDR was evoked by stimulation of the tongue (median stimulus intensity 1.9 mA) and recorded from the left digastric muscle. The FWR was elicited by stimulation at the plantar surface of the middle two toes (median stimulus intensity 7.7 mA) and recorded from the left tibialis anterior muscle. Reflexes were recorded every two min alternately, and were quantified from the voltage – time integral of the averaged response to 8 stimuli given at 1 Hz. Drugs were dissolved to 10 mM in ethanol and diluted in 5% d-glucose solution as required.

HU 210 was given i.v. in incrementing doses from 1 – 70 nmol kg^{-1} to give a cumulative total dose of 100 nmol kg^{-1}. Doses were separated by intervals of 24 min. The selective cannabinoid CB_1 receptor antagonist SR 141716A (a gift of Sanofi Recherche) was given at a dose of 100 nmol kg^{-1} 24 min after the final injection of HU 210.

HU 210 depressed the JDR only at the highest dose used (Wilcoxon test, $p < 0.02$), after which responses were a median of 18% (IQR 8 – 30%) of pre-drug control values. This effect was reversed (Wilcoxon test, $p < 0.05$) by SR 141716A to a median of 83% (IQR 34 – 127%) of pre-drug levels. In contrast, the FWR was not significantly affected by HU 210, so that after the highest dose this reflex was a median of 105% (IQR 74 – 127%) of pre-drug values. Subsequent administration of SR 141716A had no significant effect (Wilcoxon test, $p > 0.4$).

Thus, HU 210 had a selective inhibitory action against the JDR that was probably effected through CB_1 receptors. The failure of HU 210 to decrease the FWR is remarkable in view of the very potent inhibitory actions of this drug in decerebrated, spinalized rabbits (Clarke *et al.* 2001). The main differences between that and the present study are the use of pentobarbitone anaesthesia, and a less invasive preparation. Either of these may have contributed to the present results.

S.J. is supported by Merck, Sharp and Dohme.

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65P EFFECT OF WIN55, 212, A CANNABINOID RECEPTOR AGONIST, ON SENSORY NEUROTRANSMISSION IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

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Activation of capsaicin-sensitive sensory nerves in the rat mesenteric bed releases calcitonin gene-related peptide producing vasodilatation (Kawasaki *et al.*, 1988). CB_1 and CB_2 receptor mRNA has been detected in dorsal root ganglion cells and the receptor protein localised by Ross *et al.* (2001). CB_1 receptors undergo axonal flow and are likely to be inserted on nerve terminals in the periphery (Hohmann & Herkenham, 1999). Recently it has been shown that the cannabinoid receptor agonist HU210 attenuates sensory neurotransmission in the rat mesenteric bed but the receptor and the mechanisms mediating this effect are unknown (Ralevic & Kendall, 2001). The present study investigated whether a structurally unrelated cannabinoid receptor agonist WIN55, 212 can modulate sensory neurotransmission in this preparation.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused with oxygenated Krebs' solution containing guanethidine (5μM) to block sympathetic neurotransmission (Ralevic & Kendall, 2001). After 30 min equilibration, preparations were preconstricted with methoxamine (10-100μM) and three consecutive frequency response curves to electrical field stimulation (EFS; 1-12Hz, 60V, 0.1ms, 30s) (EFS control, EFSI and EFSII) were constructed in each preparation. WIN55, 212 or vehicle (ethanol; 0.01%) was added after EFS control, 15 min before EFSI. SR141716A was added at the start of the equilibration period. Data are expressed as mean±s.e.m. and analysed by ANOVA with Tukey's post hoc test.

EFS produced frequency-dependent relaxation (1-12Hz) of the rat mesenteric bed. WIN55, 212 (0.1 and 1μM) attenuated neurogenic relaxation evoked during EFSI and EFSII compared with EFS control. At a submaximal frequency (8Hz) there was a significant reduction in the presence of WIN55, 212 0.1μM (from 50.5±4.4% EFS control to 34.9±2.4% EFSI, n=6, $P<0.05$). In the presence of 1μM WIN55, 212 the response at 8Hz was reduced from 53.4±5.5%, EFS control, to 37.2±4.3%, EFSI (n=6, $P<0.05$). In the presence of the selective CB_1 receptor antagonist SR141716A (1μM) inhibition of the relaxation response by WIN55, 212 (0.1μM) was partially blocked. At 8Hz the response was not significantly different between EFS control, 49.3±2.7% and EFSI, 42.31±2.8% (n=7). There was no significant difference between EFS control, EFSI and EFSII generated in the presence of 0.01% ethanol, the vehicle for WIN55, 212 and SR141716A.

These data show that WIN55, 212 attenuates sensory neurogenic relaxation in the rat isolated mesenteric bed. The modulatory actions of WIN55, 212 were SR141716A-sensitive indicating the possible involvement of a CB_1 receptor, which is in agreement with evidence that CB_1 receptors are located on sensory nerves. However the inhibitory actions of the classical cannabinoid HU210 were not blocked by SR141716A (Ralevic & Kendall, 2001) suggesting that the two cannabinoids act at different sites on sensory nerves.

We are grateful to Servier for financial support.

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66P CHARACTERISATION OF THE EXCITATORY EFFECTS OF CAPSAICIN IN THE RAT BLADDER *IN VITRO*.

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Some categories of over-active bladder (OAB) are thought to be due to an increase in the activity of primary afferent neurones, primarily C-fibres, within the bladder wall. Capsaicin causes profound excitation and desensitisation of C-fibres, via an interaction with the VR1 receptor (Caterina & Julius, 2001). Intravesical capsaicin is used clinically to treat neuropathic patients with OAB (De Ridder & Baert., 2000), and this approach capitalises on the desensitising nature of this agent. Instillation of capsaicin into the bladder causes an initial excitation prior to desensitisation. This study investigated this process further using the rat bladder *in vitro,* as a model system.

Longitudinal bladder quarters were prepared from male rats (Sprague-Dawley, 180-230g) and mounted under 1g tension in 5ml organ baths containing standard Krebs solution at 37°C, and gassed with 5% CO_2/ 95% O_2. Tissues were stimulated electrically (EFS; 50V, 15Hz, 0.1ms pulse duration, 10s train duration, 2 minute cycle) and contractions measured as area under curve (AUC), and expressed as percent increase over control (mean ± s.e.m.). Antagonists were allowed at least 15 minutes to equilibrate with the tissue before the addition of agonists, which were added cumulatively to the bath.

In the absence of antagonists, a maximally-effective concentration of capsaicin (1μM) caused an increase in total AUC in response to EFS (15 ± 1.9%, $p<0.01$ by Students' t test). On sequentially isolating the purinergic and cholinergic components of the response to EFS using atropine (1μM) and α,β-methylene ATP (100μM) respectively, capsaicin increased the purinergic component by 77 ± 8.6% ($p=0.001$) with an EC_{50} value of 15nM (n=68), but was without effect on the cholinergic response to EFS (1.2 ± 5%, $p>0.05$). The potentiation of the purinergic response by capsaicin was antagonised by capsazepine (10μM), which caused a parallel, rightward shift in the concentration-response curve to capsaicin, yielding an EC_{50} value of 450nM (n=6). The vehicle used (0.01% DMSO) was without effect on any measured parameter. The effect of capsaicin on the purinergic response was mimicked by exogenous substance P (maximal increase 162 ± 27.4%, EC_{50} 1.4nM, n=12) and β-alanine neurokinin A (187 ± 32.3%, EC_{50} 70nM, n=12). Furthermore, NK_1 (CP-99994) and NK_2 (SR-48968) receptor antagonists together (3μM) abolished the increase in the purinergic response to EFS evoked by capsaicin, suggesting the involvement of NK_1 and NK_2 receptors. CGRP (100nM) was without effect.

These data suggest that at least in part, the excitatory action of capsaicin on the bladder is due to VR1 receptor-mediated release of substance P and NKA from primary afferent neurones within the bladder wall. In addition to the well-recognised ability of these peptides to contract smooth muscle, they also selectively enhance purinergic signalling.

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67P EUK-134 REDUCES OXIDATIVE STRESS-MEDIATED INJURY AND DEATH OF RAT PROXIMAL TUBULAR CELLS.

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Reactive oxygen species (ROS)-mediated cell injury and death has been implicated in the pathogenesis of renal ischaemia-reperfusion (I/R) and associated acute renal failure (ARF) (Weight *et al.*, 1996). Within the kidney, the proximal tubule (PT) appears to be particulary susceptible to I/R-injury (Lieberthal and Levine, 1996). Mn(3-methoxy-(N,N[1]-salicydenaminoethan))Cl (EUK-134) is a superoxide dismutase mimetic with catalase activity and acts as a ROS scavenger (Dobashi *et al.*, 2000). The aim of this study was to investigate the effect of EUK-134 on cellular injury and death in primary cultures of rat PT cells exposed to oxidant stress in the form of hydrogen peroxide (H_2O_2).

PT cells were isolated from the kidney cortex of eight male Wistar rats (250-300 g) using collagenase digestion, differential sieving and Percoll density centrifugation (Chatterjee, *et al.*, 2000). PT cells were cultured on 24 well plates in Minimum Essential Medium (MEM) containing 10% (v v^{-1}) fetal calf serum. Once confluent, cultures were divided into three groups; (i) PT cell incubated with MEM only (**'Untreated'**), (ii) PT cells treated with 1 mM H_2O_2 for 2 hours (**'H_2O_2 only'**) or (iii) PT cells treated with 1 mM H_2O_2 and 10 or 100 μM EUK-134 (**'EUK-134'**). Cellular injury and death were assessed spectrophotometrically by measurement of the mitochondrial-dependent conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan and lactate dehydrogenase (LDH) assays, respectively.

Incubation of rat PT cell cultures with 1 mM H_2O_2 for 2 hours significantly inhibited mitochondrial respiration (**Table 1**). Incubation with 100 μM EUK-134 significantly reduced the H_2O_2-mediated inhibition of mitochondrial respiration (**Table 1**) and significantly reduced percentage cell death (**Table 1**).

Table 1	**Untreated PT Cells**	**H_2O_2 only**	**EUK-134 (10μM)**	**EUK-134 (100μM)**
MTT (% viability)	$102\pm7^{+}$	15 ± 1*	17 ± 1*	43 ± 3*$^{+}$
LDH (% cell death)	$21\pm2^{+}$	51 ± 7*	59 ± 6*	$18\pm2^{+}$
N	8	8	7	7

Table 1: Effect of EUK-134 on H_2O_2-mediated injury and death of rat PT cells. Data are expressed as mean±s.e.mean for *N* rats, *$P<0.05$ *vs.* Untreated PT cells, $^{+}P<0.05$ *vs.* H_2O_2-only, analysed using one-way ANOVA and Bonferroni's testing.

Thus, EUK-134 reduces H_2O_2 (and therefore ROS)-mediated cellular injury in primary cultures of rat PT cells. Therefore, we propose that EUK-134 may be beneficial in renal disorders mediated by oxidative stress.

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68P ACTIN CYTOSKELETON AND DOPAMINE-INDUCED INHIBITION OF NA^+-K^+-ATPASE ACTIVITY IN OPOSSUM KIDNEY CELLS

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Dopamine-induced natriuresis depends mainly on its ability to inhibit tubular reabsorption of sodium. This occurs predominantly at the level of renal proximal tubules and is largely mediated through inhibition of the apical Na^+/H^+ exchanger and basolateral Na^+-K^+-ATPase (Jose et al., 1992). On the other hand, the anchoring of Na^+-K^+-ATPase to the basolateral membrane and its association to actin cytoskeleton is critical to the proper vectorial transport of sodium (Molitoris, 1991). The present study evaluated the importance of the association between Na^+-K^+-ATPase and the actin cytoskeleton on dopamine-induced inhibition of Na^+-K^+-ATPase activity.

The approach used measures the transepithelial transport of sodium in monolayers of opossum kidney (OK) cells, when the sodium delivered to Na^+-K^+-ATPase was increased at the saturating level by amphotericin B (Vieira-Coelho *et al.*, 2001). 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. Results are arithmetic means with s.e. mean or geometric means with 95% confidence limits, n=4-5.

Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

The maximal amphotericin B (1.0 μg/ml) induced increase in short-circuit current (I_{sc}) was prevented by ouabain (100 μM) or removal of apical sodium. Dopamine (1 μM) applied from the apical side significantly decreased (29±5 % reduction) the amphotericin B-induced increase in I_{sc}, this being prevented by the D_1-like receptor antagonist SKF 83566 (1 μM; Vieira-Coelho *et al.*, 2001) and the PKC inhibitor chelerythrine (1 μM). Exposure of OK cells to the actin inhibitor cytochalasin B (1 μM) from both cell sides reduced by 31±4% the amphotericin B-induced increase in I_{sc} and abolished the inhibitory effect of apical dopamine (1 μM), but not that of the PKC activator phorbol-12,13-dibutyrate (PDBu; 100 nM). The relationship between Na^+-K^+-ATPase and the concentration of extracellular sodium showed a Michaelis-Menten constant (K_m) of 44.1±13.7 mM and a V_{max} of 49.6±4.8 μA/cm^2 in control monolayers. In the presence of apical dopamine (1 μM) or cytochalasin B (1 μM) V_{max} values were significantly ($P<0.05$) reduced without changes in K_m values. These results are the first, obtained in live cells, showing that the PKC-dependent inhibition of Na^+-K^+-ATPase activity by dopamine requires the integrity of the association between actin cytoskeleton and Na^+-K^+-ATPase.

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69P THE SOD MIMETIC EUK-134 REDUCES OXIDATIVE STRESS-MEDIATED RENAL DYSFUNCTION IN THE RAT *IN VIVO*

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Generation of reactive oxygen species (ROS) has been implicated in the pathogenesis of renal ischaemia-reperfusion (I/R) injury (Weight *et al.*, 1996). Mn(3-methoxy-(N,N^1-salicydenaminoethan))Cl (EUK-134) is a salen-manganese complex with high SOD and catalase activities, thus catalytically eliminating both superoxide (O_2^-) and hydrogen peroxide (H_2O_2), respectively (Rong *et al.*, 1999). The aim of this study was to investigate the effects of EUK-134 on the renal dysfunction mediated by I/R of rat kidneys *in vivo*.

Forty-six male Wistar rats (210-330 g) were anaesthetised with sodium thiopentone (120 mg kg^{-1} i.p.). After performing a midline laparotomy, rats were divided into 4 groups; (i) **'Shams'**, in which rats were maintained under anaesthesia for the duration of the experiment, (ii) **'I/R only'**, in which rats underwent bilateral clamping of the renal pedicles for 45 min followed by reperfusion for 6 h and (iii) **'I/R + EUK-134 (pre-I/R)'**, in which rats underwent I/R, but were administered an i.v. bolus of EUK-134 (0.3 mg kg^{-1} in saline) 10 minutes prior to I/R, (iv) **'I/R + EUK-134 (pre-R)'**, in which rats also underwent I/R, but were administered an i.v. bolus of EUK-134 (0.3 mg kg^{-1} in saline) 5 minutes prior to reperfusion.

Subsequent to I/R, serum urea (Ur) and creatinine (Cr) levels were measured and used as indicators of glomerular function. Fractional excretion of Na^+ (FE_{Na}), representing the fraction of filtered sodium load excreted, was used as an indicator of tubular function. Serum aspartate aminotransferase (AST) levels were measured and used as an indicator of renal I/R injury.

Renal I/R produced significant increases in Ur, Cr and AST concentrations, which were significantly reduced by EUK-134 administered 5 and 10 minutes prior to reperfusion and I/R, respectively (**Table 1**). Increased FE_{Na} was also attenuated significantly by EUK-134 administered pre-I/R, but not when administered pre-R (**Table 1**).

Table 1	Shams	I/R only	Pre-I/R	Pre-R
Ur (μmol L^{-1})	6±0.4*	24±1	18±1*	17±1*
Cr (μmol L^{-1})	43±4*	234±13	148±7*	165±10*
FE_{Na} (%)	1±0.2*	48±11	25±4*	35±6
AST (iu L^{-1})	144±7*	2403±239	601±71*	789±101*
N	12	10	12	12

Table 1 Effect of I/R and EUK-134 on biochemical indicators of renal dysfunction. Data are expressed as mean±s.e.mean for *N* rats, **P*<0.05 vs. I/R only, analysed using one-way ANOVA / Bonferroni's test.

These results suggest that EUK-134 can reduce the glomerular and tubular dysfunction, and renal I/R injury caused by renal I/R *in vivo*. EUK-134 may therefore be beneficial against ROS-mediated renal dysfunction and injury.

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Weight, S.C., Bell, P.R. & Nicholson, M.L. (1996) *Br. J. Surg.* **83**, 162-170.

70P EFFECTS OF EUK-8, A SUPEROXIDE DISMUTASE MIMETIC WITH CATALASE ACTIVITY, ON THE CIRCULATORY FAILURE AND MULTIPLE ORGAN INJURY IN HAEMORRHAGIC SHOCK IN THE ANAESTHETISED RAT

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An enhanced formation of reactive oxygen species (ROS) contributes to the multiple organ injury and dysfunction associated with haemorrhagic shock. EUK-8 [Mn(N,N'-bis-salicydenaminoethan)Cl] is a superoxide mimetic with catalase activity (Baker *et al.*, 1998). This study investigates the effects of EUK-8 on the circulatory failure and multiple organ injury and/or dysfunction associated with haemorrhagic shock (HS) in the anaesthetised rat.

Forty-six male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.). The carotid artery was cannulated to withdraw blood, the femoral artery to measure mean arterial blood pressure (MAP) and heart rate (HR) and the jugular vein for the intravenous administration of drugs. After a 30 min stabilisation period, rats were subjected to haemorrhage to lower MAP to 50 mm Hg for 90 min. Animals were then treated with either EUK-8 at 1 mg kg^{-1}, **(HS+E(1))**, 3 mg kg^{-1} **(HS+E(3))** or saline (1 ml kg^{-1}, **HS**) and subsequently resuscitated with the shed blood plus an equivalent volume of Ringers Lactate solution. Four hours after the onset of resuscitation, blood samples were taken for the measurement of biochemical markers of organ injury.

In animals treated with saline, HS caused a delayed fall in MAP within 4 h (n=9, $p<0.05$). In addition, HS caused significant rises in the serum levels of (i)urea and creatinine (renal dysfunction), (ii) aspartate aminotransferase (AST), and alanine aminotransferase (ALT), (liver injury) (**Table 1**). EUK-8 attenuated the rise in the serum levels of creatinine as well as the liver injury, but did not affect the rise in the serum levels of urea or the delayed fall in blood pressure, caused by severe haemorrhage and resuscitation (**Table 1**).

Table 1. The effects of EUK-8 on the multiple organ injury caused by severe haemorrhage and resuscitation

Group	n	Urea (mmol L^{-1})	Creatinine (μmol L^{-1})	AST (iu L^{-1})	ALT (iu L^{-1})
Sham	7	7±1*	42±3*	173±36*	75±5*
HS	15	13±1	97±11	723±127	330±73
HS+E(1)	15	12±1	68±6*	483±77	176±15*
HS-E(3)	9	11±1	51±3*	373±35*	141±14*

Data are expressed as mean±s.e.mean, *P<0.05 vs. HS, analysed using one-way ANOVA followed by Dunnett's post hoc test.

These results demonstrate that EUK-8 reduces the organ (kidney, liver) dysfunction and injury associated with severe haemorrhage and resuscitation in the rat *in vivo*.

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71P PYRROLIDINE DITHIOCARBAMATE (PDTC) REDUCES THE RENAL DYSFUNCTION ASSOCIATED WITH ISCHAEMIA-REPERFUSION OF KIDNEY OF THE RAT *IN VIVO*

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PDTC inhibits the activation of nuclear factor kappa-B (NF-κB) and, hence, the expression of NF-κB-dependent genes (Liu *et al.*, 1999). For instance, PDTC (100 mg·kg^{-1}) reduces iNOS mRNA and protein expression and prevents the hypotension induced by LPS (Liu *et al.*, 1997). Renal failure is a common component of the multiple organ failure associated with shock. It is not known whether PDTC protects the kidney against ischaemia-reperfusion (I/R) injury. The aim of this study was to investigate whether PDTC reduces the renal dysfunction caused by I/R of the kidney *in vivo*. Forty-two male Wistar rats (200-250g) were anaesthetised using sodium thiopentone (120 mg·kg^{-1} i.p.) and tracheotomised. Following a midline laparatomy and isolation of the renal pedicles, the rats were randomly divided in three groups:(i) sham rats maintained under anaesthesia for the duration of the experiment, (ii) control rats subjected to bilateral clamping of the renal pedicles (45 min) followed by reperfusion (6h), (iii) PDTC treated rats manipulated as described for the control group, but which received (100 mg·kg^{-1} i.v.) 30 min prior to clamping. On completion of experiments, renal function and injury were assessed by measurement of the serum levels of urea, creatinine, aspartate amino-transferase (AST) and γ-glutamyl-transpeptidase (γGT). In the control group, renal I/R produced significant increase in the serum concentrations of urea. creatinine. AST. and γGT.

However, on comparison with the control group, administration of PDTC produced significant reductions in serum urea, creatinine, AST and γGT, while urine flow, the glomerular filtration rate and fractional excretion of Na^+ were not affected (Table 1).

The effects of PDTC on the renal dysfunction caused by ischaemia-reperfusion of the kidney

Group	n	Urea (mmol L^{-1})	Creatinine (μmol L^{-1})	AST (iu L^{-1})	γGT (iu L^{-1})
Sham	6	6±1	33±2	198±18	1±0.3
I/R	21	19±1°	153±8°	554±47°	3.0±0.3°
I/R PDTC	15	17±1*	110±10*	327±32*	2.0±0.4*

Table. 1 Data are expressed as mean±sem, °P<0.05 vs. Sham, *P<0.05 vs. Control, analysed using one-way ANOVA followed by Dunnet's post significance test.

These results suggest that PDTC protects the kidneys against I/R injury *in vivo*. The mechanism underlying this effect of PDTC warrants further investigation.

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Liu, S.F., Ye, X., Malik, A.B., (1999) *Circulation* **100**:1330-1337

72P DITHIOCARBAMATES ATTENUATE THE ORGAN INJURY AND DYSFUNCTION CAUSED BY ENDOTOXIN IN THE RAT

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The dithiocarbamates are a class of antioxidants reported to be inhibitors of NF-kappa B. These compounds have shown to attenuate endotoxin-induced acute lung injury (Avery *et al.*, 1997) and to prolong survival of mice in endotoxic shock model (Kishnani *et al.*, 1999). The aim of the present work was to investigate the effect of pre-treatment of rats with pyrrolidine dithiocarbamate (PDTC) or diethyldithiocarbamate (DETC) on the liver injury, renal dysfunction and nitrite/nitrate serum levels caused by LPS in the rat. Male Wistar rats (280-350g) were pre-treated with PDTC (100 mg/kg, i.p.) or DETC (100 mg/kg, i.p.) one hour before the administration of LPS (7.5 mg/kg, i.v.). Six hours after the LPS administration, animals were anaesthetised (penthobarbital sodium, 60 mg/kg, i.p.) and blood samples were obtained through a cannula placed in carotid artery. Serum levels of biochemical markers of liver injury (AST and ALT), renal dysfunction (urea and creatinine), and nitrate/nitrite were determined. Results (mean ± S.E.M.) were compared by one-way ANOVA followed by a Bonferroni post-test. Differences were considered statistically significant when $P<0.05$.

LPS caused an increase in AST, ALT, urea, creatinine and nitrite/nitrate serum levels. In endotoxemic rats, PDTC significantly reduced levels of AST, ALT and urea. DETC reduced AST, creatinine and urea. Both dithiocarbamates reduced nitrite/nitrate levels.

Table 1: Effect of pre-treatment with PDTC and DETC on renal dysfunction and hepatic injury, and nitrite/nitrate serum levels, caused by endotoxin in rat. Mean ± S.E.M (*P<0.05 *vs.* Sham; $^{+}$P<0.05 *vs.* LPS-treated)

	AST (U/l)	ALT (U/l)	Urea (mg/dl)	Creatinine (mg/dl)	NO_2^-/NO_3^- (μM)
Sham (n=11)	124±14	34±4	38±3	0.6±0.02	45±19
LPS (n=7)	312±53*	325±94*	111±13*	0.9±0.13*	447±34*
PDTC + LPS (n=8)	132±5^{+}	51±1^{+}	62±5^{+}	0.6±0.07	278±15*$^{+}$
DETC + LPS (n=6)	174±19^{+}	84±17	67±7^{+}	0.5±0.07^{+}	324±37*$^{+}$
PDTC (n=3)	110±16^{+}	51±7^{+}	36±3^{+}	0.4±0.03^{+}	27±4^{+}
DETC (n=4)	82±6^{+}	35±2^{+}	38±3^{+}	0.3±0.02^{+}	31±2^{+}

These results suggest that administration of dithiocarbamates may represent a novel approach to limiting the organ injury caused by septic shock.

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73P AIRWAY FUNCTION, HYPERREACTIVITY, CELL INFLUX AND NITRIC OXIDE, IN CONSCIOUS PARAINFLUENZA-3 INFECTED GUINEA-PIGS: EFFECT OF DEXAMETHASONE AND ROLIPRAM

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Respiratory virus infections account for 80% of asthmatic exacerbations (Nicholson *et al.*, 1993). Parainfluenza-3 (PI-3) causes airflow obstruction, airway hyperreactivity (AHR) and leukocyte influx in guinea-pigs (Folkerts *et al*, 1994). AHR and increased exhaled nitric oxide (NO) correlates with the severity of viral infection (de Gouw *et al.*, 1998). Phosphodiesterase-4 (PDE4) inhibitors and corticosteroids attenuate AHR, airways cell influx and excess NO after lipopolysaccharide inhalation (Toward & Broadley, 2001). Here, we examine their effects on these parameters in conscious, PI-3 infected guinea-pigs.

PI-3 (gift from *J.D Fox, U.W.C.M., Cardiff*) was propagated in BSC1 cells. Non-infected and PI-3 infected cells (after significant cytopathic effect) were homogenised, centrifuged (5000r.p.m., 15min), filtered (0.2μm pore) and concentrated in saline (Centriplus™, Amicon). Specific airway conductance (sGaw) was measured in groups (n=5-6) of conscious Dunkin-Hartley guinea-pigs (male, 300-350g) by whole-body plethysmography (Griffiths-Johnson *et al.*, 1988). Baseline (Bl) sGaw values were obtained and 30min later they received a nose-only exposure to a threshold bronchoconstrictor dose of nebulised (0.2ml.min^{-1}) histamine (1mM, 20s) and sGaw was recorded at 0, 5 and 10 min. 24h later, guinea-pigs were intranasally (250μl: bi-laterally) inoculated with PI-3 ($2.08x10^8$ .ml^{-1}), or the PI-3 vehicle (PI-3-deficient medium). Airway function was measured daily thereafter. Airway reactivity to histamine was re-assessed 4 days later. After assessing airway reactivity, animals were overdosed with pentobarbitone sodium (0.6mg.100g^{-1}, i.p.), the lungs lavaged (1% EDTA, 1ml.100g^{-1}, twice) and the bronchoalveolar lavage fluid (BALF) cell content was determined. Remaining BALF was centrifuged (1000r.p.m., 6min) and supernatant NO metabolites (NO_2 & NO_3 (Greiss reaction: Toward & Broadley, 2001)) assayed. In other animals, the steroid, dexamethasone (20mg.kg^{-1}), PDE4 inhibitor, rolipram (1mg.kg^{-1}), or vehicle (5%DMSO in saline) were administered (i.p.) 24 and 0.5h before inoculation and daily thereafter.

Inoculation of PI-3 or medium, with or without dexamethasone, rolipram or vehicle, did not significantly alter airway function from Bl sGaw values. Four days after PI-3, histamine caused an increased ($P<0.02$) bronchoconstriction (-32.5±8.0 % change from Bl sGaw), compared with before PI-3 (+0.1±2.6 %), indicating AHR. There was no AHR after medium ($P>0.05$). Dexamethasone and rolipram inhibited ($P<0.05$) the PI-3-induced AHR. At 4 days after PI-3 inoculation, the BALF contained increased macrophages, eosinophils and neutrophils (Figure 1), and raised ($P<0.004$) combined NO metabolites, compared with medium. Both dexamethasone and rolipram reduced the PI-3-induced macrophage (41 & 48% inhibition, respectively), eosinophil (78 & 84%) and neutrophil (96 & 96%) airways infiltration and overproduction of airways NO (33 & 38%).

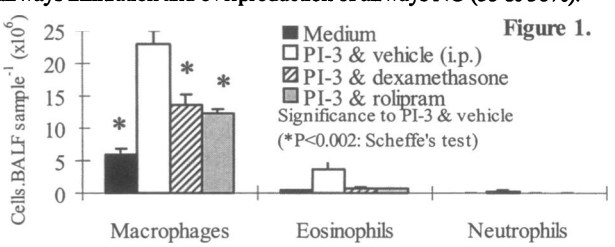

Figure 1.

This study shows that PI-3 inoculation causes AHR to histamine in conscious guinea-pigs. In addition, PI-3 induces airways infiltration of leukocytes and excess NO. The AHR, cell influx and increased NO were dexamethasone- and rolipram-sensitive. Steroids and PDE4-inhibitors may benefit PI-3-induced exacerbated asthma

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74P EXPOSURE OF A CONTRACTILE RESPONSE TO ADENOSINE IN GUINEA-PIG ISOLATED TRACHEA BY PASSIVE SENSITIZATION

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Active sensitization of guinea-pigs with ovalbumen has long been used as a technique for the study of the airways responses to ovalbumen and adenosine *in-vivo* (Pretolani *et al.*, 1994) and *in-vitro* (Lewis and Broadley, 1995). Passive sensitization has also been used to study contractile responses in tissues from non-sensitized human tissues (Rabe, 1998). This technique involves the passive transfer of antibodies from sensitized serum to non-sensitized tissues. The responses to adenosine in passively sensitized airways have not been investigated and are the subject of this study.

After cervical dislocation, blood was collected from non-sensitized or ovalbumen sensitized guinea-pigs, left to clot and centrifuged at 200G for 10 minutes at 21°C. Tracheas were removed from non-sensitized guinea-pigs, cut spirally and divided in two equal pieces. These were then placed in serum from either a non-sensitized or sensitized guinea-pig, gassed for 24hours at room temperature. Control tracheas were incubated in Krebs or not incubated. After incubation the tracheal spirals were suspended in a heated jacket (37°C) and superfused with pre-warmed (37°C) and gassed (5%CO_2 in oxygen) Krebs solution at 5ml.min^{-1}. Agonists were added in the order: adenosine (1mM), histamine, (10μM), methacholine (10μM), ovalbumen (10μg). The antagonist, 8PT (3μM), was added to the Krebs solution and perfused throughout the experiment. Increases in tension (g) ± SEM were measured and compared using a paired Students *t*-test. P values of 0.05 were taken to indicate significance.

Passive sensitization with ovalbumen sensitized serum revealed constrictor responses to adenosine (0.04±0.01g) and OA (0.40± 0.09g), which were not seen in either non-incubated or Krebs-incubated tracheas. These responses were not effected by 8PT (0.03±0.01g and 0.33±0.01g). After incubation in non-sensitized serum a similar contractile response to adenosine (0.07±0.02g) was observed. A contraction to ovalbumen was observed (0.08±0.03g) but this was significantly less than after sensitized serum incubation and was not significantly different from the adenosine response. 8PT did not affect these responses

Incubation of tissues with serum from animals sensitized to ovalbumen revealed a contractile response to the specific antigen. This is consistent with the concept that incubation of tissues with high levels of allergen-specific IgE results in the loading of high affinity IgE receptors on mast cells with the specific IgE and subsequent release of mediators by ovalbumen. Tissues incubated in allergen-specific IgE serum also constrict when exposed to adenosine in a similar way to actively sensitized tissues. Surprisingly the tissues incubated overnight in serum from non-sensitized guinea-pigs also produced contractile responses to adenosine and ovalbumen that were of similar magnitude. Thus, the presence of IgE-loaded receptors on mast cells, by passive sensitization can reveal a contractile response to adenosine, presumably mediated via mast cell degranulation.

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75P GLYCOSAMINOGLYCANS POTENTIATE ANP-EVOKED RELAXATION IN BOVINE BRONCHI

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Atrial natriuretic peptide (ANP) reverses methacholine-induced constriction in human and bovine bronchi: an effect enhanced by the addition of the neutral endopeptidase 24.11 (NEP) inhibitor phosphoramidon (Angus *et al*, 1993). Some glycosaminoglycans (GAGs) have been reported to regulate extracellular matrix enzymes (Redini *et al*, 1988) such as collagenase and elastase but no effect on NEP activity has been reported. The aim of this study was to investigate the effect of a variety of GAGs on NEP activity in bovine airway.

Bovine lungs were obtained from a local abattoir. Responses were measured isometrically in rings of bronchi (3-5mm) in 5ml vertical organ baths containing oxygenated Krebs-Henseleit solution at 37°C. Tissues were preconstricted with methacholine ($3x10^{-5}$M) and cumulative concentration-response curves were constructed for ANP (10^{-9}-$3x10^{-7}$M) alone, in the presence of phosphoramidon (10^{-4}M), GAGs, or a combination of GAGs and phosphoramidon. Statistical significance was examined by two-way ANOVA or Student's t-test.

Phosphoramidon alone potentiated ANP-evoked relaxation ($P<0.01$) as previously reported (Angus *et al*, 1993) (control relaxation at $3x10^{-7}$M ANP; 83.7±6% compared with 69.8±9% in the presence of phosphoramidon). ANP–mediated relaxations were significantly potentiated both in the presence of heparin (60μg/ml≡9.12 USP units; $n=7$, $P<0.05$) or low molecular weight hyaluronic acid (low HA) (100μg/ml≡ 6.6 $x10^{-6}$ M; $n=5$, $p<0.05$) (control relaxation at $3x10^{-7}$M ANP; 83.7±6% compared with 73.5±8.6% in the presence of heparin and 94.9±5.6% compared with 84.7±5.6% in the presence of low HA). No effect was seen with heterogeneous molecular weight HA (100μg/ml, $n=6$). The combination of phosphoramidon with either low HA or heparin evoked a similar potentiation of ANP-evoked relaxations to that seen with phosphoramidon alone (relaxation at $3x10^{-7}$M ANP; 59.4±21.3% in the presence of phosphoramidon and heparin and 57±31.3% in the presence of phosphoramidon and low HA).

In bovine airways both heparin and low HA appear to protect ANP from degradation by NEP 24.11. In each case the potentiation was of the same magnitude as that produced by phosphoramidon. The GAGs/phosphoramidon combination did not further potentiate the ANP-evoked response and this lack of synergy suggests that these two GAGs, heparin and low HA, are likely to be operating via similar mechanisms to that of phosphoramidon.

This work is supported by TENOVUS, Scotland.

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76P REGIONAL DISTRIBUTION OF DEXFENFLURAMINE MEDIATED PULMONARY ARTERIAL VASOCONSTRICTION: COMPARATIVE STUDY IN WISTAR, FAWN HOODED AND CHRONICALLY HYPOXIC WISTAR RATS.

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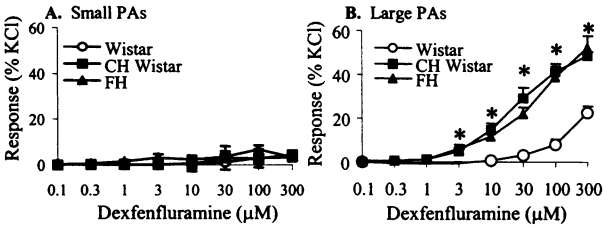
The anorectic drug, dexfenfluramine (dFen), is known to cause pulmonary hypertension (PH) by as yet an unknown mechanism. dFen interferes with intracellular storage for 5-hydroxytryptamine (5-HT) which itself has been implicated in the development of PH. Fawn hooded (FH) rats have a 'platelet 5-HT storage defect' and susceptibility to develop PH (Sato *et al*, 1992). Previous studies have shown that 5-HT_1 and 5-HT_2 receptor-mediated vasoconstriction is enhanced in intralobular large and small pulmonary resistance arteries of FH and in chronic hypoxic (CH)-induced PH Wistar rats (MacLean *et al*, 1996; Lal *et al*, 2001). We have questioned whether similar susceptibility to pulmonary vasoconstriction is seen with dFen in these rat models of PH.

Male Wistar, FH and CH (10 % O_2, 4 weeks) Wistar rats, (8-10 weeks old) were killed with an over dose of pentobarbitone sodium (120 $mgKg^{-1}$, *i.p.*), intralobular large pulmonary arteries (PAs) (i.d. ~ 1000μm) and small resistance PAs (*i.d.* ~ 200μm) were mounted onto wire-myograph in Krebs' solution (20% O_2/ 5% CO_2/ 75% N_2; 37°C). The vessels were subjected to transmural pressures equal to ~16 mmHg in normal Wistar rats and FH, and ~32 mmHg in CH Wistar rats. The concentration-response curves to dFen were constructed with or without the 5-HT_{2A} receptor antagonist, Ketanserin (1μM). The responses are expressed as % of KCl (50 mM)-induced contraction in each vessel. Data (mean ± s.e.m) were analysed by 1-way ANOVA (*P< 0.05; n= vessels, 4-9 rats/ group).

dFen (0.1-300μM) elicited only weak contractions in small PAs from normal Wistar, FH and CH Wistar rats (Figure 1A). dFen - evoked significantly greater contractions in large PAs vs. small PAs (Figure 1, P< 0.05). In large PAs only, dFen produced significantly enhanced contractions in FH and CH Wistar rats vs. normal Wistar rats (*P < 0.05, Figure 1).

Figure 1. dFen-induced contractions in small and large PAs



Ketanserin (1μM) had no effect on dFen (0.1-300μM)-mediated contractions in large PAs from Wistar rats (E_{max} to dFen 300 μM; control 22 ± 3.5 % vs. with Ketanserin 22 ± 3 %, n=5-9). In contrast, ketanserin significantly inhibited dFen (0.1-300 μM)-evoked enhanced contractions in large PAs from FH (E_{max} dFen 300 μM; control 52 ± 6 % vs. with ketanserin 30 ± 4 %, n= 5-8, P< 0.05) and CH Wistar (E_{max} dFen 300 μM; control 47 ± 2 %, n=6 vs. with ketanserin 27 ± 5 %, n= 6, P< 0.05) to values similar to those seen in large PAs from normal Wistar rats.

In summary, small PAs respond poorly to dFen, despite enhanced responses to 5-HT, in FH and CH Wistar rats (MacLean *et al*, 1996; Lal *et al*, 2001). dFen mediated augmented vasoconstriction in large PAs from FH and CH Wistar rats is obviated with ketanserin. These data show that in these rat models of PH, dFen mediated vasoconstriction is enhanced in large but not small pulmonary resistance arteries. Funded by the Wellcome trust.

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77P CHANGES IN PULMONARY VASCULAR REACTIVITY DURING AND AFTER CHRONIC HYPOXIA IN RATS.

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Chronic hypoxia (CH) induces pulmonary hypertension as a result of vasoconstriction and pulmonary remodelling. The pulmonary vasculature in CH also becomes hyper-responsive to vasoconstrictors. The aim of the present study was to investigate whether enhanced pulmonary vascular reactivity is solely attributable to vascular smooth muscle proliferation.

Male Wistar rats were exposed to normoxia or hypoxia (10% O2) for 3 weeks. For recovery, CH rats were returned to room air and utilised 3 weeks later. Lungs were perfused (Lal *et al.*, 1994). Phenylephrine (PHE) (1-30nmoles), KCl (25-400umoles) and angiotensin II (1-300pmoles) were injected into the pulmonary artery and increases in pulmonary perfusion pressure (PPP) recorded. Weights of the right ventricle (RV) and left ventricle plus septum (LV) were measured for an index of right ventricular hypertrophy. For histology, after fixation with formol saline (0.4%), transverse slices (4μm) of lung tissue were stained with haemotoxylin and eosin. Vessel diameters (range: 37 - 815 μm) and medial wall thickness were measured using a Zeiss KS300 computer image programme. The percentage wall thickness was calculated from the formula: wall thickness (%) = 2× wall thickness × 100/ external diameter (Hislop *et al.*, 1976). Data were expressed as mean ± s.e.mean and analysed by a one-way ANOVA with Dunnett's test or Student-t test.

Basal PPP increased from 6.3 ± 0.9 mmHg in control to 10.5 ±0.2 mmHg after CH (P<0.05, n = 5) but fell again after recovery, (6.4 ± 0.5mmHg, P>0.05, n=6). PPP increases to 10nmoles PHE were 2.74 ± 0.28 mmHg in control vs 15.35 ± 3.01 mmHg in CH (P<0.05, n=5-7). After 3 weeks recovery, PHE responses had declined (3.08 ± 0.52 mmHg). PPP increases to 200umoles KCl were 8.48 ± 1.20 mmHg in control vs 18.67 ± 3.76 mmHg in CH (P<0.05, n=5) and were still elevated after recovery (14.19 ± 1.04 mmHg). This was also true for angiotensin II (100pmoles), 6.83 ± 1.08 mmHg in control vs 17.33 ± 3.74 mmHg in CH (P<0.05, n=5-6) and 12.02 ± 1.26 mmHg after recovery. RV/LV ratio was increased from 0.205 ± 0.013 in control to 0.338 ± 0.010 (P<0.05) in CH. After recovery the ratio (0.270 ± 0.019) was still significantly elevated (P<0.05, n=5-6). Similarly, pulmonary arterial wall thickness was significantly increased from 17.5 ± 1% (n=84) in age-matched control to 21.8 ± 0.9% (n=106) in CH, P < 0.01 and was still higher after recovery, 18.4 ± 0.7% (n=114) in age-matched control vs 25.7 ±1% (n=89) in the recovery group, P<0.001.

In conclusion, our study shows that CH induces pulmonary hypertension, vascular re-modelling and markedly increases pulmonary vascular reactivity. During recovery from hypoxia, responses to phenylephrine decline faster than those to KCl or angiotensin II. This suggests that in CH proliferation of vascular smooth muscle alone cannot account for the observed vascular hyper-reactivity to α_1-adrenoceptor agonists.

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78P CHARACTERISATION OF MUSCARINIC RECEPTORS MEDIATING CONTRACTION IN THE RABBIT PULMONARY ARTERY

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Acetylcholine (ACh) is an endothelium-dependent vasodilator, but when pulmonary artery endothelium is damaged, it causes vasoconstriction through a direct action on smooth muscle muscarinic receptors (Dinh-Xuan *et al.*, 1989). Although M_1, M_2 and M_3 receptors have been identified in the pulmonary artery (Hislop *et al.*, 1998), the receptor responsible for vasoconstriction has not yet been determined. This study therefore characterised the muscarinic receptors mediating vasoconstrictor responses to ACh in the endothelium-denuded rabbit main pulmonary artery. Arteries were obtained from male New Zealand rabbits (2-2.5 kg) killed by lethal injection of sodium pentobarbitone (140 mg kg^{-1} i.v.). Isometric tension was recorded from artery rings bathed in physiological salt solution at 37°C. The abilities of selective muscarinic receptor ligands to either activate contraction or inhibit the contractile response to ACh were determined. Contraction was measured as a percent of the response to 50 mM K^+, activated by substituting KCl for equimolar NaCl in the bath solution. Results are given as mean ± s.e.mean. Statistical comparisons employed student's t-test, $P<0.05$ indicating significance.

In the presence of neostigmine (2 μM) to prevent metabolism, concentration-dependent contraction to ACh (3nM–1mM) reached a maximum at 38 ± 8% (n=8) of the response to K^+. The M_2/M_4 selective agonist, oxotremorine sesquifumarate (Oxo-S), (3nM - 100μM), produced a significantly smaller maximum contraction (15±3%, n=8, $p<0.05$), whilst the M_1-agonist, McN-A-343 (3nM - 100μM) had no effect. In the absence of extracellular calcium, a higher concentration of ACh was required to initiate contraction and the maximum response was significantly ($p<0.05$) reduced to 29±1% (n=6) of control, whereas the response to Oxo-S was abolished (n=6).

Contractions evoked by Oxo-S were followed by prolonged desensitization, functionally removing M_2 and M_4 receptors. In these conditions, responses to <100μM ACh were unaltered, but responses to 1mM ACh were significantly reduced ($p<0.05$; n=5). Repeated application of 300μM ACh also resulted in significant (44±9%, n=6, $p<0.05$) reduction of the response to 1 mM ACh and abolished the response to Oxo-S. A range of muscarinic antagonists inhibited the ACh-induced contraction with the following pA_2 values: atropine 10.4; telenzepine 9.0; tripitramine 7.8; *p*-f-HHSiD 8.5; tropicamide 8.3. Responses to Oxo-S were abolished by 30nM atropine (n=4).

Based mainly on the results with Oxo-S, we suggest that M_3 receptors are the main muscarinic receptors mediating ACh-induced vasoconstriction, but that M_2 receptors also participate. A mechanism proposing M_2 and M_3 receptor co-operativity was proposed to underlie the ACh-induced contraction in colon (Sawyer and Ehlert, 1999). A similar mechanism may exist here.

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79P RELAXATION TO BRADYKININ IN BOVINE PULMONARY SUPERNUMERARY ARTERIES: ROLE OF NITRIC OXIDE AND A GUANYLYL CYCLASE.

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This study examined the effect of the eNOS inhibitor L-NAME, the nitric oxide scavenger hydroxocobalamin and the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo [4,3a]quinoxalin-1-one (ODQ) on the vasorelaxant responses to bradykinin in bovine pulmonary supernumerary arteries.

Bovine lungs were obtained fresh from the local abattoir. Segments of supernumerary arteries (diameter 0.5 - 1 mm) were dissected from the lung and freed of surrounding connective tissue. The vessels were then weighed and suspended between stainless steel hooks in Krebs-Henseleit buffer (37°C) under a tension of 1 g and gassed with a mixture of O_2:CO_2 95%/5% v/v). In some rings the endothelium was removed by abrading the luminal surface with forceps. The tissues were allowed to equilibrate for 1 hour then contracted with U46619 (0.3 μM). Concentration response curves for bradykinin-induced relaxation were constructed in the presence of indomethacin. Paired tissues acted as time controls. Relaxations are expressed as % decrease of the U46619-induced tone. Results are means ± s.e. mean. The significance of differences was determined using Student's t-test.

In endothelium-intact, but not denuded rings, bradykinin (100 pM-100 nM) produced a concentration-dependent relaxation (pEC_{50}, 9.6±0.1; maximum relaxation ($R_{max.}$), 100.5 %, n=25). Hydroxocobalamin (200 μM) produced a significantly greater rightward shift of the bradykinin concentration response curve than L-NAME (100μM)(pEC_{50}, L-NAME, 8.7±0.1, n = 30, $p>0.001$; pEC_{50} hydroxocobalamin 8.3±0.2, n= 5, $p > 0.001$). The effect of combining hydroxocobalamin with L-NAME was similar to hydroxocobalamin alone (pEC_{50}, 8.1±0.3, n= 8, $p> 0.001$). ODQ (10 μM) did not alter the bradykinin concentration response curve (pEC_{50}, 9.8±0.4, n=5) but abolished the vasorelaxation induced by sodium nitroprusside (10nM-100μM).

When ODQ was combined with hydroxocobalamin or L-NAME the reduction in tissue sensitivity, observed with hydroxocobalamin or L-NAME alone, was not observed (pEC_{50} hydroxocobalamin + ODQ, 9.4±0.3, n=4; pEC_{50} L-NAME + ODQ, 9.4±0.4, n=4).

The present study indicates that nitric oxide appears to be the dominant relaxing factor in this tissue. Since hydroxocobalamin produced a significantly greater reduction in tissue sensitivity than L-NAME, this suggests that L-NAME may not fully block nitric oxide production by eNOS or that nitric oxide arises from a source other than eNOS.

Inhibition of a guanylyl cyclase appears to be able to compensate for the loss of the component of relaxation mediated by nitric oxide. We have previously reported the presence of an EDHF in this tissue (Tracey *et al.*, 2001). It is possible that guanylyl cyclase inhibition upregulates the EDHF component of the bradykinin-relaxation.

Tracey A., Bunton D., MacDonald A. *et al.*, Br. J. Pharmacol. 133, 277P.

80P USE OF EPIBATIDINE TO PROBE THE BINDING SITE OF THE DESENSITISED FOETAL MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR

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The foetal muscle nicotinic acetylcholine receptor (nAChR) is a pentameric structure whose ligand-binding sites are situated at the interface between the two alpha subunits and their neighbouring delta or gamma subunits. A number of ligands, including ACh, d-TC and carbamylcholine, have been identified that differ in their affinities for these two sites. Since this effect is due to sequence differences in the gamma and delta subunits, these ligands are extremely useful tools for identification of the important residues in the non-alpha subunits that contribute to ligand binding.

When the receptor is desensitised, none of the classical nAChR ligands exhibit site selectivity. However, the novel agonist (-)-epibatidine has been shown to have an approximately 200-fold higher affinity for the gamma site compared to the delta site in the desensitised receptor (Prince & Sine, 1998). Recent studies have identified the residues 104-117 in gamma and 106-119 in delta as important determinants of this affinity difference (Prince *et al.*, 2000).

In this study, chimeras and point mutants of gamma and delta were used to examine the region γ110-115/δ112-117. Chimeras were constructed in the form γxδ225γ, which consists of a γ sequence for the first *x* residues, followed by a δ sequence until residue 225 with the remainder being γ. Subunit omitted complexes of the form $\alpha_2\beta\chi_2$ (where χ is wild type, chimera or point mutant) were transiently expressed in HEK 293 cells. The receptors were desensitised with 100 μM proadifen, and the affinity of epibatidine was determined by competition against the initial rate of binding of [^{125}I]-α-bungarotoxin. Analysis of chimeras was performed on the log shift of the binding curves normalised to a γ curve, using one-way ANOVA with Tukey's post-hoc test. Analysis of the point mutants was performed on the log shift of their binding curves with respect to a wild-type curve, using a paired t test. Data are expressed as mean ± SEM of at least 3 curves.

With the chimeras, a significant increase in affinity was apparent as the γ sequence was extended from γ110δ225γ through to γ114δ225γ ($p<0.01$); these chimeras exhibiting EC_{50}s of 67.5 ± 16.8 and 10.5 ± 1.56 nM, respectively. However, extending the sequence by one further residue produced a significant decrease in affinity compared to γ114δ225γ (73.3 ± 21.3 nM; $p<0.01$).

The γ point mutants γS111Y, γP112D and γC115Y exhibited significantly lower affinities for epibatidine compared to wild-type γ (2.64 ± 1.00 nM); having EC_{50}s of 4.67 ± 1.12 ($p<0.05$), 3.50 ± 0.24 ($p<0.05$) and 3.53 ± 1.63 nM ($p<0.01$), respectively. Of the δ equivalents, only δY113S (135.7 ± 21.1 nM; $p<0.05$) and δY117C (82.46 ± 17.8 nM; $p<0.005$) showed marked shifts in affinity, compared to δ (471.0 ± 66.9 nM). γ111/δ113 and γ115/δ117 may be important determinants of epibatidine selectivity in the desensitised nAChR.

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81P TOPIRAMATE ENHANCES AND PROLONGS THE SLOW POST-STIMULUS AFTERHYPERPOLARIZATION (sAHP) IN RAT OLFACTORY CORTICAL NEURONES *IN VITRO*.

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Topiramate (TPM) is a novel marketed antiepileptic drug. Thus far, the cellular mechanisms underlying its anti-convulsant and antiepileptic activities include blockade of voltage-activated Na^+ channels, HVA Ca^{2+} channels, potentiation of $GABA_A$-evoked Cl^- currents and inhibition of AMPA/KAI glutamate inward currents (Shank *et. al.*, 2000). We tested TPM on transverse *in vitro* brain slices of olfactory cortex prepared from 200-250g Wistar rats (either sex) as previously described (Constanti *et al.*, 1993). This 'limbic' area of the brain is known to be important in the development and maintenance of experimental kindled seizures and also has a potential role in the genesis and spread of certain forms of human epilepsy *e.g.* temporal lobe epilepsy with partial seizures.

Stable intracellular recordings were made from neurones in the deep cell layer II-III using 4M K acetate-filled microelectrodes (60-80 MΩ). Data are presented as mean ± S.E.M. In 20/25 recorded neurones maintained at –70 mV membrane potential by constant current injection, bath-application of TPM (20 μM; 20-50 min) induced a slow membrane hyperpolarization (mean peak amplitude = 9 ± 2mV). This hyperpolarization was accompanied by a decrease in membrane input resistance (26 ± 7 %). TPM also produced a dose-dependent decrease (~ 70%) in the number (but not amplitude) of action potentials elicited during a brief (160 ms) depolarizing current pulse; these effects of TPM were usually fully reversed after a 40 min washout period. When a longer (1.5s) depolarizing current stimulus was applied, a spike burst and slow after hyperpolarization (sAHP) were induced, due to activation of a Ca^{2+}-activated K^+ conductance. TPM (20 μM; 20 min) caused a noticeable increase in spike accommodation during the stimulus and an enhancement (9 ± 2 %) and dramatic prolongation (53 ± 9 %) of the sAHP. This effect was not always fully recoverable even after 1 hr washout. Under 'hybrid' voltage clamp, a slow outward tail current (I_{AHP}) underlying the sAHP was revealed, that was progressively enhanced in amplitude (by up to 30 %) and prolonged in duration (up to 50 %) during 20 μM TPM application. In 5/25 cells, TPM hyperpolarized the membrane potential, but *reduced* (27 ± 8 %) the sAHP (with no effect on spike firing), suggesting that the action of the drug may be dependent on neurone-type or, possibly, cell metabolic status (phosphorylation state).

In conclusion, TPM hyperpolarizes and inhibits repetitive spike firing in olfactory cortical neurones (*c.f.* Kawasaki *et. al.*, 1998), and also enhances and dramatically prolongs the sAHP, possibly by selectively modulating an underlying Ca^{2+}-activated K^+ conductance or other Ca^{2+}-dependent mechanisms. Such effects could contribute to the clinical anti-epileptic efficacy of TPM.

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82P CALCITONIN GENE-RELATED PEPTIDE AND ADRENOMEDULLIN MODULATE SYNAPTIC TRANSMISSION IN PURKINJE CELLS

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Calcitonin gene-related peptide (CGRP) and adrenomedullin receptors are present in the cerebellum. Of the two, CGRP receptors show a higher level of expression (Oliver *et al.*, 1998). To date, the role that these peptides play within the cerebellum remains unclear although CGRP has been shown to modulate Purkinje cell (PC) excitatory post-synaptic currents (EPSCs) (Cater *et al.*, 2001). We have used electrophysiological techniques to assess the effects of CGRP, adrenomedullin and the selective antagonist $CGRP_{8-37}$ on synaptic transmission between parallel fibres (PF) and PCs.

14 – 21 day old male Wistar rats were decapitated under halothane anaesthesia and the cerebellum rapidly removed. 200μm thick sagittal slices were prepared and maintained at room temperature. Whole cell voltage clamp recordings were made from Purkinje cells whilst stimulating parallel fibres at 0.2Hz. All experiments were performed in the presence of 1μM leupeptin and E-64, 150nM aprotinin and 0.5mM AEBSF (Calbiochem Protease Inhibitor Kit III). A 10 minute exposure to 50 or 10nM CGRP resulted in a significant, irreversible depression of EPSC amplitudes compared to baseline levels (50nM 76.95 ± 7.11%, n = 10; 10nM 77.38 ± 7.11%, n = 8; $P<0.01$ Mann-Whitney U Test). In contrast, 50nM adrenomedullin significantly and reversibly potentiated the EPSC amplitudes (143.58 ± 24.5%, n = 7, $P<0.01$ Mann-Whitney U).

10nM CGRP failed to produce depression when applied in the presence of the selective antagonist $CGRP_{8-37}$ at a concentration of 1μM (106.03 ± 9.29%, n = 6, $P<0.01$ vs. 10nM CGRP alone, Mann-Whitney U) but not with 100nM $CGRP_{8-37}$ (79.91 ± 5.61%, n = 7). However, 10nM $CGRP_{8-37}$ alone significantly decreased EPSC amplitudes to a similar level as CGRP (48.34 ± 6.55%, n = 5, $P<0.01$ vs. control, Mann-Whitney U). Both CGRP and adrenomedullin are known to exert some of their effects through the modulation of cAMP. In the presence of the selective PKA inhibitor, H-89 (200nM), the EPSC depression produced by 10nM CGRP was not significantly affected (69.32 ± 5.01%, n = 6). However, the adrenomedullin-induced potentiation was not only reduced but reversed so that 50nM adrenomedullin in the presence of H-89 decreased the EPSC amplitude (69.10 ± 3.47%, n = 8, $P<0.01$ vs adrenomedullin in the absence of H-89, Mann-Whitney U).

Our results demonstrate that both CGRP and adrenomedullin modulate PF-PC synaptic transmission and these effects are likely to occur through distinct signalling pathways. The selective antagonist $CGRP_{8-37}$ can inhibit the CGRP response in a concentration-dependent manner, although low concentrations of $CGRP_{8-37}$ can also act as an agonist. The potentiating effect of adrenomedullin is likely to occur through the activation of cAMP but the mechanism for the action of CGRP is yet to be determined.

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83P MODULATION OF SYNAPTIC TRANSMISSION BY VIP IN THE CA1 AREA OF THE HIPPOCAMPUS IS DEPENDENT ON GABAERGIC TRANSMISSION AND ON BOTH PKA AND PKC ACTIVITIES.

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Vasoactive intestinal peptide (VIP) modulates both GABA release from hippocampal nerve terminals as well as synaptic transmission in the CA1 area of the hippocampus (Cunha-Reis *et al.*, 1999, 2000). VIP action on GABA release requires both protein kinase A (PKA) and protein kinase C (PKC) activity (Cunha-Reis *et al.*, 2000). We investigated if the action of VIP on synaptic transmission in the CA1 area of the hippocampus is dependent on GABAergic transmission as well as if it is dependent on PKA and/or PKC activity.

Field-excitatory post-synaptic potentials (fEPSPs) were recorded from the CA1 area of hippocampal slices of male Wistar rats (5-6 weeks old). The slices were kept under continuous perfusion (4 ml/min) with gassed (95% O_2 / 5% CO_2) Krebs solution (mM: NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; glucose 10). Responses were evoked by stimulation (rectangular 0.1 ms pulses, once every 15 s) of the Schaffer collateral/commissural fibres through a concentric bipolar electrode. fEPSPs were recorded through a microelectrode (4M NaCl, 3-5MΩ) placed at the *stratum radiatum*. Specificity of VIP responses was tested using the VIP receptor antagonist [Ac-Tyr^1, D-Phe^2]GRF 1-29. The dependency of VIP effect on GABAergic transmission was tested by blocking simultaneously $GABA_A$ and $GABA_B$ receptors with the selective antagonists, picrotoxin and CGP55845 respectively. Involvement of PKA activation on VIP action was tested using the PKA inhibitors, HA1004 and H-89. Involvement of PKC activation on VIP action was tested using the PKC inhibitors, chelerythrine and GF-109203x.

VIP (0.3-30nM) increased fEPSP slope with maximal excitatory effect of 23.7±1.1% (n=16, $P<0.05$) for 1nM VIP. This effect was blocked in the presence of the VIP receptor antagonist [Ac-Tyr^1, D-Phe^2]GRF 1-29 (100nM). Blockade of GABAergic transmission with picrotoxin 50μM together with CGP55845 1μM strongly attenuated ($P<0.01$) the excitatory effect of VIP on fEPSP slope (7.4±1.2% (n=4, $P<0.05$). Both PKA (HA1004, 10μM, H-89 1μM), and PKC (chelerithrine 6μM, GF-109203x 1μM) inhibitors, were able to significantly attenuate ($P<0.01$) the excitatory effect of 1nM VIP on fEPSP slope that became 12.3±1.4% (n=3, $P<0.05$) in the presence of HA1004, 9.0±0.7% (n=4, $P<0.05$) in the presence of H-89, 8.5±1.0% (n=4, $P<0.05$) in the presence of chelerythrine and 8.3±2.6% (n=4, $P<0.05$) in the presence of GF-109203x. Simultaneous PKA and PKC inhibition with H-89 (1μM) together with GF-109203x (1μM) abolished the excitatory action of VIP on synaptic transmission.

In conclusion, VIP excitatory action on synaptic transmission in the rat hippocampus is dependent on GABAergic transmission and requires both PKA and PKC activities.

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84P REGIONAL LOCALISATION OF LOW AFFINITY KAINATE RECEPTORS IN MURINE BRAIN VIA [^{3}H](2*S*,4*R*)-4-METHYLGLUTAMATE AUTORADIOGRAPHY

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Kainate receptor subunits are divided into two groups based on their relative affinity for [^{3}H]kainate, termed "low" affinity (GluR5-GluR7) and "high" affinity (KA-1 and KA-2) subunits. Previous studies have employed [^{3}H]kainate to map low affinity kainate receptors at concentrations which label both high and low affinity kainate receptor subunits. The present study employs [^{3}H](2*S*,4*R*)-4-methylglutamate (Toms *et al.*, 1997) in homogenate binding and autoradiography to selectively map low affinity kainate receptors in mouse brain.

Adult (20-25 g) male BALB/c mice were decapitated and their brains processed for autoradiography as previously described (Kitchen *et al.*, 1997). Adjacent coronal sections (20 μm) were cut for receptor mapping at 300 μm intervals for determination of total and non-specific [^{3}H](2*S*,4*R*)-4-methylglutamate (10 nM) binding. Non-specific binding was determined in the presence of 1 mM L-glutamate. Slides were apposed to [^{3}H] hyperfilm (Amersham) for five weeks and resultant films analysed by video-based computerised densitometry (MCID, Imaging Research). Homogenate binding was performed (60 min, 4°C) using cerebrocortical membranes (75 μg protein assay^{-1}) as described previously (Toms *et al.*, 1997).

[^{3}H](2*S*,4*R*)-4-Methylglutamate labelled a single site in murine cerebrocortical membranes (K_d= 9.9 ± 2.7 nM, B_{max}= 296.3 ± 27.1 fmol mg protein^{-1}, n=4). The binding of 8 nM [^{3}H](2*S*,4*R*)-4-methylglutamate was displaced by several non-NMDA receptor ligands (K_i ± s.e.m., n = 3): domoate (1.1 ± 0.2 nM) > kainate (7.1 ± 1.1 nM) >> L-glutamate (187.6 ± 31.9 nM) >> (S)-AMPA (> 50 μM).

[^{3}H](2*S*,4*R*)-4-Methylglutamate autoradiography (n=4) revealed a widespread regional distribution of low affinity kainate receptors. Highest binding densities (fmoles mg protein^{-1}) occurred within deep layers of the cerebral cortex (prelimbic cortex = 339 ± 13.5 and cingulate cortex = 332 ± 18.0). Intense binding was also observed in the hippocampal CA3 subregion (290 ± 50.2) and the external plexiform layer of the olfactory bulb (296 ± 27.3). Dense labelling was also observed in the nucleus accumbens (211 ± 28.4), caudate-putamen (233 ± 15.7), hypothalamus (147 ± 9.3), vertical limb of diagonal band (185 ± 16.1) and septum (199 ± 18.5). In the cerebellum, the granule cell layer displayed marked (169 ± 9.8) binding with moderate binding in the adjacent molecular layer (102 ± 3.7).

These data are comparable with previously reported [^{3}H]kainate binding in mouse brain (Garcia-Ladona and Gombos, 1993) and [^{3}H](2*S*,4*R*)-4-methylglutamate in monkey brain (Carroll *et al.*, 1998). Therefore, [^{3}H](2*S*,4*R*)-4-methylglutamate represents a useful radioligand, selective for low affinity kainate receptors.

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85P THE ROLE OF KAINATE (GluR5) RECEPTORS IN SENSORY RESPONSES OF RAT VENTROBASAL THALAMUS (VB) NEURONES

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Sensory vibrissa input activates ventrobasal thalamus (VB) neurones via AMPA (GluR1-4) receptors and NMDA receptors (Salt & Eaton, 1996). The aim of this study was to investigate the role of kainate receptors using the GluR5-selective agonist ATPA (Clarke *et al.*, 1997) and antagonist LY382884 (Bortolotto *et al.*, 1999).

Extracellular single-cell recordings were made from rat VB with multibarrel iontophoretic electrodes under urethane anaesthesia (Salt, 1989). Excitatory responses to vibrissa stimulation (1s air-jet) were reduced by iontophoresis of LY382884 to 50±5.7% of control (n=15, P<0.05; Wilcoxon Rank Sum test) while excitatory responses to AMPA, NMDA and ATPA were unaffected (105±12.4%, 95±4.7%, 104±9.8%, respectively). Sensory afferent input also drives recurrent GABAergic inhibition via the thalamic reticular nucleus (TRN) (Salt, 1989). Thus, iontophoretic application of the GABAa antagonist SR95531 enhanced responses to sensory stimulation (173±21% of control). However, under these conditions, LY382884 did not reduce sensory responses (94±7% of control, n=5), indicating that intact GABAergic inhibition is necessary for the GluR5 antagonist to affect sensory responses.

Intracellular current clamp recordings were made from VB cells with sharp microelectrodes in horizontal thalamic slices bathed with AP5 (50uM), MK-801 (3uM) and GYKI52466 (100uM) in order to block NMDA and AMPA-receptor excitatory transmission (Turner & Salt, 1998). Under these conditions, TRN stimulation evoked IPSPs which were reduced from -3.2 ±0.6mV (peak amplitude) to -2.5±0.5mV by the agonist ATPA (10-20uM, n=14, P<0.001). This effect was reversed from 74 ±4.9% to 98 ±2.7% of control by LY382884 (10uM, n=4).

Stimulation of a vibrissa in the centre of the receptive field evokes both excitation and inhibition, whereas stimulation of an adjacent (non-centre) vibrissa only drives inhibition. We thus compared the effect of LY382884 on responses to adjacent-vibrissa responses with responses to centre-vibrissa stimulation on 8 VB neurones in further *in vivo* experiments. Iontophoretic LY382884 reduced responses to stimulation of the central vibrissa as before (63±5% of control), but did not reduce inhibitory responses to stimulation of an adjacent whisker, indicating that activation of the central vibrissa is necessary to reveal GluR5-mediated effects.

These data suggest a role for GluR5 in sensory transmission which could be pre-synaptic (Clarke *et al.*, 1997), reducing GABAergic input onto VB relay cells. Furthermore, release of glutamate upon stimulation of the central vibrissa may be required for activation of GluR5 located presynaptically on GABAergic terminals to modulate inhibition. GluR5 may therefore be a key element in a circuit that allows important sensory input to overcome inhibition.

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86P N^3-SUBSTITUTED WILLARDIINE ANALOGUES ACT AS KAINATE RECEPTOR ANTAGONISTS IN THE NEONATAL RAT DORSAL ROOT PREPARATION

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The natural product willardiine is a selective agonist for AMPA over kainate receptors yet some 5-substituted analogues of willardiine show potent agonist activity at kainate receptors (Wong *et al.*, 1994). We have previously demonstrated that increasing the inter-acidic group chain length of willardiine by adding N^3-substituents to the uracil ring can convert its agonist action at AMPA receptors into that of an antagonist (More *et al.* 2001a, 2001b). The aim of the current study is to determine whether N^3-substituted willardiine analogues can also act as antagonists at GluR5-containing kainate receptors. Using the neonatal rat dorsal root preparation, the activity of (*S*)-3-(4-carboxybenzyl)willardiine (3-CBW), a compound that showed selectivity for AMPA over kainate receptors on neonatal rat motoneurones (More *et al.*, 2001a), has been assessed for activity at GluR5-containing kainate receptors. In addition, the activities of both the (*R*)- and (*S*)-enantiomers of 3-(2-carboxyethyl)willardiine (CEW) and 3-(3-carboxypropyl)-willardiine (CPW) have been investigated.

Recordings were made from dorsal roots of spinal cords taken from 2-5 day old Wistar rats of either sex (4-13 g). This allowed measurement of depolarisations evoked by the exogenously applied agonist, kainate (1 min applications) (Agrawal & Evans, 1986). Non-cumulative concentration-response curves (CRCs) were constructed to kainate in the absence and presence of the (*S*)-enantiomers of the antagonists (100 μM; 30 min pre-incubation). For the (*R*)-enantiomers the percentage antagonism of triplicate 10 μM kainate applications was calculated in the presence of 200 μM antagonist (30 min pre-incubation).

Parallel shifts in the CRCs to kainate were observed in the presence of 100 μM (*S*)-3-CBW, (*S*)-3-CEW and (*S*)-3-CPW giving apparent K_D values of 9.25 ± 0.54, 73.1 ± 4.5 and 60.5 ± 4.1 μM, respectively (n=3; mean ± s.e.m.). 200 μM (*R*)-CEW and (*R*)-CPW reduced responses to 10 μM kainate by 14.4 ± 1.0 and 24.2 ± 3.0 %, respectively (n=3; mean ± s.e.m.).

The rank order of potency of the antagonists tested was (*S*)-3-CBW> (*S*)-3-CPW ≥ (*S*)-3-CEW suggesting that the kainate receptors on the dorsal root can accommodate an extended chain length at the N^3-position and that the lipophilic phenyl ring in 3-CBW improves antagonist potency. The (*R*)-enantiomers were relatively inactive as antagonists suggesting (*S*)-stereochemistry is optimal for antagonism.

The weak antagonism by CBW of kainate-induced responses on motoneurones (More *et al.*, 2001a) contrasts with the moderately potent antagonism of kainate responses on dorsal roots suggesting that the subunit composition of kainate receptors in these two tissues is different.

Willardiine derivatives with N^3-substituents antagonise GluR5-containing kainate receptors on neonatal rat dorsal roots. The order of potency of the three (*S*)-enantiomers tested differs from their order of potency for AMPA receptor antagonism. Thus, further structure activity studies for willardiine derivatives may lead to optimisation of compounds for selective antagonism of either AMPA or kainate receptors.

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87P INTERACTION OF AMPA RECEPTOR MODULATORS IN THE CHICKEN RETINA

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AMPA/kainate receptors have major role in learning and memory processes (Bliss and Collingridge, 1993). Drugs that positively modulate AMPA receptors can facilitate the AMPA receptor mediated processes and may have potential therapeutic utility in diseases associated glutamatergic disfunction such as memory deficits in Alzheimers or after brain insults (Yamada, 2000). Chicken retinal spreading depression (RSD) was described as a suitable and relatively specific *in vitro* model for testing glutamate receptor antagonists (Sheardown *et al.*, 1993).

We elicited RSD in the posterior eyecups of day-old chicks (Shaver Reedbrow) by 5 μM S-AMPA (for antagonists) and 2 μM RS-AMPA (for positive modulators). AMPA antagonists (NBQX, EGIS-9637, GYKI-53655, EGIS-8332, GYKI-53405, GYKI-52466) concentration-dependently blocked the initiation of RSD (IC_{50}s are 0.2, 1.1, 1.4, 5.3, 7.0 and 16.6 μM, respectively). These effects were in good correlation with patch clamp experiments on isolated cerebellar Purkinje cells (IC_{50}s are 0.2, 2.5, 2.3, 7.6, 7.6 and 21.7 μM, respectively). We compared the effects of positive modulators (cyclothiazide, S-18986, IDRA-21 and aniracetam) which compounds accelerated the initiation and propagation of RSD. This potentiating effect reflected in a decrease of the latency of the RSD (EC_{50}s are 8.5, 78, 126 μM and 1.43 mM). The tested positive modulators also shifted the concentration response-curve of GYKI-52466 to the right in a near parallel fashion indicating a strong allosteric interaction between the modulatory sites. When drugs have been added in equiactive concentrations potencies of positive modulators were different regard by the reversal of GYKI 52466-induced inhibition (Table 1.).

Table 1.	% potentiation	IC_{50} of GYKI-52466
control	-	16.6
cyclothiazide 10 μM	58.0 ± 3.6	25.3
aniracetam 2 mM	58.7 ± 4.8	38.2
S-18986 100 μM	54.8 ± 2.6	53.0

We suppose that AMPA positive modulators can act at multiple allosteric sites and might differently interact with the negative modulatory site. When positive modulators were co-administered, aniracetam increased the effect of cyclothiazide, however, we found significant additive effects ($p<0.01$) only at the lower concentrations (300 μM aniracetam: 14.9 ± 2.7 %, n=8 and 2.5 μM cyclothiazide 9.9 ± 4 %, n=10, co-application: 32.4 ± 5.5 %, n=10). Effects of 30 μM S-18986 (49.4 ± 5.5 %, n=9) and 3 μM cyclothiazide (39.9 ± 4.9 %, n=9) were also additive (71.9 ± 2.5 %, n=11) ($p<0.001$).

These results may confirm the existance of multiple positive modulatory binding sites on the AMPA receptor. Additivity can also be explained by the heterogeneity of the AMPA receptors, i.e. positive modulators act differently on the various receptor subunits and splice variants. We conclude that retinal spreading depression is a suitable pharmacological test to predict the efficacy of AMPA positive modulators.

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88P PHARMACOLOGICAL CHARACTERISATION OF THE SUBUNIT SELECTIVE NMDA RECEPTOR ANTAGONIST PPDA ON NEONATAL RAT MOTONEURONES

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The *N*-methyl-D-aspartate (NMDA) receptor is a multisubunit channel complex, which is involved in a variety of neuronal processes (Jane *et al.*, 2000). Five genes encode for the NMDA receptor subunits termed NR1 and NR2A-D. It has been proposed that NMDA receptors are tetrameric or pentameric complexes comprised of at least two NR1 receptor subunits with the remainder being NR2 subunits. The differential distribution of NMDA receptors of different subunit compositions throughout the central nervous system (CNS) is thought to underlie distinct physiological and pathological roles for these subtypes.

As the glutamate binding site is on the NR2 subunit development of subunit specific competitive antagonists should be possible. To date, most competitive antagonists have a similar subunit selectivity profile: NR2A > NR2B > NR2C > NR2D (Jane *et al.*, 2000). Recently, we have reported that (±)-cis-1-(phenanthren-2-yl-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA), has a different subunit selectivity profile NR2C > NR2D > NR2B > NR2A (Hrabetova *et al.*, 2000).

In this study we sought to characterise the actions of PPDA using the neonatal rat spinal cord preparation. All experiments were carried out in 1-4 day old Wistar rats of either sex as described previously (Evans *et al.*, 1982). To examine the effects of PPDA (5 μM) on direct depolarisations of motoneurones induced by (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA, 6 μM), kainate (50 μM), NMDA (50 μM) and (1*S*,3*R*)-1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD, 30 μM), experiments were conducted in standard medium containing TTX (10 μM for 2 min then 0.1 μM continuously). Agonists were applied for 1 min and the degree of depolarisation of the motoneurone was measured by peak amplitude. The ability of PPDA (5 μM, 15 min pre-incubation) to selectively reduce the peak amplitude of the agonist-induced responses was then measured. All agonist-induced responses recovered after a 30 min washout of the antagonist.

In the presence of PPDA, NMDA-, AMPA- and kainate-induced responses were antagonised by 87 ± 4, 20 ± 4 and 7 ± 7 % of control responses, respectively (n=3). Responses to ACPD were unaffected suggesting that PPDA has no effect on group I metabotropic glutamate receptors.

In order to calculate the pA_2 value for the antagonism of NMDA-induced depolarisations by PPDA, non-cumulative concentration-response curves were constructed both in the absence and presence of 0.5, 0.7, 1, 2 and 3 μM PPDA and dose ratios estimated. Schild analysis was then performed. A pA_2 value of 6.45 and a slope of 0.96 ± 0.07 (n=15) was obtained suggestive of competitive antagonism.

Thus PPDA has been shown to potently and selectively antagonise NMDA receptors on rat motoneurones. Further development of compounds based on the PPDA structure is likely to lead to antagonists with even greater subunit selectivity. These will be useful tools to probe the physiological roles of NMDA receptor subunits and may lead to novel therapeutic agents with improved side effect profiles to treat a range of CNS disorders.

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89P EVIDENCE FOR TWO CLASSES OF NR2B –DIRECTED NMDA RECEPTOR ANTAGONISTS

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CP-101,606 represents a new class of subtype-selective NMDA receptor antagonists, which appear to be targeted to receptors comprising the NR2B subunit. The compound is beginning to show promise in the clinic, which is likely to reflect a combination of its subtype selectivity and its novel activity-dependent mode of action (Chazot, 2000; Chenard & Menniti, 1999).

The subtype-selectivity of [^{3}H] racemic CP-101,606, was determined using defined recombinant NMDA receptor sub-units expressed in HEK 293 cells. The assay was performed in 50 mM Tris-citrate, pH 7.1, containing 5 mM EGTA and 5 mM EDTA. Aliquots (50 μg protein) of the preparations was incubated in the presence of 0.1 to 20 nM [^{3}H] radioligand (saturation studies) or 2 nM radioligand (competition studies) at 4°C for 2 h. Non-specific binding was defined by 10 mM spermidine. The reaction was terminated by rapid filtration using a Brandell Cell Harvester.

No significant specific [^{3}H] racemic CP-101,606 (at 2nM) binding was detected to NR1, NR2A, NR2B subunits expressed alone or NR1/NR2A receptors. In contrast, [^{3}H] CP-101,606 bound to NR1/NR2B receptors expressed in HEK 293 cells with K_D = 6.0 ± 0.9 nM, which compares well with adult rat forebrain membranes, K_D = 4.2 ± 1.0 nM (mean ± SD, for three separate determinations).

HEK 293 cells were transfected with NR1, NR2A and NR2B receptor subunits and complexes comprising all three subunits were isolated by anti-NR2A immunoaffinity chromatography as previously described (Hawkins *et al.*, 1999). Based on immunoblotting with NMDA receptor subunit-selective antibodies, the immunopurified material contained all three NMDA receptor subunit polypeptides, NR1, NR2A and NR2B. However, in contrast to previous studies in which high affinity [^{3}H] Ro-25,6981 binding activity was observed (Hawkins *et al.*, 1999), no significant high affinity [^{3}H] CP-101,606 binding sites were detected to the immunopurified material (up to 10nM radioligand concentration).

This study provides further evidence for two distinct classes of NR2B-directed NMDA receptor antagonists. The first binds with high affinity, irrespective of whether another NR2 subunit type is present (Ro-25,6981). A second class is significantly affected by the presence of another NR2 subunit type within the receptor complex, exemplified by CP-101,606.

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90P PHARMACOLOGICAL CHARACTERISATION OF POSITIVE MODULATORY METABOTROPIC GLUTAMATE AUTORECEPTORS IN THE RAT CEREBRAL CORTEX

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Group I mGlu receptors are involved in a number of excitatory amino acid (EAA)-mediated responses in the CNS, both physiological and pathological. However, the mechanisms underlying these responses are unclear and much of the current data is controversial, largely due to the poor selectivity and potency of the ligands used. We have previously shown that presynaptic GroupI mGlu receptors provide positive modulatory control of EAA release in the rat cerebral cortex (Fazal *et al.*, 2001). Here, we further define the specific mGlu receptor subtype mediating this response by investigating the effects of selective Group I ligands on basal and depolarisation -evoked efflux of pre-accumulated D-[^{3}H]aspartate (D-[^{3}H]ASP) from superfused rat cerebrocortical minislices.

Minislices were prepared from male Sprague-Dawley rats (350-380 g) as previously described (Palmer & Reiter, 1994) and superfused (1.6 ml/min; 37°C) with Krebs-Ringer Medium. All results are means of duplicate determinations of at least 4 independent observations. Transient elevations in extra-cellular [K^+] produced a concentration-dependent increase in the efflux of pre-accumulated D-[^{3}H]ASP. 50 mM K^+ evoked a submaximal efflux and was used in all subsequent experiments. Both the non-selective mGlu receptor agonist 1S, 3R-ACPD (max. 129% of control at 100 μM; EC_{50} 17.8 μM; Graph Pad Prism Software) and the selective GroupI agonist (S)-3,5-DHPG (max. 128% at 3 μM; EC_{50} 0.46 μM) potentiated K^+-evoked D-[^{3}H]ASP efflux in a concentration-dependent fashion. The selective mGlu5 receptor agonist (RS)-CHPG evoked similar responses (max. 122% at 100 μM; EC_{50} 7.3 μM). The broad-spectrum mGlu receptor antagonist (S)-MCPG markedly inhibited (S)-3,5- DHPG (3 μM)-enhanced efflux (98.5% inhibition at 200 μM; IC_{50} 88.5 μM). The proposed mGlu1 receptor-selective antagonist AIDA (10-300 μM), however, was inactive. A much higher, non-selective concentration (1 mM) caused a 78% inhibition of the response ($P<0.001$). The potent, competitive and highly mGlu1-selective antagonist LY367385 (1-100 μM) also failed to significantly influence responses to (S)-3,5-DHPG (3 μM), as did CPCCOEt ($\lesssim$30 μM), another recently developed potent, non-competitive mGlu1-selective antagonist. However, the novel mGlu5-selective antagonist MPEP powerfully inhibited (S)-3,5-DHPG (3 μM)-evoked responses (99% inhibition at 10 μM; IC_{50} 0.55 μM). Moreover, two other highly selective, non-competitive mGlu5 receptor antagonists, SIB-1757 and SIB-1893, at 10 μM, significantly inhibited (S)-3,5-DHPG-enhanced efflux ($P < 0.05$). None of the agonists or antagonists significantly altered basal or K^+-evoked efflux of D-[^{3}H]ASP when superfused alone.

These results confirm that presynaptic Group I mGlu auto-receptors are present in the rat cerebral cortex and suggest that the mGlu5 receptor subtype plays a key role in the modulation of EAA release in this brain region.

AF is a BBSRC CASE Award student.

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91P PHARMACOLOGICAL CHARACTERISATION OF THREE 2-OXOPYRIDYLALANINE ANALOGUES ON GLUTAMATE RECEPTORS EXPRESSED ON NEONATAL RAT MOTONEURONES

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It has previously been shown that (*RS*)-*1H*-2-oxo-6-pyridyalanine (OHPA) produced a depolarisation of motoneurones in the neonatal rat spinal cord preparation (Davies *et al.*, 1982). Since selective antagonists were not available at the time all that could be stated was that its action was mediated mainly by non-*N*-methyl-D-aspartate (non-NMDA) receptors. With the development of selective antagonists, able to distinguish between NMDA, α-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA)/kainate and group I metabotropic glutamate (mGlu) receptors we have characterised three compounds; OHPA and two structurally related compounds recently synthesised in our laboratory, UBP1133 and UBP1134 (Figure 1).

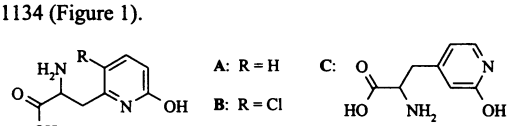

Figure 1. Structure of the 3 novel compounds.
A: OHPA, **B**: UBP1133, **C**: UBP1134.

All experiments were performed on isolated spinal cords from neonatal rats (2-5 days old) bathed in a physiological saline (Evans *et al.*, 1982) with tetrodotoxin (TTX: 10 μM for 2 min, then 0.1 μM continuously) added to the perfusate. Saline flow was 1ml min^{-1} throughout the experiment. Recordings were made from the ventral root. The three compounds were then applied for 1 min and the effects on the motoneurones examined.

OHPA (300 μM) and UBP1133 (3 mM) produced a depolarisation of the motoneurones. In contrast UBP1134 (1 mM) did not. Next, equi-effective concentrations of AMPA (1 μM), NMDA (10 μM), (S)-3,5-dihydroxyphenylglycine (DHPG, 20 μM, a selective group I mGlu receptor agonist), OHPA (300 μM) and UBP1133 (3 mM) were applied in the absence and presence of the AMPA/kainate receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]-quinoxaline-7-sulphonamide (NBQX, 1 μM, 15 min pre-treatment). The responses to AMPA, OHPA and UBP1133 were abolished in the presence of NBQX but recovered after washout. The responses to NMDA and DHPG were not significantly reduced (paired t-test, $p>0.05$) at 95.4 ± 6.5 % and 76.8 ± 11.7 % of control values, respectively (n=3). The responses to AMPA, OHPA and UBP1133 partially recovered to 58.9 ± 13.5 %, 67.8 ± 8.6 % and 56.4 ± 9.1 %, respectively, after a 30 min washout.

UBP1134 was then tested for antagonist activity. Equi-effective concentrations of NMDA (10 μM), AMPA (1 μM), DHPG (20 μM) and kainate (4 μM) were applied in the absence and presence of 1 mM UBP1134 (15 min pre-treatment). In the presence of UBP1134 the NMDA response was reduced to 38.3 ± 3.1 % of control (n=3). None of the other agonist responses were reduced (AMPA: 132.2 ± 6.3 %, DHPG: 100.5 ± 2.2 % and kainate: 117.0 ± 20.2 %, all n=3). The NMDA response recovered to 109.0 ± 9.9 % of the control value after a 30 min washout.

These results highlight that small changes in the structure of the pyridylalanine can lead to a large change in its activity. In this case moving the alanine side chain from the 6 position (OHPA) to the 4 position (UBP1134) altered the activity of the compound from an AMPA receptor agonist to an NMDA receptor antagonist. Contrary to expectations, 5-Chloro-substitution of OHPA (to give UBP1133) reduced agonist activity at AMPA receptors, most probably due to the steric hindrance imposed by the chlorine atom.

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92P A COMPARISON OF GROUP III METABOTROPIC GLUTAMATE RECEPTOR AGONISTS AND THE ABILITY OF LY341495 TO ANTAGONISE THEIR RESPONSES ON NEONATAL RAT PRIMARY AFFERENTS

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It is known that group III metabotropic glutamate (mGlu) receptors are present on primary afferents in the neonatal rat spinal cord. Activation of these receptors has been shown to mediate a reduction in the amplitude of the fast component of the dorsal root evoked-ventral root potential (fDR-VRP), most likely via attenuation of glutamate release (Jane *et al.*, 1994). Here we examine two group III mGlu receptor agonists; (*S*)-2-amino-4-phosphonobutyrate (L-AP4) and (1*S*,3*R*,4*S*)-1-aminocyclopentane-1,2,4-tricarboxylic acid (ACPT-1, an mGlu4α receptor agonist; Acher *et al.*, 1997). We have also examined the ability of (2*S*,1'*S*,2'*S*)-2-(9-xanthenthenyl-methyl)-2-(2-carboxycyclopropyl)-glycine (LY341495), a broad spectrum mGlu receptor antagonist (Schoepp *et al.*, 1999), to block the depression of the agonist-induced depression of the fDR-VRP.

All experiments were performed on isolated hemisected spinal cords from neonatal rats (2-3 days old) bathed in a physiological saline (Evans *et al.*, 1982) with 2 mM $MgSO_4$ and 50 μM D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) added. Recordings were made from the ventral root following stimulation of the corresponding dorsal root (16x threshold, 2 pulses min^{-1}). Non-cumulative concentration-response curves (CRCs) were then constructed to L-AP4 or ACPT-1 by changing the perfusate to one containing the agonist for 5 min. After the first CRC was constructed the perfusate was then changed to one containing 20 nM LY341495, left 90 min to equilibrate, and a second CRC constructed.

Both the agonists tested were able to completely abolish the fDR-VRP and were therefore full agonists. The EC_{50}s for L-AP4 and ACPT-1 were 1.04 ± 0.52 μM (4) and 7.10 ± 0.63 μM (7), respectively (number of determinations in parentheses). The EC_{50} value for L-AP4 found in this study was similar to that found in previous studies (Howson and Jane, 2000).

ACPT-1, previously untested in the neonatal rat preparation, has been shown to have an EC_{50} of 7.2 μM at the mGlu4α receptor (Acher *et al.*, 1997).

CRCs constructed to the agonists in the presence of 20 nM LY341495 were parallel to the first CRC but shifted rightwards. Apparent K_D values were calculated and found to be 8.86 ±1.53 nM (4) and 4.01 ± 0.74 nM (3) against L-AP4 and ACPT-1, respectively.

The high potency of LY341495 against the agonists used was unexpected as previous studies using LY341495 have shown the potency at mGlu4α, and mGlu8 receptors to be 22, and 0.173 μM, respectively (Schoepp *et al.*, 1999). However, these results were from studies using cloned human receptors and the differences in preparations and protocols may account for the higher potencies of LY341495 seen in this study.

ACPT-1 has previously been reported as a mGlu4a selective agonist but was not tested on mGlu8 in this study (Acher *et al.*, 1997). We suggest that ACPT-1 is more likely to be primarily acting at mGlu8 rather than mGlu4 receptors in the spinal cord, as LY341495 is over 100 times more potent at cloned mGlu8 receptors than mGlu4α and yet was almost as potent against ACPT-1 as L-AP4.

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93P INFLUENCE OF LOCALLY APPLIED GROUP I mGlu RECEPTOR LIGANDS ON NEURONAL 5-HT RELEASE IN THE RAT FRONTAL CORTEX *IN VIVO*

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A number of studies have recently emerged to address the neuromodulatory roles of presynaptic group I metabotropic glutamate (mGlu) receptors. Initial evidence suggests that these receptors mediate a facilitatory effect on the release of a variety of central neurotransmitters (Cartmell & Schoepp, 2000). However, very little is known on the regulation of serotoninergic function by this receptor subtype, especially *in vivo*. Thus, we now investigate the effect of group I mGlu receptor ligands on neuronal 5-HT release in the frontal cortex of freely-moving rats, using intracerebral microdialysis.

Under halothane anaesthesia, concentric dialysis monoprobes (4mm, Gambro-Hospal GFE-9 membrane) were stereotaxically implanted into the frontal cortex of male Wistar rats (300-400g). On the following day, probes were perfused at 1μl/min with artificial CSF (composition in mM: NaCl 145; KCl 2.7; Na_2HPO_4 2.0; $MgCl_2$ 1.0; $CaCl_2$ 1.2) containing 1μM citalopram. Following a 1 h equilibration period, samples were collected every 30 min. Ligands were administered over two collections (60 min) by introduction into the perfusion stream following at least three basal samples. Dialysate 5-HT was evaluated by HPLC separation with electrochemical detection. Data were calculated as the maximum percentage change compared to the basal sample immediately preceding drug treatment and expressed as mean ±s.e.mean of *n* =3-4 independent observations. Significance of differences was assessed using Student's paired two-tailed t-test.

Basal 5-HT was readily detectable in the frontal cortex and was consistent between animals. Initial experiments showed a basal level of 40.5 ±3.9 fmol/20μl. This increased by 622.7 ±137.5% in the presence of 100mM KCl, whilst removal of Ca^{2+} from the perfusion medium reduced basal efflux by 53.9 ±1.9%. The mGlu receptor agonist (1S,3R)-ACPD (3mM) failed to significantly ($P>0.05$) modify basal release of 5-HT (108.6 ±14.2% of basal levels). Similarly, the group I mGlu receptor-selective agonist, (RS)-3,5-DHPG did not ($P>0.05$) influence release at either 1mM or 3mM (96.4 ±12.6% and 92.5 ±2.2% of basal levels, respectively). In accord with these results, neither the group I mGlu receptor antagonist (RS)-MCPG (3mM) nor the selective $mGlu_5$ antagonist MPEP (100μM) showed any effect ($P>0.05$) on basal 5-HT levels (116.0 ±20.9% and 111.1 ±9.7% of basal levels, respectively).

The current data provide no evidence that neuronal 5-HT release in the rat frontal cortex is subject to regulation by group I mGlu receptors, either by endogenous or exogenous activators. A similar absence of group I mGlu receptor activity has recently been reported for 5-HT release in the rat periaqueductal gray matter (Maione *et al.*, 1998). The central facilitatory action of group I mGlu receptors may not, therefore, be a universal phenomenon but may instead be region-specific.

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94P EFFECT OF ANTAGONISTS AT THE NMDA RECEPTOR COMPLEX ON CHANGES IN AMINO ACID EFFLUX INDUCED BY GlyT-1 INHIBITION

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The NMDA receptor is modulated by glycine (GLY) at a strychnine insensitive binding site on the receptor complex. Recent studies have reported positive NMDA receptor modulation *in vivo* by agonists of this site (*e.g.* Kolhekar *et al.*, 1994) despite theoretical saturation by the extracellular GLY concentration. It has been suggested that GLY concentrations within the glutamatergic synapse could be maintained at sub-saturating levels by the selective GLY uptake site GlyT-1 which is co-localised with the NMDA receptor (see Suplisson & Bergman, 1997). In this study, we have used microdialysis in the spinal cord of the rat to investigate whether the novel GlyT-1 inhibitor ORG 24598 (Walker *et al.*, 1999) can enhance NMDA receptor activity. This was assessed by determining the effect of ORG 24598 on GLY and citrulline (CIT) efflux in the presence of the non-competitive NMDA receptor antagonist MK-801 and the antagonist of the GLY site on the NMDA receptor 7-chlorokynurenic acid (7-CK).

Male Wistar rats (250-350g; Charles Rivers Ltd.) were anaesthetised under halothane/N_2O throughout. A burr hole was created in the dorsal aspect of Th_{13} through which a microdialysis probe was inserted (at an angle of 16° to the horizontal) to place a 1.5 mm length of dialysis membrane (Hemophan® o.d. 218 μm; Gambro Hospal U.K. Ltd.) unilaterally into the dorsal horn of the L3/4 lumbar region of the spinal cord. The probe was perfused at a rate of 2 $\mu l.min^{-1}$ with an artificial extracellular fluid solution (aECF) for 120 min post implantation before 10 min samples of dialysate were collected. After 30 min to estimate basal efflux, separate groups received 40 min perfusion with aECF containing MK-801 (500 μM) or 7-CK (1mM) followed by 60 min with ORG 24598 (10 μM) plus either MK-801 or 7-CK. Separate time matched control groups received either MK-801, 7-CK or ORG 24598 alone. Dialysate GLY and CIT, as a marker of NMDA receptor activation, were quantified by HPLC coupled with fluorescence detection. The effect of antagonists on ORG 24598 induced increases in GLY and CIT efflux was assessed on the basis of the absolute efflux over basal during the 60 min period of ORG 24598 administration.

Perfusion with ORG 24598 increased GLY to a sustained maximum of 187-201 % of basal levels after 20 min ORG 24598 administration. CIT efflux increased gradually reaching a maximum of 142-149 % following 50 min ORG 24598 administration ($P<0.01$, one way ANOVA with repeated measures, n=4). Neither MK-801 nor 7-CK had any significant effect on basal GLY or CIT efflux or on the ORG 24598 induced efflux of GLY. However, the ORG 24598 induced increase in CIT efflux was reduced from 7.7 ± 2.4 $pmol.60min^{-1}$ to 0.77 ± 0.82 and 0.06 ± 3.17 $pmol.60min^{-1}$ by MK-801 and 7-CK respectively ($P<0.01$, one-way ANOVA followed by *post-hoc* Dunnet's test compared to ORG 24598 alone, n=6 for both).

These results demonstrate that the extracellular concentration of GLY and CIT is increased by GlyT-1 inhibition in the rat spinal cord *in vivo*. Further, the present study indicates that the increase in CIT resulting from GlyT-1 inhibition is dependent on NMDA activation. Taken together with a previous study, which showed the ORG 24598 induced increase in CIT to be dependent on nNOS activation (Pearce *et al.*, 2000) this study supports the hypothesis that GlyT-1 inhibition results in positive modulation of NMDA receptor activity *in vivo*.

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95P EFFECT OF SELECTIVE GABA UPTAKE INHIBITION ON BASAL GABA AND HIGH K^+-EVOKED RELEASE IN THE RAT SPINAL CORD *IN VIVO*

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Inhibitory regulation of primary afferent transmitter release is likely to be an important role for GABAergic neurotransmission in the spinal cord. Rapid inactivation of released GABA within the synaptic cleft is achieved via selective uptake mechanisms, the majority of which is through the action of the predominately neuronal uptake site, GAT-1 (Borden, 1996). A recent report has shown that selective inhibition of this transport protein by tiagabine is antinociceptive in rodent models of acute pain (Ipponi *et al.*, 1999). However it is unclear whether this effect is mediated at the spinal or supraspinal level. Here, using *in vivo* microdialysis, we have characterised the effect of local administration of a more selective and potent tiagabine analogue NO-711 on GABA efflux in the rat spinal cord dorsal horn and its effect upon high K^+-evoked amino acid release.

Male Wistar rats (250-350g, Charles Rivers U.K. Ltd) were used and anaesthetised under halothane/N_20 throughout. The spinal cord was exposed through a burr hole in the dorsal aspect of Th_{13} vertebrae. A microdialysis probe was implanted 0.4 mm lateral to the midline blood vessel at an angle of 16° to the horizontal, such that 1.5 mm of dialysis membrane was within the dorsal horn at the L3-4 region of the spinal cord. The probe was perfused at a rate of 2 $\mu l.min^{-1}$ with an artificial extracellular fluid solution (aECF). Following a 120 min recovery period, 10 minute samples were collected and assayed for aspartate (ASP), glutamate (GLU), glycine (GLY) and GABA content by HPLC coupled to fluorescence detection. To investigate the effect of NO-711 on non-stimulated efflux, separate groups received a 60 min period of perfusion with aECF containing 10, 100 or 300 μM NO-711 preceded by a 40 min basal period. To determine the effect of NO-711 on stimulated efflux, efflux was stimulated by perfusion with aECF containing 45 mM K^+ for 6 min during the fifth (S1) and eleventh (S2) samples both in the presence and absence of 300 μM NO-711, included in the perfusate for 30 min preceding S2. S1 and S2 values were calculated as the difference from mean of the preceding 3 samples and S2/S1 ratios determined.

NO-711 produced a concentration dependent increase in extracellular GABA, maximal within 30 min at 326 ± 14% of basal (mean ± s.e. mean, n = 3). ASP efflux was increased only on the highest concentration to a maximum of 160 ± 9% (mean ± s.e. mean, n = 3). There was no significant change in GLU and GLY basal efflux ($P>0.05$, two way ANOVA). Perfusion of NO-711 significantly reduced high K^+ evoked GLU release and tended to reduce ASP efflux with no effect on GLY release (Table 1).

Table 1: S2/S1 ratio of high K+ evoked amino acid release.

	Control	NO-711
ASP	1.45 ± 0.38	0.59 ± 0.26
GLU	1.02 ±0.09	0.53 ± 0.16*
GLY	1.15 ± 0.12	1.24 ± 0.27

Mean ± s.e. mean, n = 3-5 *$P<0.05$ Student's t-test (one sided).

GAT-1 inhibition in the dorsal spinal cord increased extracellular GABA and inhibited K^+-evoked glutamate efflux. Inhibition of primary afferent transmitter release may therefore contribute to the reported antinociceptive effect of GAT-1 inhibition through increased GABAergic tone at the spinal level.

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96P THE EFFECT OF GAP JUNCTION INHIBITORS ON GABA UPTAKE INHIBITOR-INDUCED DEPOLARIZATIONS IN MOUSE CORTICAL SLICES.

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The predominant role of GABA within the CNS concerns fast, inhibitory synaptic transmission. However, it has been shown that GABA can also elicit depolarization in a number of experimental preparations (Perkins & Wong, 1996). For example, perfusion of mouse cortical slices with the GABA-uptake inhibitors tiagabine or 1-(2-((diphenyl-methylene) imino)oxy)ethyl)-1,2,5,6,-terahydro-3-pyridine carboxylic acid hydrochloride (NO-711) induces slow depolarizations which continue for 4-5 hours (Davies & Shakesby, 1999). The mechanism(s) underlying these depolarizations has yet to be determined and this present study investigated the possible involvement of gap junctions.

Coronal cortical slices (500 μm) were prepared from male or female BALB/c mice and placed in a two-compartment bath with a grease seal separating the cortex from the callosum as described by Burton *et al*, (1987). Drugs were perfused over the cortical portion of the preparation in artificial cerebro-spinal fluid at 2ml min^{-1} at room temperature. NO-711 was perfused for 15 minutes and once stable depolarizations were recorded the gap junction inhibitors, carbenoxolone or octanol were perfused for 60 or 30 minutes respectively. In some experiments the potassium channel antagonist 4-amino-pyridine (4-AP, 50 μM) was perfused prior to the gap junction inhibitors. Electrical activity was monitored between the two portions of the bath with Ag/AgCl electrodes and recorded on a MacLab computer system. Results were analysed by one-way ANOVA followed by Bonferroni's post-hoc test.

NO-711 (25 μM) elicited depolarizations which were stable in frequency and amplitude over 3-4 hours. Carbenoxolone (10-300 μM) and octanol (10-30 μM) both significantly reduced the depolarizations in a concentration-dependent manner. Carbenoxolone (100 μM) increased the time interval between depolarizations from 9.1 ± 0.5 to 35.5 ± 8.7 minutes ($P<0.01$, n= 7), while octanol (20 μM) increased the interval from 11.5 ± 0.6 to 21.1 ± 4.4 minutes ($P< 0.05$, n= 6). At low concentrations of carbenoxolone (10-100 μM) and octanol (10-20 μM) the effects were reversible but at higher concentrations the depolarizations were abolished. Perfusion with the potassium channel blocker, 4-AP, significantly increased the frequency of the NO-711-induced depolarizations ($P<0.01$ n=17) and these were also blocked by carbenoxolone (100 μM). However, in 8 out of 13 slices with carbenoxolone, the depolarizations were converted into hyperpolarizations.

The ability of both carbenoxolone and octanol, at relatively low concentrations, to inhibit GABA uptake blocker-induced depolarizations suggests that gap junctions contribute to the initiation and/or spread of these paradoxical physiological events.

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97P THE DEVELOPMENT OF DIFFERENTIAL CONTINGENT NEGATIVE VARIATION POTENTIALS TO REINFORCED AND NON-REINFORCED SIGNALS IN RATS ARE ENHANCED BY PRETREATMENT WITH NICOTINE.

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The contingent negative variation (CNV) is a slow negative wave that can be recorded from the scalps of conscious human subjects or rats during the interval between a warning stimulus (S1) and a response stimulus (S2) (see Ebenezer, 1986a), and is sensitive to psychoactive drugs, such as nicotine (Ebenezer, 1986b). The magnitude of the CNV is attenuated in people with learning and memory dysfunction (Donald, 1980). A number of behavioural studies have shown that nicotine can enhance memory-related performance in mammalian species (Bovet et al., 1967). The aim of the present study was to investigate the effects of pretreatment with nicotine on the development of differential CNVs in the rat.

A method, similar to that described by Pirch (1977), was used. Male Wistar rats (n=8) were chronically implanted under Equithesin anaesthesia with silver-silver chloride electrodes. The active electrode was located over frontal cortex and the reference electrode was located over ipsilateral occipital cortex (Ebenezer, 1986a). EEGs were recorded by means of a.c. amplifiers (time constant = 15s). The rats were divided into 2 equal groups. Each rat was fasted for 22h a day and placed separately in an operant chamber. Tone S1A (100ms, 1400Hz) was paired with the automatic delivery of food reinforcement (S2), while tone S1B (100ms, 400Hz) was not reinforced. Thirty trials (15 S1A and 15 S1B) were presented to a rat in a single session and one session was conducted every day for 16 days. The CNVs that developed to S1A and S1B were averaged using the technique of signal averaging. The rats in Group 1 (Control; n=4) received an s.c. injection of saline 10 min prior to each session, while those in Group 2 (n=4) received nicotine (0.4 mg kg^{-1}; s.c.).

Control rats (Group 1) developed differential CNVs to tones S1A and S1B (see Fig. 1A) by the 10th training session, whereas rats that were pretreated with nicotine (Group 2; see Fig. 1B) developed differential CNVs by the 4th training sessions. In addition, the magnitude of the CNVs to the reinforced tone (S1A) were generally larger in rats that had been pretreated with nicotine compared with control animals over the 16 day training period.

The results show that pretreatment with nicotine enhances the ability of rats to generate differential CNVs. The mechanisms involved are not know, but may be related to the effects of the drug on level of arousal (Ebenezer, 2001). These findings suggest that nicotine may be useful in the treatment of learning and memory disorders.

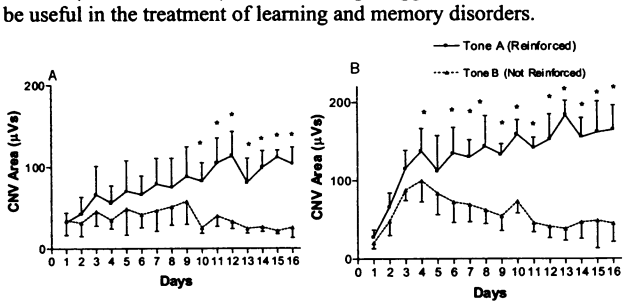

Fig. 1. Learning curves for rats trained in a discrimination CNV paradigm under (A) saline or (B) nicotine (4 mg kg^{-1}; s.c.). *p<0.05

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98P EFFECTS OF SOCIAL DEFEAT ON REGIONAL BRAIN CORTICOSTERONE CONCENTRATIONS

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The experience of social defeat causes behavioural changes in rodents, including increased intravenous self-administration of cocaine (Tidey and Miczek, 1997) and increased alcohol consumption (Croft et al., this meeting). Neurochemical effects include prolonged increases in blood corticosterone concentrations in defeated animals. We examined the effects of social defeat, using the resident/intruder paradigm, on regional brain corticosterone concentrations.

Male TO strain mice were used throughout, housed 10 per cage, except for resident animals that were housed singly and screened previously for aggression. For each defeat session, an 'intruder' mouse was placed in the cage of a resident for a maximum of 5 min. When an unambiguous display of upright submissive posture was seen the intruder was immediately removed to its home cage. The confrontation was watched throughout and the intruder removed if there was any risk of tissue damage, but such confrontations between mice are almost entirely ritualistic, actual fighting being rare. Further groups of mice were either kept in their home cage or exposed to a novel environment for corresponding times. The mice were killed by cervical dislocation 1h after either the single or the last of 5 daily exposures to either defeat or novel environment (Novelty). Brain regions were homogenised and extracted into 100% ethanol. Brain, and free and total blood, corticosterone concentrations were measured by radioimmunoassay (ICN). Statistical analysis was one-way analysis of variance and Bonferroni post hoc test; n=6 per treatment group.

Single defeat and single novelty (Table 1) significantly ($P<0.01$) increased corticosterone concentrations in all three brain regions, compared with home cage values. The increase after defeat was significantly greater ($P<0.05$) than after novelty for hippocampus (Hipp), but not for other regions. Repeated experiences (Table 2) gave significant increases in hippocampus and striatum, $P<0.01$ for comparisons between home cage and repeat novelty or repeat defeat and $P<0.01$ for comparison between repeated defeat and repeat novelty for hippocampus.

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 with different experiences and that these do not exactly parallel the blood concentrations.

Table 1. Corticosterone concentrations (nM) after single experiences of novelty or defeat, mean± s.e.m.

Treatment	Cortex	Striatum	Hipp.	Total blood	Free blood
Home cage	14±2.5	25±2.2	35±4.0	530±11.0	102±2.2
Novelty	19±0.9	48±0.1	50±2.0	1122±11.9	218±2.2
Defeat	19±0.5	46+2.3	61±3.4	1126±9.2	218±1.9

Table 2. Corticosterone concentrations (nM) after repeated experiences of novelty or defeat, mean± s.e.m.

Treatment	Cortex	Striatum	Hipp	Total blood	Free blood
Home cage	16±1.5	21±2.8	28±1.8	555±11.9	105±2.1
Novelty	19±0.3	31±1.2	35±0.7	810±12.5	222±0.9
Defeat	19±0.7	33±2.2	47±3.0	961±19.4	202±0.9

Tidey JW and Miczek KA 1997 Psychopharmacology 130, 203-212

99P LAMOTRIGINE PHARMACOKINETIC PARAMETER ESTIMATION IN AN INPATIENT POPULATION

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Lamotrigine (LTG) is a new anticonvulsant drug currently used in partial and generalized epilepsies, both as adjunctive treatment and as monotherapy (Goa *et al.*, 1993). Tentative target ranges of 1 to 4 mg/L of LTG have been proposed (Brodie, 1992). Recently, Morris *et al.* (1998) established a LTG range of 3 to 14 mg/L. The aim of our study was to assess the pharmacokinetic profile of LTG in Portuguese epileptic patients submitted to video electroencephalographic monitoring (VEEG).

The study involved 28 adult inpatients (15F;13M), aged 30.7±10.8 (m.±s.d.) years old, 163.1±8.7 cm tall and weighing 64.4±10.5 kg, submitted to LTG therapy associated with an inductor, such as carbamazepine (CBZ), and/or to an inhibitor, such as valproic acid (VPA), which was discontinued in accordance with a protocol. In each patient we collected 4 to 7 plasma samples of LTG (90 levels: 66 trough; 24 peak) quantified by HPLC (Castel-Branco *et al.*, 2001). Patients were divided into three groups according to the presence of an inhibitor (Group 1), an inductor (Group 2) or both (Group 3). The non-linear regression program WinNonlin® was used to determine individual pharmacokinetic parameters of LTG (Figure 1). The analysis assumed a one-compartment open model with first order absorption and first order elimination.

The final estimates (m.±s.d.) for clearance (CL) and volume of distribution (V_d): Group 1 (n=13): CL=0.012±0.003L/h/kg (C.V.=26%) and V_d=0.72±0.32L/kg (C.V.=45%); Group 2 (n=7): CL=0.072±0.031L/h/kg (C.V.=42%) and V_d=1.05±0.52

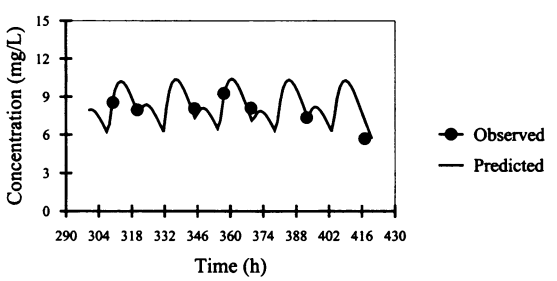

Figure 1 - Concentration profile of the patient n° 37 receiving LTG (150mg/day) in the presence of VPA (CL=0.013L/h/kg).

Patients receiving inductor agents revealed clearance values twice higher than those observed in healthy volunteers given LTG alone (0.021-0.035L/h/kg). Our results are identical to those reported by Goa *et al.* (1993). Concomitant therapy with VPA, a known inhibitor of glucuronidation, showed low LTG clearance values, similar to those observed by Gilman (1995). Thus, the obtained results demonstrated the influence of comedication on LTG metabolism, which may be of clinical relevance in epilepsy management.

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100P RELATIONSHIP BETWEEN LAMOTRIGINE IN PLASMA AND BRAIN OF RATS

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Lamotrigine (LTG) is a new antiepileptic drug which was shown to be effective against partial and secondarily generalized tonic-clonic seizures either on adjunctive treatment in patients with refractory epilepsy or when received as monotherapy (Goa *et al.*, 1993). A linear relationship appears to exist between the doses of LTG administered and the respective plasma concentrations. However, its inter-relation with the induced pharmacological response remains unknown (Bartoli *et al.*, 1997). The aim of the present work is to establish the relationship between plasma and brain concentrations of LTG through 72 hours after its administration to the rat by intraperitoneal (i.p.) route.

Adult male Wistar rats (250-320g) received by i.p. route 5, 10 or 20 mg/kg of a freshly prepared aqueous solution of LTG isethionate (2.5, 5.0 or 10.0 mg (LTG) /ml). The blood samples were obtained by open cardiac puncture and collected in citrated tubes at 7.5, 15 or 30 min, 2, 12, 24, 48 or 72 h post dose. Immediately after, the animals were decapitated and the brains removed to be homogeneized in 5 ml of phosphate buffer (pH=7.4) per g of tissue at 4° C. Blood collection and brain removal were carried out under anaesthesia (7.7 mg/kg ketamine hydrochloride + 2.3 mg/kg chlorpromazine intramuscularly injected 10 min before the above referred procedures). LTG in plasma and brain homogenates were determined by HPLC (Castel-Branco *et al.*, 2001).

The obtained plasma and brain LTG concentrations are shown in the figure. The plasma concentration versus time profiles of LTG demonstrated rapid absorption, with peak concentrations at 15 min post dose; thereafter there was a biphasic fall in plasma concentrations, with a prolonged elimination phase.

LTG brain peak concentrations were reached 0.5-2.0 h after LTG administration.

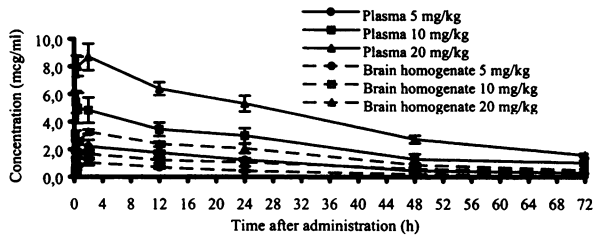

Figure 1. Concentrations of LTG in plasma and brain homogenate of rats after a single i.p. dose of LTG (m±sd; n=5)

LTG plasma and brain concentrations exhibited linear correlations with a dose over the range 5–20 mg/kg (r^2>0.92 after peak values). Linear correlations between LTG in plasma and LTG in brain were observed after the absorption phase for the three doses studied (r^2>0.93). The average brain : plasma ratios were 0.39, 0.33 and 0.30 for 5, 10 and 20 mg/kg, respectively. The variation of the partition coefficients with time and dose was not statistically significant beyond 2 h post dose (ANOVA; p>0.05).

The present study may improve the understanding of the relationship between LTG in blood and brain by establishing the relationship between the two types of curve in rats.

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Acknowledgments: GlaxoWellcome Research Laboratories; M.M. Castel-Branco was supported by PRAXIS XXI BD/18351/98.

101P LACK OF DEVELOPMENT OF TOLERANCE IN GENETICALLY EPILEPSY-PRONE RATS (GEPR -9S) FOLLOWING REPEATED TREATMENT WITH TOPIRAMATE OR CFM-2.

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Topiramate (TPM) is a novel antiepileptic drug recently licensed for clinical use in many European countries. Previous studies of the mechanisms responsible for its anticonvulsant activity have revealed an unusual variety of actions *e.g.* on voltage-activated Na^+ channels, HVA Ca^{2+} channels, as well as ligand-gated $GABA_A$ and AMPA/kainate glutamate receptors (Shank et al, 2000). In contrast, CFM-2 (1-(4'-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3benzodiazepin-4-one), is a new AMPA receptor antagonist with anticonvulsant activity (De Sarro et al., 1999).

We used genetically epilepsy-prone rats (GEPR-9s; a strain derived from Sprague-Dawley rats), sensitive to sound-induced seizures, to compare the anticonvulsant activity of TPM and CFM-2 following acute and chronic treatment. The audiogenic seizure response was assessed using a previously reported scale (De Sarro et al., 1999) and the anticonvulsant doses (ED_{50} ± 95% confidence limits) were calculated using the Litchfield and Wilcoxon test on an IBM computer. Sixty minutes after i.p. administration, both compounds exhibited a good antiseizure activity. The ED_{50} values against clonic seizures for the two compounds were similar, ranging from ~5 to 60 mg/kg. In particular, the ED_{50} values (in mg/kg) for suppression of clonic seizures (score>2 at the time of anticonvulsant effect) for TPM were 36.7 (21.06-63.82) (0.5 h), 26(18-37.6) (1 h), 21 (16-27.6) (2 h), 56 (42-74.7) (4 h) and 61 (45-82.7) (8 h), with at least 32 animals being used to calculate each ED_{50} value. By comparison, the ED_{50} values for CFM-2 (in mg/kg) were 5.42 (2.76-11.31) (0.5 h), 4,29 (2.09-9.42) (1 h), 5.45 (2.73-11.58) (1.5) and 8.15 (4.59-15.46) (2 h). The time of maximum anticonvulsant effect was at 2 h after administration of TPM and 1 h for CFM-2, whereas the duration of anticonvulsant activity was about 6 h for TPM and 3 h for CFM-2. The animals used for the chronic study were treated twice a daily at 7:00 a.m. and 7:00 p.m. for 6 weeks with i.p. TPM (30 mg/kg) and CFM-2 (5 mg/kg). The duration of anticonvulsant activity observed between 0.5 and 1, 2, 3, 4 or 8 h after the administration of the compounds studied was quite similar for acute and chronic treatment. In particular, the ED_{50} values (in mg/kg), for suppression of clonic seizures, after 14 days of treatment were 40.6 and 12.1 (0.5 h) for TPM and CFM-2, respectively. After the administration of the anticonvulsant compounds on days 1, 14, 28, and 42 of repeated treatment, motor impairment was studied with a rotarod apparatus. The TD_{50} values for TPM or CFM-2-induced impairment of locomotor activity showed that TPM was less toxic than CFM-2 following acute and repeated treatment. In fact, the TD_{50}'s for TPM and CFM-2 were 312.2 and 17.9 for the acute administration and, for repeated treatment, 316.3 and 18.2 (14 days), 321.9 and 18.3 (28 days) and 357.2 (42 days), respectively.

In conclusion, we found no evidence of tolerance to the anticonvulsant effects of TPM or CFM-2 in epilepsy-prone rats.

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102P WEAK ANTI ABSENCE ACTION OF ETHOSUXIMIDE INFUSED DIRECTLY INTO THE RETICULAR THALAMIC NUCLEUS (NRT) OF THE GENETIC ABSENCE EPILEPSY RAT FROM STRASBOURG (GAERS)

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Ethosuximide (ETX) is used clinically for its selective effect on absence seizures. Generation of the spike and wave discharges (SWD) that characterise absence epilepsy requires functional connectivity of thalamocortical circuitry. Recent electrophysiological studies (Leresche *et al.*, 1998; Pinault *et al.*, 1998) have questioned the hypothesis that ETX exerts its therapeutic effect by reducing I_T (low voltage Ca^{2+} current) (Coulter *et al.*, 1989). We previously reported that when infused directly into the ventrobasal thalamus (VB), the maximal reduction of SWD by ETX was ~50% and this only occurred in the second 30 min period after drug administration (Richards et al., 2000), perhaps reflecting diffusion of the drug to other thalamic areas. In the present study we investigate the effects of ETX directly infused into the reticular thalamic nucleus (NRT).

GAERS (275±75g) were anaesthetised with medetomidine/ketamine (0.5 & 75 mg/kg i.p. respectively) and implanted with a bipolar EEG electrode in the frontal cortex (AP, 2.2:L, 2.4; V, 2.6) and, bilaterally, with guide cannulae in the NRT (AP, -2.6;L, 3.5; V, 5.4). The following day, the EEG signal was amplified, filtered and recorded (Neurolog NL 824/820/135/530) for a 30 min basal period. There were four experimental groups (n=4 each group); (a) ETX (20 nmol/side) infused directly into the NRT, (b) ETX (200 nmol/side) as in (a), (c) CGP 36742, a $GABA_B$ antagonist (27 nmol/side) as in (a), and (d) vehicle (saline) as in (a). Following drug administration, EEG was recorded for a further 2 x 30 min periods. SWD are expressed as the percentage of each 30 min period. Drug effects are assessed by one-way ANOVA, with post-hoc comparison to basal values using Dunnett's test when significant ($p<0.05$) differences were found.

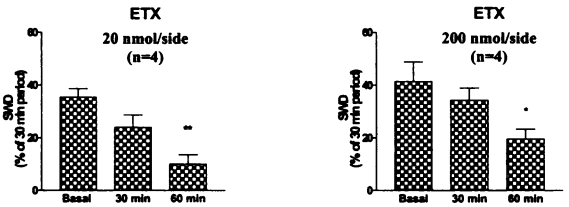

Figure 1. Effect of ETX infused into NRT in GAERS (results shown as mean ± s.e.mean, *p<0.05; **p<0.01 compared to basal) Saline control showed no significant change.

In agreement with our previous findings in the VB, direct infusion of CGP 36742 into NRT (27 nmol/side) (data not shown) produced an immediate and almost complete elimination of SWD. However, as with VB, infusion of ETX into the NRT had no effect on SWD within the first 30 min and produced only around a 60% reduction in SWD within 1h at a dose of 20 nmol/side ($p<0.01$) and slightly less reduction at 200 nmol/side ($p<0.05$). This is in marked contrast to the immediate and dramatic reduction of SWD produced by systemic administration of ETX (100mg/kg i.p.; Richards et al., 2000). These results suggest that ETX is not acting within the thalamus, either at the VB or NRT alone. It is feasible however, that ETX may need to target both sites simultaneously in order to produce a decrease in SWD comparable to that seen when given systemically, and we are currently using reverse microdialysis which can distribute ETX over a larger area and will provide a method of simultaneous delivery to both thalamic areas.

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103P KAINIC ACID-INDUCED EPILEPTIFORM ACTIVITY AND NEURONAL CELL DEATH IN HIPPOCAMPAL ORGANOTYPIC SLICES

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Kainic acid (KA) administration *in vivo* causes chronic spontaneous recurrent seizures and produces widespread patterns of neuronal damage (Ben-Ari, 1985). These features are similar to those observed in human temporal lobe epilepsy (Meldrum and Corsellis, 1984), and is commonly used as an experimental model of this condition. This study investigated the effect of kainic acid-induced epileptiform activity and associated necrotic or apoptopic mediated cell death in hippocampal organotypic cultures.

Organotypic hippocampal slice cultures were prepared from 6 day old male Wistar rats and cultured for 14 days *in vitro* (Gähwiler *et al*, 1997). Cultures were treated with KA (1μM) at 14 days *in vitro* for 24hrs to induce epileptiform-like activity. Organotypic hippocampal slice cultures were either stained with terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) or with propidium iodide (2μM) to estimate apoptotic or necrotic neurones, respectively, and examined with confocal microscopy. Single-unit extracellular activity was recorded from CA1 neurones at 37°C using *Spike-2* software (CED, UK) and analysed with *NeuroEXplorer* (Plexon Inc., USA). All data are presented as mean±SEM; statistical significance levels were determined using an unpaired t-test.

Cells exhibited spontaneous discharge activity, which was significantly higher in KA-treated organotypic hippocampal slice cultures (13.81±4.40 Hz, n=4) compared with controls (2.55±1.13 Hz, n=4; $p<0.05$). Both KA-treated and control organotypic hippocampal slice cultures exhibited bursting activity (control 6.05±3.96 bursts/min, n=4; KA 24.27±13.22 bursts/min, n=4; $p>0.05$). A burst was defined as a minimum of three spikes occurring with interspike intervals <300ms and a minimum duration of 10ms.

Morphologically, propidium iodide uptake (measured as % fluorescence density over the CA1 region) was significantly greater in KA-treated organotypic hippocampal slice cultures (4.0 ±1.87%, n=7) than controls (0.08±0.06%, n=9; $p<0.05$). Both control (n=4) and KA-treated (n=6) organotypic hippocampal slice cultures were TUNEL negative.

These preliminary findings suggest that KA-induced epileptiform activity in organotypic hippocampal slice cultures is accompanied by neuronal death, which appears to be associated with necrosis rather than apoptosis. Similar observations have been reported *in vivo*, concluding that KA-induced status epilepticus produces neuronal necrosis (Fujikawa *et al.*, 2000). This early seizure-induced neuronal death may contribute to the development of a reduced seizure threshold later in life.

LJ is supported by a BBSRC-GlaxoWellcome Studentship.

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104P CORRELATION OF ISATOGEN DERIVATIVES AND SPIN TRAPS AS ANTAGONISTS OF ATP RECEPTORS AND AS NEUROPROTECTIVE AGENTS: COMPARISON WITH AMPA ANTAGONISTS

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2,2'-Pyridylisatogen tosylate (PIT) is a selective antagonist of P2Y responses in taenia caeci preparations from the guinea-pig (Spedding et al., 1975) with allosteric effects (King *et al.*, 1996) which has been reported to be neuroprotective (Volonté *et al.*, 1998). The compound is an immine oxide, acting as a spin trap for free radicals, and has neuroprotective effects.

The objectives were to test whether the neuroprotective effects of PIT were due to P2Y antagonism or due to the immine oxide moiety, and to discover P2Y antagonists without the immine oxide group. Analogues were tested as antagonists of responses to ATP in the taenia preparation of the guinea-pig caecum at a concentration of 50 μM (Spedding et al., 1975). 2,3-Pyridylisatogen was also a potent antagonist (dose ratio, 20 ± 6, n=5), but not 2,3-nitrophenylisatogen (dose ratio <3). The reactive immine oxide group could be substituted by a keto moiety (N-(2'pyridyl)phthalide (dose ratio, 53 ± 10, n=5)), while maintaining antagonism of responses to ATP, equivalent to PIT. Thus, antagonism of P2Y receptors was not restricted to the isatogen nucleus. Other spin traps (DMPO) did not interact with ATP receptors.

PIT (10 $mg.kg^{-1}$, i.p.) was found to be a powerful neuroprotective agent in protecting against the lesions induced by 15 μg S-bromo-willardiine injected into the cortex or white matter of 5-day old mice (>70% reduction of lesion size, $p<0.001$, n=10) pups. Lesions were measured at 10 days. Lesions were also reduced in a dose-dependent manner by the AMPA antagonists GYKI 52466 and GYKI 53655 (1, 3, 10 $mg.kg^{-1}$, i.p.) administered at the same time as the excitotoxic agent. However, in motoneurones obtained from rat embryos, low concentrations of PIT (0.1 μM) had no protective effect against cell death induced by domoic acid (10 μM), whereas high concentrations of PIT (10 and 100 μM) exacerbated cell death. The AMPA antagonists NBQX (10 μM), GYKI 52466 and GYKI 53655 (1-100 μM) protected against cell death in these conditions.

Thus the multiple effects of PIT may induce both beneficial and deleterious effects, whereas AMPA antagonists were protective in both models. N-(2(pyridyl)phthalide was less neuroprotective ($p<0.05$) than PIT (10 $mg.kg^{-1}$, i.p.) in protecting against the lesions induced by 15 μg S-bromo-willardiine injected into the cortex or white matter of 5-day old mice pups, which implies that the immine oxide moiety is important for the neuroprotective effects and not antagonism of ATP.

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105P CHANGES IN BRAIN MITOCHONDRIAL FUNCTION AFTER HEAD TRAUMA: EFFECT OF MECHANOGATED MEMBRANE ION CHANNEL BLOCKERS

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Previous findings suggest a protective role of mechanogated membrane ion channel blockers (MMICB) in blood-brain barrier permeability increase induced by the Marmarou's closed head trauma model in the rat (Vaz et al., 1998). The aim of this study was to evaluate the effect of MMICB on brain mitochondrial function induced by trauma (Fiskum, 2000). For this purpose, the same trauma model (Marmarou et al., 1984) was used and mitochondrial reducing capacity measured by staining brain slices with 2,3,5-triphenyltetrazolium chloride (TTC). Briefly, male Wistar rats, weighing 340-360g, were i.p. anaesthetised with diazepam (6 mg.kg-1), ketamine (60 mg.kg-1) and atropine (0.5 mg.kg-1) and allowed to breathe spontaneously. Procedures were carried out in accordance with EU guidelines for animal experiments. The control group of animals was sham operated.

Twenty-four hours later, the animals were anaesthetized again and the brain was removed. Two other groups of rats were submitted to the same procedures but 15 minutes before trauma, gadolinium (70 mg.kg-1 i.v.) or amiloride (20mg.kg-1 i.p.) were injected. The removed brain was submitted to -80 °C during 15 min.

A 2.5 mm thick coronal slice was cut between 5.0 and 7.5 mm posterior to the bregma. The slices were immersed in TTC for 14 minutes. Under standard light conditions, a picture was obtained from the anterior surface of each brain slice using a digital camera. Each picture was analysed by an image analysis software. The threshold level for distinguishing red from white points was previously determined and was the same for all images. For each image, a percentage of white points was calculated by the program. This percentage was 23.9±2.3, n=9 for controls, 12.1±1.7, n=9 for the trauma group, 21.5±1.0, n=4 for the gadolinium and 21.9±2.5, n=6 for the amiloride treated groups (results are presented as means and S.E.M.). The value of the trauma group was significantly different from all the other groups ($p<0.05$, Newman-Keuls test) and no other differences were found.

These results show that MMICB prevented the increase in TTC staining induced by trauma, in agreement with the hypothesis of mechanogated channels involvement in the pathogeny of brain trauma.

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106P ATTENUATION OF EXCITOTOXIC OLIGODENDROCYTE PROGENITOR CELL DEGENERATION BY THE CASPASE INHIBITOR, Z-VAD-FMK.

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Recent data suggest that oligodendrocytes (macroglial cells responsible for CNS axonal myelination) are vulnerable to excitotoxic insults (Matute *et al.*, 2001). However, the apoptotic and/or necrotic mechanisms underlying excitotoxic oligodendrocyte lineage cell degeneration remain unclear. The current study examined the ability of the selective caspase inhibitor, Z-VAD-fmk to influence both excitotoxic- and staurosporine (an inducer of apoptosis)-mediated oligodendrocyte progenitor cell (OPC) degeneration.

Mixed glial cell cultures were prepared from Wistar rat cerebral cortices (2-3 day old, mixed sex) as described by (Liu *et al.*, 1997). OPCs (25,000 cells well^{-1}) were maintained for 3 to 5 days in Bottenstein-Sato N2 medium, supplemented with basic Fibroblast Growth Factor (10ng ml^{-1}) and Platelet-derived Growth Factor-AA (10ng ml^{-1}). OPC identity was confirmed via A_2B_5 immunoreactivity. Percentage control OPC viability was determined via fluorescein diacetate and propidium iodide fluorescence microscopy (Jones & Sneft, 1985). Statistical analyses were performed using either one-way ANOVA (Dunnett's post-test) or paired Student's *t*-test, where appropriate.

After a 24h exposure, kainate (pEC_{50}=3.9 ± 0.3, E_{max}= 54.5 ± 7.1%, n=5) and 100μM (S)-AMPA/100μM cyclothiazide (65.2 ± 9.3%, p<0.05, n=4) induced significant OPC death. Alone, 100μM (S)-AMPA was ineffective. Kainate (300μM)-induced OPC death was abolished by the selective non-competitive AMPA receptor antagonist 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466, 50μM).

A 24h exposure to either 300 μM kainate (49.2 ± 4.0%, n=8, p<) or 500 nM staurosporine (20.4 ± 1.1%, n=4, p<) induced OPC death. However, 100 μM Z-VAD-fmk significantly limited both kainate- (67.2 ± 6.8%, n=8, p<) and staurosporine- (53.8 ± 4.0%, n=4, p<) induced OPC death. However, neither 100 μM trolox (a vitamin E analogue), 100 μM N-t-butyl-α-phenylnitrone (PBN, a spin trap reagent) nor 10μM forskolin influenced kainate (24h, 300 μM) toxicity. A 6h exposure to 300 μM kainate also significantly decreased OPC viability (59.8 ± 4.9%, n=5, p<), an effect reduced by 100μM Z-VAD-fmk (80.0 ± 5.3%, n=5, p<).

These data suggest that AMPA-receptor mediated OPC death involves caspase activation, but is insensitive to the free radical scavengers trolox and PBN. Therefore as well as AMPA receptor antagonists, selective caspase inhibitors may be useful in limiting white matter degeneration during excitotoxic CNS insults.

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Funded by the MRC and the Royal Society.

107P TRANSFORMED ASTROCYTES ARE VULNERABLE TO AMPA- AND KAINATE-INDUCED EXCITOTOXIC INJURY.

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Cortical "protoplasmic" astrocytes are pharmacologically inert cells, exhibiting limited responses to neurotransmitters including glutamate (glu), released under ischaemic conditions. However, in gliosis transformation of protoplasmic astrocytes to fibrous cells takes place, dramatically changing cell morphology and pharmacological responsiveness.

We have studied acute and delayed cell death induced by ionotropic glu receptor agonists in astroglia. Cell death was estimated by determining release of the cytoplasmic marker, lactate dehydrogenase (LDH) (Koh & Choi, 1986). In acute toxicity studies, effects of glutamate receptor agonists were compared with that of ionomycin (10μM), while in chronic toxicity studies, comparison was made with the effects of staurosporine (100nM). Cortical astrocytes were obtained from P1 male Wistar rats and maintained for 15 days in DMEM/F12 medium (McCarthy & De Vellis, 1980). Fibrous astrocytes were produced by switching protoplasmic cells to DMEM medium, supplemented with growth factors (Miller *et al.*, 1993). Data were analysed by two way ANOVA.

AMPA, (0.1-1.0 mM) for 1 hour, in the presence of 100μM cyclothiazide, elicited only weak toxicity (20–35 % cell death) in protoplasmic astrocyte cultures. In contrast, 24 hours after 1 hour exposure to agonists, up to 80 % cell death was observed, with EC_{50} values of: glu, 7.4 ± 4.2μM; glu + L-*trans*-2,4-PDC (10μM), 1.6 ± 0.25μM; AMPA, 897 ± 36nM; AMPA + cyclothiazide (CTZ) (100μM), 629 ± 29nM; kainate (KA) 24.6 ± 6.3μM, and KA + concanavalin A (Con A)(1μM) 16.7 ± 4.8μM. Fibrous cells were highly vulnerable to glu, AMPA and KA toxicity in both chronic (EC_{50} values (μM): glu, 9.7 ± 6.4; glu + PDC, 1.7 ± 0.3; AMPA, 10.3 ± 9.3; AMPA + CTZ, 3.5 ± 0.47; KA, 5.6 ± 2.6; KA + Con A, 0.76 ± 18), and acute (EC_{50} values (μM): glu, 0.74 ± 0.02; glu + PDC, 0.52 ± 0.01; AMPA, 1.47 ± 0.3; AMPA + CTZ, 14 ± 6; KA, 5.4 ± 4.3; KA + Con A, 1.46 ± 0.26) studies.

Acute and chronic excitotoxic responses to AMPA and KA were inhibited by the selective AMPA receptor antagonist NBQX. Preincubation with 100nM NBQX decreased acute AMPA and KA toxicity similarly (25-30% and 20-25%). Delayed AMPA toxicity was reduced by 50-65% and that to KA by 30-40%. NBQX (1μM) decreased acute AMPA toxicity by 60-70% and that to KA by 30-35%. This concentration of NBQX abolished the delayed toxic response to AMPA, while decreasing that to KA by 60-70%. These observations are consistent with the toxic effects of KA in fibrous astrocytes being mediated partially *via* activation of AMPA receptors.

In conclusion, this study challenges the perception that astrocytes are largely invulnerable to excitotoxic impact, and demonstrates that, in astroglia, the presence of growth factors may exacerbate, rather than prevent, excitotoxic damage.

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108P EFFECT OF CYTOCHROME P4502E1 INHIBITION ON FREE RADICAL FORMATION AND DOPAMINE EFFLUX IN THE RAT SUBSTANTIA NIGRA

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Susceptibility to develop Parkinson's disease (PD) has been linked to abnormalities of cytochrome P450 (CYP) function. Multiple P450 enzymes are expressed in the brain, but the relationship of these to PD is unclear. In particular CYP2E1 is localised in the substantia nigra, co-localising with tyrosine hydroxylase (Watts *et al.*, 1998). Its functional role is unknown although it may contribute to oxidative stress thought to underlie PD (Jenner 1998). CYP2E1 inhibition does not alter striatal dopamine efflux or radical formation in the normal rat brain (Kulkarni *et al.*, 2001). The current study investigates the role of CYP2E1 in free radical formation and DA efflux in the substantia nigra (SN) *in vivo* using diallyl sulphide (DAS) to inhibit 2E1 activity.

Microdialysis probes were implanted into the right SN of male Wistar rats (250-350g) and perfused as previously described (Kulkarni *et al.*, 2001). Following basal determination of DA, 2,3-DHBA and 2,5-DHBA, either vehicle or DAS was administered (after 120min of basal collection). Levels of hydroxyl radicals and DA were determined by HPLC coupled with electrochemical detection (Kulkarni *et al*, 2001). Immunohistochemical labelling of CYP2E1 was performed on sections of SN from DAS (200mg/kg i.p. for 5 days) or vehicle-treated rats by the ABC method (Watts *et al.*, 1998). Levels of 2E1 antibody staining were determined by optical densitometric analysis.

DAS had no effects on levels of DA (Figure 1), 2,3-DHBA or 2,5-DHBA when compared to vehicle controls (mean±s.e.m, one-way ANOVA with *post hoc* Dunnet's test, $p>0.05$). Immunohistochemical studies confirmed that chronic treatment with DAS significantly reduced 2E1 antibody staining in nigral tissue. One hour after treatment, density of staining was 0.12±0.01 and 0.07±0.004 in vehicle and DAS treated groups respectively (arbitary units, mean±s.e.m, two-tailed unpaired t-test, n=6, $p<0.05$).

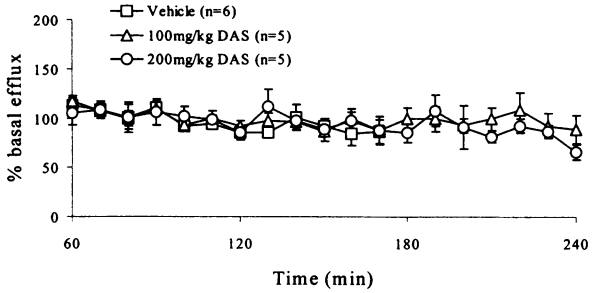

Figure 1 The effect of diallyl sulphide on nigral DA efflux

The data suggest that there is no link between DAS-induced CYP2E1 inhibition and DA efflux in the rat SN contrary to previous reports (Nissbrandt *et al.*, 2001). The findings also indicate that CYP2E1 inhibition does not alter hydroxyl radical production in the normal rat SN.

RK is supported by the Parkinson's Disease Society

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109P EFFECTS OF GLUCOCORTICOID MANIPULATION ON OREXIN-A-INDUCED FOOD INTAKE IN RATS

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Orexin-A increases food intake (Sakurai *et al*, 1998) and hypothalamo-pituitary-adrenal axis activity (Al-Barazanji *et al.*, 2001). We investigated whether manipulation of endogenous corticosterone (CORT) concentrations might influence orexin-A induced food intake.

Adult male Sprague-Dawley rats (250±20g, Charles River, UK) were kept on a 12:12h light:dark cycle (lights on at 0600h). Prior to surgery, animals were anaesthetised (Domitor 0.03ml/100mg im + Sublimaze 0.6ml/100g ip) and unilaterally implanted with a stainless steel cannula into the lateral ventricle of the brain (icv). Guide cannulae were secured to the skull using jeweller's screws and dental acrylic. Thirty-two rats were bilaterally adrenalectomised (ADX) while 16 rats were subjected to sham ADX. After surgery, anaesthesia was reversed by Antisedan (0.02ml/100g, ip) and Nubain (0.02ml/100g, ip). Animals were allowed 7 days post-operative recovery period and handled daily. During this period, all ADX rats were given 0.9% NaCl to drink while sham animals were given tap water. Two groups of ADX rats were given 0.9% NaCl containing CORT, either 25mg/L (Low CORT) or 125mg/L (High CORT). One week post-surgery, cannulae were checked by a polydipsia response to angiotensin II (100ng/5ul). On the day of experiment, animals, water and food pellets were weighed prior to icv injection of either 30ug orexin-A (GlaxoSmithKline, Harlow, Essex) or saline 5ul/rat between 0900 and 1000h. Food intake was determined at 1, 2, 3 and 4h after injections. After 4h all rats were decapitated and trunk blood was collected into 5ml EDTA tubes on ice for ACTH and CORT measurement by radioimmunoassays. All ADX rats had low to undetectable plasma CORT (around 5ng/ml) compared to the sham vehicle group (31.4 ± 6.6ng/ml). Plasma ACTH in the ADX vehicle group was 796 ± 131pg/ml compared to the sham vehicle group (146 ± 43pg/ml

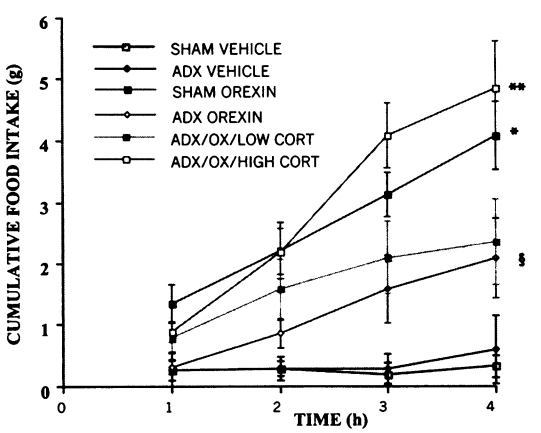

Figure 1. Cumulative food intake over 4h. Values are mean ± SEM (n=7-8/group); *$p< 0.05$ compared to ADX Vehicle and ShamVehicle; **$p < 0.05$ compared to ADX Orexin; §$p <0.05$ compared to ADX vehicle (ANOVA followed by Fisher's PLSD post hoc test).

Orexin-A had no effect on ACTH or CORT at the 4h time point compared to the sham vehicle control group. After 4h, food intake was significantly greater in the sham rats injected with orexin-A (4.09±0.55g) compared to the sham vehicle treated group (0.33±0.18g) (Figure 1). ADX significantly reduced orexin-A-induced food intake (2.10 ± 0.65g), a reduction which was reversed by high CORT replacement (4.87± 0.78g). These data provide evidence that glucocorticoids exert an important influence on orexin-A-induced food intake.

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110P INVESTIGATION OF THE ACUTE ADMINISTRATION OF IMIDAZOLINE LIGANDS ON FOOD INTAKE

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Idazoxan and 2-(2-benzofuranyl)-2-imidazoline have been shown to increase feeding behaviour in rats, implicating a role for imidazoline-2 sites (I_2) in the control of food intake (Jackson & Nutt, 1996). This study investigates whether the selective I_2 ligands, BU224 (2-(4,5-dihydroimidaz-2-yl) quinoline) and BU239 (2-(4,5-dihydroimidazol-2-yl) quinoxaline), produce similar effects on food intake to idazoxan. In addition, we have characterised the effects of harmane on feeding behaviour. This β-carboline has recently been proposed to act as an endogenous ligand at I-sites (Hudson *et al.*, 1999).

Male Sprague-Dawley rats (Charles River; 200-350 g at the start of the experiment) were individually housed in cages with metal grid floors. Trays were placed below each cage to detect food spillage. Animals were maintained on a 12 h light:dark cycle (lights on 07.00 - 19.00 h) to which they were acclimatised for at least 2 weeks prior to experimentation. Rats were allowed free access to a standard powdered rodent diet and tap water at all times. Food intake was monitored by weighing feeding jars (to 0.1 g) at the time of drug administration and after 2, 4, 6 and 24 h. Drugs were administered by intraperitoneal (i.p.) injection at approximately 09.00 h. Food intake was calculated in terms of g/kg body weight. Statistical comparisons were by one-way analysis of variance followed by Williams' test to compare drug treatment to the control group.

BU224 and BU239 (10 mg/kg i.p.) significantly increased cumulative food intake for up to 6 and 4 h following drug administration, respectively (Table 1). These hyperphagic responses were relatively short-lived as food intake was not significantly altered over 24 h. Conversely, harmane (10 mg/kg i.p.) had no effect on food intake in the 6 h following treatment but significantly decreased food intake over 24 h. Lower doses (1 and 3 mg/kg i.p.) of all three drugs were administered, but had no significant effect on food intake.

	Time (hours)			
	2	4	6	24
Treatment	Food intake (g/kg body weight)			
Vehicle	1.4±0.5	2.9±0.6	3.4±0.6	64.2±1.0
BU224	6.5±1.2***	6.5±1.3*	7.3±1.5*	62.4±2.1
Vehicle	1.9±0.8	2.5±1.0	3.2±1.4	72.5±1.9
BU239	5.6±1.3**	6.0±1.4*	6.1±1.5	72.1±5.0
Vehicle	3.6±1.1	4.1±1.3	4.7±1.4	58.6±0.9
Harmane	3.8±0.5	4.8±0.4	5.2±0.5	48.3±2.0***

Table 1. Effect of BU224, BU239 and harmane (10 mg/kg i.p.) on cumulative food intake in rats. Values are means±s.e.mean (n=7). *P<0.05 **P<0.01 and ***P<0.001 versus control group.

These data show that the selective I_2-ligands, BU224 and BU239, have similar effects on food intake in rats to idazoxan (Jackson & Nutt, 1996) confirming that compounds acting at I_2 sites can modulate feeding behaviour. In contrast, harmane decreased food intake. The effects of harmane *in vivo* may be complicated by its actions at other sites. Alternatively, harmane may have different functional effects at I-sites to idazoxan and the other I_2-ligands.

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111P LEPTIN AND INSULIN RESISTANCE IN GOLD THIOGLUCOSE-TREATED MICE

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The ventromedial hypothalamic lesion produced by acute goldthioglucose (GTG) treatment is a well established means of inducing hyperphagia and maturity-onset obesity and diabetes in rodents (Debons *et al.*, 1977). It is now known that leptin released from adipose tissue acts centrally as a key regulator of appetite and body mass and that defects in leptin signalling lead to hyperphagia and obesity (Friedman & Halaas, 1998). We have therefore investigated the effect of GTG on leptin levels with the aim of exploiting the GTG lesion as a model for studying the development of insulin resistance in obesity and diabetes.

Male CBA/Ca mice were given GTG (400mg/kg i.p.) or saline as control at 4-5 weeks of age; by 16-17 weeks, GTG-treated mice had reached the static phase of their obesity. Blood glucose levels were determined using glucotest strips and triglyceride levels using the Sigma diagnostics kit. Plasma insulin and leptin levels were measured by radioimmunoassay. Tritiated water incorporation into fatty acids and triglycerides *in vivo* was used to measure basal and insulin-stimulated adipose tissue lipogenesis (Mercer, Denton & Taberner, 1992). At 17 weeks old, obese mice were defined as those with body weight > 40g (control: 31.26 ± 0.57, n=8), non-fasted plasma glucose level > 14mM (control: 6.17 mM ± 0.20, n=8), and non-fasted triglyceride levels >3.2 mM (control: 2.34 mM ± 0.184, n=8). Results are shown as means ± S.E. mean of n. Differences between groups were analysed by Student's t test; the time course of GTG effects by 2-way ANOVA.

In the GTG-treated group, circulating leptin levels showed highly significant increases with time compared to age-matched controls ($F_{<6,53)}$=6.71, p<0.0001) and with GTG-treatment (F (1,53), p<0.0001). At around 20 weeks in GTG-treated mice, the lipogenic activity (expressed as μg H inc.hr^{-1}.g $tissue^{-1}$) of brown adipose tissue (26.26 ± 0.66, n=5) was significantly reduced compared to control (89.23 ± 1.70, n=3) (p<0.05) and there was no increase of lipogenesis in response to acute insulin (22.82 ± 5.11, n=5), indicating insulin resistance in the tissue. In contrast, no insulin resistance was observed in white adipose tissue or the liver. At 20 weeks, plasma insulin levels were 42.3 ± 9.46 ng/ml (n=8) which was significantly different from control (8.76 ± 2.72 ng/ml, n=8); leptin levels were 30.95 ± 3.09 ng/ml (n=3) which were significantly different from control (3.07 ± 0.19 ng/ml, n=7). Body weight and insulin levels in GTG-treated mice showed a correlation coefficient of 0.40 (p<0.05). The ratio of the slope for the insulin levels was 2.62 ± 0.80.

In conclusion, the GTG-treated mice with increased body weight and blood glucose also showed elevated insulin and leptin levels and selective insulin resistance in brown adipose tissue. We propose to use this model to investigate the efficacy of potential insulin-sensitizing drugs.

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112P PROTEIN KINASE A DEPENDENCY OF THE EFFECT OF PHOSPHODIESTERASE 4 INHIBITION ON HUMAN NEUTROPHIL ELASTASE AND MYELOPEROXIDASE RELEASE *IN VITRO*

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Neutrophils have been implicated in the pathophysiology of a range of inflammatory diseases, including chronic obstructive pulmonary disease (COPD) and are thought to contribute to tissue destruction through release of proteolytic enzymes, such as elastase. We have demonstrated previously (Jones *et al.*, 2001) that inhibition of phosphodiesterase (PDE) 4 attenuates the release of elastase from neutrophils, stimulated both in the absence of and following priming with tumour necrosis factor-alpha (TNF-α), a cytokine which augments the neutrophil response to stimulants such as the chemotactic peptide f-met-leu-phe (fMLP) and is elevated in the lungs of patients with COPD (Keatings *et al.*, 1996). The anti-inflammatory effects of raising intracellular cyclic AMP (cAMP) are thought to be mediated through protein kinase A (PKA) activity (Kammer, 1988). Therefore, we have investigated the PKA dependency of the effects of PDE4 inhibition on neutrophil elastase (NE) release and myeloperoxidase (MPO) activity *in vitro*.

Human neutrophils were isolated by density-dependent centrifugation from the venous blood of healthy volunteers (n=6), obtained in accordance with local ethical approval. Neutrophils were placed in Eppendorf tubes (final cell density 2.5 x 10^6 per ml), in Hank's balanced salts solution. Cells were incubated for 30 minutes with either of the PKA inhibitors Rp-cAMPS (10^{-4} M) or KT5720 (1 μg ml^{-1}), or a vehicle control, in the absence or presence of a PDE inhibitor. Following drug treatment, TNF-α (100 U ml^{-1}), or medium control, was added for a further 30 minutes, prior to cell stimulation (fMLP, 10^{-7} M; with cytochalasin B, 5 μg ml^{-1}; 45 minutes). Cells were removed by centrifugation and smples of supernatant from each tube transferred to 96-well plates. NE and MPO were quantified colorietrically, using the chromogenic substrates N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide and K-blue, respectively. Data were analysed by ANOVA, followed by Dunnett's test.

Priming of neutrophils with TNF-α enhanced ($P<0.05$) NE and MPO release, upon fMLP stimulation (50.7 ± 6.0% increase in NE release, compared to non-primed cells, mean ± s.e. mean), without affecting basal responses. Treatment with the PDE4 inhibitor, rolipram (0.03-0.3 μM), reduced ($P<0.05$) NE and MPO release from both non-primed and primed cells (55.5 ± 4.1% and 63.6 ± 3.2% of controls, maximum inhibition of NE release, respectively; 62.4 ± 4.1% and 75.9 ± 4.1% of controls, maximum inhibition of MPO release, respectively). Pretreatment of cells with Rp-cAMPS or KT5720 led to a significant ($P<0.05$) reduction in the effects of rolipram, on NE and MPO release, under each of the experimental conditions investigated. In accordance with other data suggesting PDE4 to be the predominant cAMP PDE subtype in human neutrophils, the selective PDE3 inhibitor, milrinone, had no effect in our assays, irrespective of the presence of a PKA inhibitor. These data suggest the effects of PDE4 inhibitors on release of neutrophil granule contents, from cells activated with or without prior TNF-α priming, to be PKA dependent.

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113P THE EFFECT OF PROTEIN KINASE A INHIBITION ON THE ANTIPROLIFERATIVE ACTIONS OF PHOSPHODIESTERASE INHIBITORS IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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The anti-inflammatory effects of increased intracellular cyclic AMP (cAMP) are thought to be mediated through protein kinase A (PKA) activity (Kammer, 1988). The increase in intracellular cAMP and the subsequent antiproliferative effects brought about by PDE inhibitors, are also thought to involve this PKA dependent pathway. However, it has been shown that, in T lymphocytes, pharmacological elevation of intracellular cAMP inhibits proliferation through a PKA independent mechanism (Bryce *et al.*, 1999). In the present study, we have investigated the effect of PKA inhibition on the antiproliferative actions of PDE inhibitors in human peripheral blood mixed mononuclear cells (HPBMNC) from healthy, non-atopic volunteers (n=8). HPBMNC were isolated by density-dependent centrifugation from peripheral blood, obtained in accordance with local ethical approval. Cells were washed before being resuspended in RPMI 1640 containing 10% foetal calf serum. Proliferation was induced by phytohaemagglutinin (2 μg ml^{-1}) and assessed by [^{3}H]-thymidine incorporation (3.7 kBq per well) added 24hr prior to cell harvesting at 72 hr. Data were analysed by ANOVA, followed by Dunnett's test.

Incubation with the PKA inhibitor Rp-cAMPS (10^{-4} M) had no significant effect on proliferation in cells not treated with PDE inhibitors ($P>0.05$). Rolipram (PDE 4 inhibitor; 1-10 μM), zardaverine (mixed PDE 3/4 inhibitor; 0.3-1 μM) and IBMX (non-selective PDE inhibitor; 10-100 μM) were able to reduce ($P<0.05$) proliferation of HPBMNC (54.5 ± 29.5%, 70.9 ± 9.0% and 17.7 ± 5.2% of vehicle control treated cells, maximum reduction, respectively, mean ± s.e. mean). The PDE3 inhibitor milrinone had a small but significant effect on proliferation at the highest concentration used ($P<0.05$). Co-incubation with Rp-cAMPS did not affect the actions of any of the PDE inhibitors in our hands ($P>0.05$).

Cells treated only with KT5720 (1 μg ml^{-1}), a highly selective PKA inhibitor, showed a significant decrease in proliferation ($P<0.05$) when compared to vehicle control treated cells (79.8 ± 14% of control). Co-incubation of cells with KT5720 (1 μM) and rolipram, zardaverine or IBMX led to significant augmentation of the antiproliferative actions of these PDE inhibitors, at all concentrations tested (26.8 ± 11.1%, 42.5 ± 7.1% and 15.2 ± 4.6% of vehicle control treated cells, maximum reduction, respectively). Furthermore, in the presence of KT5720, the PDE3 inhibitor milrinone significantly inhibited proliferation (48.2 ± 9.3% of control) at all concentrations tested ($P<0.05$; 0.1-3 μM). KT5720 was confirmed to lack cytotoxicity in our assays.

Our data support the work of Bryce *et al.* (1999) and further suggest that the antiproliferative effect of phosphodiesterase inhibition in mixed mononuclear cell preparations proceeds *via* a mechanism independent of PKA. Moreover, these results raise the possibility that KT5720 may, itself, have inhibitory effects on mononuclear cell proliferation through a mechanism which, at present, remains undetermined.

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114P BINDING OF WARFARIN ENANTIOMERS TO HUMAN PLASMA PROTEINS

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Warfarin is an oral anticoagulant extensively used in numerous thromboembolic disorders (Mungall et al., 1985; Sawyer, 1983). It is clinically administered as a racemic mixture of two active enantiomers, despite the higher potency of S-warfarin in comparison to its optical antipode stressed by several studies (He et al., 1997; Hignite et al., 1980).

Since the magnitude of the pharmacological effect of any drug is always related to its unbound plasma concentration (He et al., 1997), the present work intended to determine whether the binding characteristics of R and S-warfarin could influence the observed clinical differences in pharmacological response.

The binding study used the equilibrium dialysis technique, carried out at 37ºC, for 3 hours. Drug solutions were prepared from racemic warfarin sodium salt in Sörensen phosphate buffer pH 7.4, in a 0.4-22.7x10^{-4}M concentration range (n=13), and dialysed against a pool of blank human plasma. Free enantiomer levels were determined by reverse-phase H.P.L.C., a modification from Pais et al.'s technique (1999). Experimental data were fitted to different binding models by a non-linear regression program (WinNonlin): a non-linear model considering one (model I) and two (model II) classes of binding sites, and a mixed model with nonlinear binding and linear non-specific and non-saturable binding (model III).

The analysis of variance (ANOVA) did not reveal any statistically significant differences ($p>0.05$) between R and S-warfarin binding percentages (for example, 98.9 %(R) and 99.0%(S) for 1.5 x10^{-4} M level) and, in all the studied models, these isomers showed similar binding capacities. The curvilinear shape of the Rosenthal plot (figure 1) suggested the existence of more than one class of binding sites, which was stressed by the best goodness of fit presented by models II and III. However, model III showed lower values of the coefficient of variation for the estimated binding parameters.

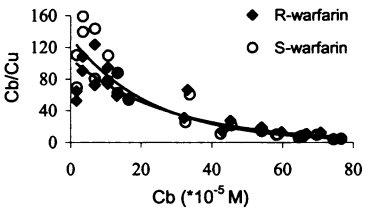

Figure 1 – Rosenthal plot of warfarin enantiomers
(Cb= concentration of drug bound to proteins; Cu=unbound concentration)

This study concluded that R and S-warfarin presented similar binding behaviour, with two classes of binding sites. Model III was the best one to describe their binding profile. No significant differences were found that may contribute to explain the observed differences in pharmacological response.

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115P AN INVESTIGATION OF THE ROLE OF CYCLIC NUCLEOTIDES AND POTASSIUM CHANNELS IN THE ADP-INDUCED RELAXATION OF THE PORCINE ISOLATED CORONARY ARTERY

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In the porcine isolated coronary artery, the purine nucleotides ATP and ADP elicit endothelium-independent smooth muscle vasorelaxation (Alexander *et al.*, 2000). This study investigated the possible mechanism(s) of the endothelium-independent smooth muscle relaxation elicited by ADP in this tissue through the use of potassium channel blockers and isoform-selective phosphodiesterase (PDE) inhibitors. Thus, rolipram was investigated as an inhibitor of the cyclic AMP-selective PDE, PDE4, while zaprinast was used to test the involvement of PDE5, a cyclic GMP-selective PDE (Beavo, 1995).

Segments of coronary artery (dissected from whole hearts transported on ice from the abattoir) from pigs (either sex) were mounted for isometric tension recordings, as described previously (Alexander *et al.*, 2000). Tissue viability was assessed by eliciting contractions in the presence of 60 mM KCl. The thromboxane A_2 analogue U46619 (11α,9α-epoxymethano-PGH_2) was utilised (up to 100 nM) to elicit a contraction to about 60% of that to KCl. Thereafter, concentration-relaxation curves were constructed in the presence of increasing cumulative concentrations of ADP in the absence or presence of modulators. Data reported are means ± s.e.m. of results from at least six separate experiments and were initially compared for statistical significance by ANOVA.

After contraction in the presence of U46619, ADP evoked a complete, concentration-dependent relaxation (pEC_{50} value 5.63 ± 0.41, R_{max} 102 ± 7 %). In the presence of tetraethylammonium (10 mM), concentration-response curves to ADP were not significantly different compared to controls (pEC_{50} value 6.12 ± 0.11, R_{max} 92 ± 3 %). In the presence of glibenclamide (10 μM) or charybdotoxin (100 nM), inhibitors of K_{ATP} and K_{Ca2+} channels respectively, response curves were also not significantly different compared to the controls (glibenclamide pEC_{50} value 5.59 ± 0.16, R_{max} 98 ± 4 %; charybdotoxin pEC_{50} value 5.95 ± 0.09, R_{max} 87 ± 4 %).

In a second series of experiments, the presence of a concentration of rolipram insufficient to elicit direct relaxation (30 nM) evoked a significant ($P<0.05$, t-test) leftward shift in the ADP concentration-response curve (pD_2 values: control 4.65 ± 0.20, rolipram 5.92 ± 0.22; R_{max} values: control 92 ± 8%, rolipram 104 ± 4 %). The presence of the same concentration of zaprinast, however, failed to elicit a significant effect on ADP concentration-relaxation curves compared to the controls (pD_2 value 4.68 ± 0.41; R_{max} value 111 ± 10 %).

These data suggest that, in the porcine coronary artery, ADP evokes an endothelium-independent relaxation primarily through elevation of cyclic AMP levels. The mechanism of relaxation appears not to involve cyclic GMP or potassium channels, since zaprinast and potassium channel inhibitors are ineffective.

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116P INHIBITORS OF THE PORE-FORMING SUBUNIT OF THE K_{ATP} CHANNEL PARTIALLY REVERSES ENDOTOXIN-INDUCED VASCULAR HYPOREACTIVITY IN RAT SUPERIOR MESENTERIC ARTERY

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Vascular hyporeactivity is well described in sepsis. We recently described an organ culture model of vascular hyporeactivity in rat superior mesenteric artery (RSMA) where inhibitors of inducible NO synthase (iNOS) substantially reversed (80%) the effects of lipopolysaccharide (LPS) *in vitro* (O'Brien *et al.* 2001). In animal models, the ATP-sensitive K^+ (K_{ATP}) channel inhibitor, glibenclamide reverses LPS-induced hypotension *in vivo* but not vascular hyporeactivity *in vitro* (*e.g.* Wu *et al.* 1995). This difference may relate to its antagonism of contractions to thromboxane, a potent vasoconstrictor released during sepsis. Using our *in vitro* model of hyporeactivity, we compared the effects of various K_{ATP} channel inhibitors, which bind to either the pore forming subunit or the sulphonylurea receptor (SUR).

Male Sprague-Dawley rats (300g) were humanely killed and the RSMA removed. Rings were incubated in culture media ± 1.0 μg ml^{-1} LPS (*S. typhosa;* 20 hr) and mounted under 0.8g isometric tension in 25ml organ baths. K_{ATP} channel inhibitors were added 25 min before concentration-response curves to PE (10^{-9} -10^{-5} M) were constructed. At the end of each experiment, the thromboxane-A_2 mimetic, (U46619; 10^{-7} M) was added. For electrophysiology, K_{ATP} current was generated by transiently expressing Kir6.2ΔC26 in HEK cells. Recordings were made in 140/140 mM K^+ with 0.1 mM ATP in the pipette. Data are expressed as mean±s.e.m and statistical analysis performed using 1 or 2-way ANOVA with the appropriate post-hoc test.

LPS induced significant hyporeactivity to PE, reducing the maximum contraction (E_{max}) from 1.51±0.06 g to 0.13±0.05 g (P<0.001; n=10). Incubation with $BaCl_2$ (300 μM) or PNU-37883A (1 μM; from BIOMOL) significantly reversed LPS-induced hyporeactivity (P<0.05, n=8), but only partially compared to control (P<0.001). The maximum response (E_{max}) was increased to 0.9±0.1g and 0.86±0.1g, respectively. PNU-37883A inhibited K_{ATP} current carried through the truncated pore-forming subunit Kir6.2ΔC26 with a pD_{50} value of 5.34 ± 0.2 (n=5). The SUR inhibitors, glibenclamide (10 μM), tolbutamide (1 mM) or the pinacidil derivative, PNU-99963 (1 μM; Khan *et al.*1997) had no effect upon LPS-induced vascular hyporeactivity. Concentration-response curves in controls were unaffected by the K_{ATP} inhibitors. In LPS tissues, contractions to U-46619 were significantly (P<0.05, n=5) inhibited by glibenclamide and tolbutamide, but not PNU-99963.

Based on our observations with the pore inhibitors, we conclude that K_{ATP} channels contribute to LPS-induced vascular hyporeactivity *via* the iNOS pathway. SUR inhibitors do not reverse hyporeactivity and this appears unrelated to the inhibition of thromboxane receptors.

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117P STRUCTURALLY DISSIMILAR ATP-SENSITIVE K^+ CHANNEL INHIBITORS HAVE VARIABLE EFFECTS ON RELAXATION TO L-ARGININE IN LPS-TREATED RAT AORTIC RINGS

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K_{ATP} channels are formed from pore-forming and sulphonylurea receptor (SUR) subunits, the latter being the target of the anti-diabetic agents, glibenclamide and tolbutamide, although at higher concentrations these agents also interact with the pore. In contrast, Ba^{2+} and PNU-37883A are thought to interact directly with the pore (Surah-Narwal *et al.*, 1999). K_{ATP} channels have been implicated in the pathogenesis of endotoxic shock, although glibenclamide has inconsistent effects in many *in vitro* and *in vivo* models. In the present study, we investigated whether NO derived from inducible NO synthase (iNOS) modulates K_{ATP} channels in endothelium-denuded, lipopolysaccharide (LPS)-treated rat aorta.

Male Sprague-Dawley rats (250-300g) were humanely killed and the thoracic aorta removed and cleaned. Aortic rings were mounted under 1g isometric tension in 25ml organ baths containing physiological salt solution, and incubated in the presence of *S. typhosa* LPS (1 μg ml^{-1}; 4 hrs). Rings were contracted with phenylephrine (PE, 1 μM), and preincubated for 20 mins with inhibitors of K_{ATP} channels. For nitrite (NO_2^-) measurements, rings were incubated in cell culture media with LPS (1 μg ml^{-1}; 18 hrs) and NO_2^- determined using the Griess reaction. Data are expressed as mean±s.e.m. Differences were calculated using ANOVA with appropriate post-hoc tests.

Application of L-arginine (0.3 – 300 μM) to LPS-treated aortic rings, caused concentration-dependent reversal of PE-induced tone with a maximum relaxation (E_{max}) of 67.2±2.8% (n=21). The guanylyl cyclase inhibitor, ODQ (3 μM), and the selective arginine analogue inhibitor of iNOS, 1400W (10 μM) both significantly inhibited (P<0.001, n=3-4) E_{max} to 4.8±2.9% and 31.4±5.3%, respectively. A variety of K_{ATP} channel blockers also significantly (P<0.01) inhibited relaxation, shifting the E_{max} to 32.1±8% for $BaCl_2$ (300 μM), 44.1±4.3% for PNU-37883A (1 μM), 36.2±3.2% for 4-aminopyridine (5 mM), and 24.9±4.9% for tolbutamide (1 mM). Interestingly, glibenclamide (10 μM) had no effect on the concentration-response curve, the E_{max} being 66.2±5.1% (n=5-8).

NO_2^- levels were measured to assess whether the K^+ channel inhibitors were non-specifically inhibiting iNOS. LPS increased nitrite levels from 2.94±0.1 μM to 5.36±0.23 μM (n=7, P<0.001) which was fully reversed (2.71±0.1 μM, n=7) when tissues were incubated with 1400W. $BaCl_2$, glibenclamide, PNU-37883A, or ODQ had no significant effect on nitrite accumulation (n=7). In contrast, tolbutamide did significantly inhibit LPS-induced increases in nitrite levels by 69.6±6.7% (P<0.001, n=7).

These results suggest that the pharmacology of K_{ATP} channels in endotoxaemia may be altered, since K_{ATP} channel inhibitors that bind to the pore appear significantly more effective than those that bind preferentially to the SUR. This phenomenon appears largely unrelated to inhibition of NO synthesis, and may imply that molecular communication between the SUR and the pore becomes deranged during endotoxaemia.

Surah-Narwal, Xu, McHugh *et al.* Br. J. Pharmacol., **128**:667-672.

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118P POTENT INHIBITION OF CLONED K_{ATP} CHANNELS STABLY EXPRESSED IN HUMAN EMBRYONIC KIDNEY (HEK) 293 CELLS BY THE PINACIDIL DERIVATIVE, PNU-99963

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ATP-sensitive potassium (K_{ATP}) channels are composed of pore-forming subunits of the Kir6.0 family and sulphonylurea receptors (SUR). It is believed that Kir6.2/SUR1, Kir6.2/SUR2A, Kir6.2 (Kir6.1)/SUR2B correspond to the native K_{ATP} channels in pancreatic β-cells, heart and smooth muscle, respectively (Seino, 1999). The aim of the present study was to investigate the mechanism of action of PNU-99963, a novel vascular K_{ATP} channel inhibitor derived from pinacidil (Khan *et al.*, 1997), on these four subtypes of K_{ATP} channel.

K_{ATP} channels were generated by stable coexpression of the SUR and Kir6.0 subunits in HEK cells (Cui *et al.*, 2001). Current was recorded in a symmetrical potassium solution (140 mM) using the whole-cell patch clamp technique. 100 ms voltage steps from a holding potential of 0 mV were applied to the cell to evoke the current between −100 mV and +50 mV. Inhibition of the current was calculated as the percentage block of $BaCl_2$-sensentive (10 mM) current measured at −50 mV. Dialysis of the cell with 1 mM ATP caused a time-dependent increase in basal current in cells expressing Kir6.2/SUR1, Kir6.2/SUR2A, or Kir6.2/SUR2B. PNU-99963 (1 μM) significantly inhibited basal current generated by all three types of K_{ATP} channel by 87.8±3.4% (mean±s.e.m, n=4), 78.0±4.4% (n=5) and 86.2±3.7% (n=12), respectively. The inhibition was dose-dependent with IC_{50} of 46.1 nM (Kir6.2/SUR1), 53.2 nM (Kir6.2/SUR2A) and 40.2 nM (Kir6.2/SUR2B), respectively. In cells where Kir6.1/SUR2B, the putative nucleotide diphosphate (NDP)-sensitive potassium channel, was expressed, the current was activated by dialysis of cells with 1 mM GDP in the pipette solution. PNU-99963 (1 μM) inhibited the current by 96.6±1.6% (n=10), which is greater ($P<0.05$, ANOVA) than the inhibition observed with the other 3 clones. Inhibition was dose-dependent, with IC_{50} of 15.7 nM. However, when the channel was generated by transiently expressing Kir6.2ΔC26, a carboxy terminus truncated pore-forming subunit Kir6.2, that expresses in the absence of SUR, PNU-99963 up to 3 μM caused no significant inhibition of the current (14.7±5.0% of control, $P>0.05$, n=6)

Our results show that PNU-99963 is a potent inhibitor of all K_{ATP} channel subtypes, although it has a greater effect against Kir6.1/SUR2B compared to either Kir6.2/SUR1, Kir6.2/SUR2A or Kir6.2/SUR2B. We conclude that inhibition is through binding to the SUR receptor.

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119P INTERCELLULAR CALCIUM SIGNALLING VIA GAP JUNCTIONS IN RESPONSE TO MECHANOSENSITIVE SIGNALS IN RAT AORTIC ENDOTHELIAL CELLS

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A mechanical stimulus applied to a single cell has been shown to increase the free intracellular calcium concentration ($[Ca^{2+}]_i$) in many cell types including endothelial cells (Demer *et al.*, 1993). Such increases in cytosolic Ca^{2+} have been demonstrated to propagate intercellularly as Ca^{2+} waves and may provide a mechanism for the co-ordination of multicellular function. The expression of gap junction proteins by endothelial cells provide a potential pathway for the direct intercellular communication of Ca^{2+} signals in this cell type (Domenighetti *et al.*, 1998). This study investigated the role of gap junction channels in intercellular Ca^{2+} wave propagation in cultured rat aortic endothelial cells and demonstrated the importance of these channels for the intercellular communication of mechanosensitive signals.

Male Wistar rats (200-250g) were killed by cervical dislocation. Thoracic aortae were dissected, cleaned of fat and connective tissue and cut into small strips. Endothelial cells were isolated by digestion with papain, and used after 3-4 days of primary culture. The cultured cells were superfused with MOPS buffer (2ml/min) at 37°C. All experiments were imaged by confocal laser scanning microscopy (Olympus FluoView 500) and analysed using Fluoview software.

The fluorescently-labelled acetylated low density lipoprotein (DiI-Ac-LDL) which is selectively taken up into endothelial cells (Voyta *et al.*, 1984) , demonstrated a pure population of cells (98.2 ± 0.6%).

The cell-impermeant fluorescent dye, Lucifer yellow, was introduced into a number of cells by scrape loading (Domenighetti *et al.*, 1998). Intercellular spread of the fluorescence signal demonstrated the cultured cell monolayers were dye coupled (n=5).

When loaded with the fluorescent Ca^{2+} indicator fluo-4, the cells responded to a bolus application of either bradykinin or ATP (100μM) with a 206 ± 35% (n=15) and 137 ± 17% (n=9) increase from basal fluorescence intensity, respectively.

A mechanical stimulus applied to a single cell with the tip of a glass micropipette evoked a rapid rise in $[Ca^{2+}]_i$ which spread to 4.8 ± 1.2 neighbouring non-stimulated cells (n=5). In the presence of the gap junction uncoupler palmitoleic acid (50μM), the stimulated rise in $[Ca^{2+}]_i$ was restricted to 0.8 ± 0.4 adjacent cells (n=5), demonstrating a significant inhibition of the spreading Ca^{2+} response ($P<0.05$). This inhibition was not attributable to an effect of palmitoleic acid on the stimulated cells, as the increase from basal fluorescence intensity was unaltered between control (160 ± 28%, n=5) and palmitoleic acid treated groups (195 ± 41%, n=5).

In conclusion, gap junctions provide a pathway for the direct intercellular communication of Ca^{2+} signals in response to mechanical stimulation in rat aortic endothelial cells.

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120P RELAXATION OF RAT ISOLATED MESENTERIC SMALL ARTERIES IN RESPONSE TO FLUOXETINE

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In vivo, fluoxetine (a 5-HT reuptake inhibitor) or nisoxetine (a selective noradrenaline reuptake inhibitor) produce significant vasoconstriction in the mesenteric vascular bed of conscious rats (Woolard *et al.*, 2001). The purpose of this study was to compare the direct effects of fluoxetine and nisoxetine in small resistance arteries isolated from male, Sprague Dawley rats.

Animals, weighing 400-500 g, were killed by stunning and exsanguination and the mesentery was removed. A segment of second order mesenteric resistance artery was isolated, cleaned of fat and connective tissue, and set up in a Halpern pressure myograph. Vessels were pressurised to 60 mmHg and allowed to equilibrate for a period of 30 min. Subsequently, U46619, a thromboxane A_2-receptor agonist, was added to the superfusion solution (physiological salt solution (PSS)), in order to produce an approximate 40% reduction in vessel diameter. This allowed scope for the vessel to dilate or further constrict in response to an additional agents. Following the establishment of a steady baseline with U46619, concentration-response curves were generated for fluoxetine or nisoxetine. Results are presented as mean±se mean and differences were considered statistically significant if $P<0.05$.

The mean starting diameters of the vessels in the fluoxetine or nisoxetine groups (n=6) were 304±24 μm and 318±28 μm, respectively. Prior to the administration of fluoxetine, U46619 ($3x10^{-7}$ M) reduced vessel diameter to 194±21 μm. Fluoxetine had little effect on vessel diameter at low concentrations ($1x10^{-8}$ M - $3x10^{-6}$ M); however, a significant vasodilatation occurred at $1x10^{-5}$ M (Wilcoxon Test). The maximum vasodilator response (fluoxetine ($3x10^{-5}$ M)) was 88±4% of the relaxation determined in Ca^{2+}-free PSS (diameter 301±23 μm). In the nisoxetine experiments, U46619 ($3x10^{-7}$ M) reduced vessel diameter to 203±19 μm. No significant changes in vessel diameter were noted except at $3x10^{-5}$ M nisoxetine, where a vasodilatation was observed (38±10% of the relaxation in Ca^{2+}-free PSS (diameter 314±27 μm). The maximum responses to fluoxetine and nisoxetine were significantly different (Mann-Whitney test).

The results of this study indicate that fluoxetine causes vasodilatation in mesenteric resistance arteries, as previously shown for cerebral arteries (Pacher *et al.*, 1999) and skeletal muscle arterioles (Ungvari *et al.*, 1999). However, the mechanism by which fluoxetine evokes a dilator response in mesenteric resistance vessels is unknown. In contrast, nisoxetine had only modest dilator effects on mesenteric small arteries. Neither nisoxetine nor fluoxetine caused vasoconstriction *in vitro*, in contrast to their effects *in vivo*. Thus, the latter are likely to be due to indirect actions of the drugs.

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Ungvari, Z., Pacher, P., Kecskemeti, V., *et al.* (1999). Stroke, **30**, 1949-1954.

Woolard, J., Gardiner, S.M. & Bennett, T.B. (2001). Br. J. Pharmacol., **133**

121P MYOENDOTHELIAL GAP JUNCTIONS PROVIDE A PATHWAY FOR EDHF IN THE MESENTERIC ARTERY OF THE MOUSE

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There is mounting evidence for the dependence of EDHF (endothelium-derived hyperpolarizing factor) activity on the electrotonic coupling of the endothelium and smooth muscle, which involves the spread of hyperpolarization from the endothelium to the adjacent smooth muscle. In mouse mesenteric arteries, acetylcholine can evoke an EDHF-mediated vasodilation, although the mechanism of this activity in unclear (Ding *et al.*, 2000). We investigated whether the EDHF-mediated dilation was associated with hyperpolarization, and identified the most likely pathway for the transfer of the hyperpolarization to the smooth muscle cell layers.

Male BALB/C mice (10-12 wks) were stunned and killed by cervical dislocation. First and second order mesenteric arteries (100-200 μm diameter) were dissected and mounted in a wire myograph. In the presence of L-NAME (100 μM), sharp microelectrodes were used to measure changes in smooth muscle membrane potential. Cyclopiazonic acid (CPA) was used to activate the EDHF response. Perfusion fixed tissues (n=3) were processed using standard serial section electron microscopy methods (Sandow and Hill, 2000).

CPA (10 μM) evoked smooth muscle hyperpolarization of 9.9 ± 0.4 mV (from -53 ± 1 mV, n=4). In arteries stimulated with 0.1-3 μM phenylephrine, CPA stimulated 94 ± 10% repolarization (11.8 ± 1.5 mV, n=3), associated with 84 ± 3% relaxation (n=18). Relaxation was not significantly inhibited by antagonists of EDHF (apamin, 50 nM, or charybdotoxin, 100 nM) alone, but was totally abolished by the combined toxins or by removal of the endothelium (n=12 and 5, respectively).

Raising $[K^+]_o$ to 10.8 mM only evoked small maximal relaxations (28 ± 10%, n=7). Arteries fixed on completion of experiments and visualized with confocal microscopy showed a high density of perforations through the internal elastic lamina, which corresponded with a relatively large number of myoendothelial gap junctions at the ultrastructural level. An approximate 3-fold increase in the number of myoendothelial gap junctions (MEJs) and a 50% decrease in the number of medial smooth muscle cell layers was found in the mouse, compared to the same region of the rat mesenteric arterial tree (Sandow and Hill, 2000).

The endothelium-dependence of CPA-induced smooth muscle hyperpolarization and relaxation, together with the blockade of EDHF by apamin and charybdotoxin in combination, indicate a primary action on the endothelial cells. Together with a high density of MEJs and relatively weak relaxations to exogenous K^+, these data indicate that the predominate pathway for EDHF relaxation is by spread of a hyperpolarizing signal from the endothelium by direct spread of current to adjacent smooth muscle cells via MEJs.

Ding, H., Kubes, P. & Triggle, C. (2000) *Brit J Pharmacol.* 129, 1194-1200.

Sandow S.L., Hill C.E. (2000) *Circ Res.* 86;341-346.

122P NON-SELECTIVE CATION CURRENTS IN ENDOTHELIAL CELLS FRESHLY ISOLATED FROM SMALL PULMONARY ARTERIES OF THE RAT

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Vasoactive substances produced by the vascular endothelium are known to play an important role in modulating blood vessel diameter. The release of many of these vasoactive substances requires an increase in the intracellular Ca^{2+} concentration. In turn the endothelial cell membrane potential is thought to play an important role in regulating Ca^{2+} influx, ultimately controlling release of vasoactive substances and vessel tone. It has been demonstrated that endothelial cells are characterised by the presence of a K^+ conductance (Hogg *et al.*, 1999), however the nature of the background currents and their Ca^{2+} permeability remains to be elucidated. The aim of this study was to characterise the nature of the background currents in these cells and their Ca^{2+} permeability.

Male Wistar rats (~250 g) were killed by an overdose with pentobarbitone sodium and the small pulmonary arteries (200-400 μm) removed. Single pulmonary arterial endothelial cells were isolated as previously described (Hogg *et al.*, 1999). To investigate the nature of the background current, the perforated-patch voltage-clamp technique was used with a Cs^+ pipette solution and K^+-free bath solutions (K^+ substituted for Na^+, Cs^+ or $NMDG^+$). Statistical comparisons were made using a paired or unpaired Student's *t*-test as appropriate.

In response to voltage-ramps from −100 mV to +50 mV, membrane currents showed little voltage-dependence. Replacement of the extracellular Na^+ solution with $NMDG^+$ caused a reduction of the magnitude of inward current at -90 mV (I_{90}) from −13.9 ± 3.8 pA.pF^{-1} (mean ± standard error of the mean throughout; n=8) to −4.7 ± 1.5 pA.pF^{-1} (n=8; P < 0.05). However, replacement of extracellular Na^+ with Cs^+ caused an increase in the I_{90} to -16.0 ± 4.4 pA.pF^{-1} (n=4). Ionic substitution of extracellular Na^+ for NMDG or Cs^+ also induced shifts in the experimental reversal potential from −7.2 ± 1.1 mV (n=17) to −37.4 ± 6.9 mV (n=8; P < 0.05) and −1.1 ± 0.9 mV (n=7; P < 0.05), respectively. To assess the pharmacology of the background currents, the trivalent cations Gd^{3+} and La^{3+} were utilised. In response to depolarising voltage-ramps, Gd^{3+} (100 μM) and La^{3+} (100 μM) significantly reduced I_{90} by 70.1 ± 7.9 % (n=3; P < 0.05) and 58.0 ± 11.3 % (n=3; P < 0.05), respectively. In order to determine the Ca^{2+} permeability of the background current, extracellular Ca^{2+} was increased from 1.5 mM to 10 mM. In response to depolarising voltage-ramps, I_{90}, in the presence of 1.5 mM extracellular Ca^{2+}, was found to be −13.0 ± 2.7 pA.pF^{-1} (n=5). However, I_{90} in the presence 10 mM Ca^{2+} was not significantly altered (-11.4 ± 3.3 pA. pF^{-1}; n=5).

Together these data suggest that, at least in part, non-selective cation currents, with a selectivity of $Cs^+ > Na^+ >> NMDG^+$, may conduct the background current in pulmonary arterial endothelial cells. However, the background current recorded under these conditions does not appear to provide a Ca^{2+} influx pathway, at least under non-stimulated conditions.

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Hogg *et al.* (1999) *Biochem Biophys Res Commun* **263**, 405-9.

123P VASOACTIVE RESPONSES TO NOVEL ORPHAN RECEPTOR LIGANDS HEXARELIN AND GHRELIN IN HUMAN ARTERIES *IN VITRO*

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The 28 amino acid endogenous peptide ghrelin has recently been paired with the orphan growth hormone secretagogue receptor GHS-R (Kojima *et al.*, 1999). We have identified high affinity binding sites for [^{125}I]ghrelin (human) in the medial smooth muscle layer of human arteries (Katugampola *et al.*, 2001), suggesting a vasoactive role. In healthy volunteers ghrelin elicited a, presumed, growth hormone-independent reduction in mean arterial pressure (Nagaya *et al.*, 2001) and we have observed an endothelium-independent relaxation of endothelin-1 (ET-1) vasoconstriction by ghrelin in human mammary artery *in vitro* (Wiley & Davenport, 2001). Interestingly the synthetic GHS-R ligand hexarelin did not produce a hypotensive response *in vivo* in man, however it increased coronary perfusion pressure in rat heart (Bodart *et al.*, 1999). We have determined, therefore, the effect of hexarelin on human arteries *in vitro*, and compared this to ghrelin and the potent vasoconstrictor ET-1.

With local ethical approval, coronary arteries (CA) were obtained from 7 patients transplanted for ischaemic heart disease or dilated cardiomyopathy. Radial (RA) and internal mammary arteries (IMA) were from 15 patients receiving coronary artery bypass grafts. Endothelium-denuded rings (4mm) of each artery were set up in organ baths, containing oxygenated Krebs' solution (37°C) for isometric force recordings. Cumulative concentration-response curves were obtained to hexarelin, ghrelin and ET-1 (10^{-10}-10^{-6}M). The maximum possible contractile response was determined by addition of 100mM KCl and agonist responses were expressed as a percentage of this. Data were analysed using the iterative curve-fitting program Fig P (Biosoft, Cambridge, UK) to determine values of pD_2 and E_{max}. Data are mean±s.e.mean and n-values are the number of patients from whom tissue was obtained.

ET-1 contracted all arteries tested, from each vessel group, with nanomolar potency (Table 1). Responses to the GHS peptides were more variable with 20-50% of human arteries not responding. In those arteries that did respond, hexarelin and surprisingly ghrelin, exhibited comparable constrictor potency to ET-1 but the maximum response to these peptides was much less than to ET-1 (Table 1).

Table 1 Relative potency and efficacy of hexarelin, ghrelin and ET-1 in human arteries *in vitro* (x=responders)

	Hexarelin		Ghrelin		ET-1	
	pD_2	E_{max}	pD_2	E_{max}	pD_2	E_{max}
CA	8.6±0.7	22±11%	8.7±0.4	15±5%	8.3±0.2	76±14%
x/n	3/6		4/7		4	
RA	8.6±0.4	31±14%	8.4±0.2	17±5%	8.2±0.2	51±7%
x/n	4/5		5/8		7	
IMA	7.5±0.2	14±2%	7.8±0.3	27±9%	8.5±0.1	82±4%
x/n	3/5		4/8		4	

These data show that hexarelin and ghrelin are potent, low efficacy constrictors of human arteries. The lack of response in some patients and low efficacy in others may reflect the low receptor density determined for [^{125}I]ghrelin in, for example, human CA (Katugampola *et al.*, 2001). The observed vasoconstriction to ghrelin contrasts with its hypotensive effect *in vivo* and its ability to oppose ET-1-mediated vasoconstriction *in vitro*. One possible explanation may be that the presence, or absence, of significant vascular tone influences the vascular response to GHS-R activation in human arteries.

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124P DIETARY SUPPLEMENTATION WITH CANNED SARDINE (C.S.) IMPROVES THE PARAMETERS OF RISK OF CARDIOVASCULAR DISEASE. STUDY IN OLD MALE RATS.

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Dietary supplementation with ω-3 fatty acids (f.a.) has been shown to improve several parameters of risk of cardiovascular disease (Simopoulos, 1999). In most studies fish oils were used as a source of fatty acids for supplementation. Sardine oil is also rich in ω-3 fatty acids.

In this study we have investigated in male *Wistar* rats the effects of dietary supplementation with portuguese canned sardine on some markers of cardiovascular function, like blood pressure (B.P.), serum lipid profile and vascular contractility.

Two groups of 5 animals aged 1 year, with 650-950g-body weight were maintained during 4 months with a diet of standard pellets (2.8%fat) only (control group) or supplemented with 13.6 g of canned sardine (containing 0.58 g of ω-3 fatty acids, 2% w/w) five days *per* week (supplemented group). Serum lipids profile and blood pressure were measured before (M0) and by the end of each month of supplementation, in both groups. By the end of month 4 (M4) the animals were sacrificed, aortic rings were collected and phenilephrine (Phe)-induced dose-response curve (d.r.c) were analysed in the absence (control) and presence of L-nitroarginine (L-Noarg; 100μM) and L-Noarg plus Indometacine (Ind.; 10μM). The results were analysed using the Student's unpaired *t* test; $P<0.05$ was taken to be statistically significant.

The mean blood pressure increased along time only in control animals. at M4 the values were: control 151.5 mmHg±3.71 *vs* suppl: 127 mmHg±10,06).
Serum triglycerides (TG), cholesterol (CH) and total lipids (TL) were significantly lower in the suppl. group at M4 (TG (mg/dl): control 226.8±29.3 *vs* suppl. 124±9.27; CH (mg/dl): control 256.8±25.9 *vs* suppl. 133±8.27; TL (mg/dl): control 703.6±50.2 *vs* suppl. 545±27.7). The results of E_{max} (mg) of Phe dose-response curve in aortic rings from non-supplement and supplement animals are shown in the table.

	Control (mg)	L-Noarg (mg)	L-Noarg+Ind (mg)
Non-supplement	1373.4±362	1411±120	1404±312
Supplement	1084±69	1340±84*	1287±135

(*P *vs* control<0.05)

The results suggest that, like fish oil, dietary canned sardine had a beneficial effect on cardiovascular function, namely on blood pressure, lipid profile and vascular contractility. Also the endothelial responsiveness seemed to be improved. The protective effect of ω-3 fatty acids (Scharky, 2000) seems therefore to persist in canned sardine.

Simopoulos, A. P. (1999). *Am J Clin Nut.* 70S: 560-569.
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125P ANTIHYPERTENSIVE EFFECTS OF LOSARTAN AND ATENOLOL ON 1,3-DIPROPYL-8-SULFOPHENYLXANTHINE (DPSPX)-INDUCED HYPERTENSION

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In the rat, the prolonged infusion of DPSPX (1,3-dipropyl-8-sulfophenylxanthine), a non-selective antagonist of adenosine receptors, causes hypertension (Albino-Teixeira *et al*, 1991), accompanied by an increase in plasma renin activity (Albino-Teixeira & Osswald, 1994). Marked morphological changes similar to those found when angiotensin II is infused at subpressor doses are also found in DPSPX-treated rats. This facts suggest an involvement of the renin-angiotensin system in this experimental model of hypertension.

The aim of this study was to evaluate the effects of losartan (an AT_1 antagonist) and atenolol (a β-adrenoceptor antagonist) on DPSPX-induced hypertension.

The study was divided in two parts: Part 1) six groups of male Wistar-kyoto rats weighing 250-300 g were treated for a week: A-saline i.p.; B-DPSPX (90 $\mu g.kg^{-1}.h^{-1}$) i.p.; C-losartan (15 $mg.kg^{-1}.d^{-1}$) p.o.; D-atenolol (25 $mg.kg^{-1}.d^{-1}$) p.o.; E-DPSPX (90 $\mu g.kg^{-1}.h^{-1}$) i.p+losartan (15 $mg.kg^{-1}.d^{-1}$) p.o.; F-DPSPX (90 $\mu g.kg^{-1}.h^{-1}$) i.p.+atenolol (25 $mg.kg^{-1}.d^{-1}$) p.o. Part 2) rats were treated for a month. Saline or DPSPX was infused through Alzet 2ML1 minipumps. Losartan or atenolol was administred in drinking water. Systolic blood pressure (SBP) was determined by the tail-cuff method. At the end of the study, rats were killed and fragments of the rat tail artery were processed for morphological study. Statistical analysis was done by ANOVA, Newman-Keuls test.

The results of the study are summarized in table 1.

Neither systolic blood pressure nor diameter of smooth muscle cells (SMC) were not changed by losartan (SBP: 118.5±6.2; SMC diameter: 7.10±0.31) or atenolol (SBP: 117.4±0.9; SMC diameter: 7.04±0.37) alone.

Table 1. Systolic blood pressure and diameter of smooth muscle cells of each group. * *vs* correspondent saline; $^{+}$ *vs* correspondent (DPSPX+losartan); $^{\#}$ *vs* correspondent DPSPX; $p<0.05$; n=6-12 rats, 600-800 cells.

Group	SBP (mmHg)	Diameter of SMC (μm)
Saline 1 week	110.5±5.0	6.72±0.21
Saline 1 month	115.2±1.2	6.87±0.19
DPSPX 1 week	150.0±5.3*	9.61±0.31*
DPSPX 1 month	155.4±7.1*	10.38±0.15*
(DPSPX+losartan) 1 week	109.2±4.7$^{\#}$	7.10±0.42$^{\#}$
(DPSPX+losartan) 1 month	111.8±2.9$^{\#}$	6.43±0.21$^{*,\#}$
(DPSPX+atenolol) 1 week	116.9±1.3$^{\#}$	8.53±0.44$^{*,+,\#}$
(DPSPX+atenolol) 1 month	111.4±1.7$^{\#}$	7.46±0.18$^{*,\#,+}$

We can conclude that both losartan and atenolol counteract the rise in blood pressure induced by DPSPX. However, while losartan completely prevents the hypertrophy of the DPSPX-hypertensive rat tail artery, atenolol exerts a partial protection.

Albino-Teixeira, A., Matias, A., Polónia, J., *et al*, (1991) *J. Hypertens.*, 9 (suppl 6), S196-S197.
Albino-Teixeira, A. and Osswald, W. (1994) *J. Autonom. Pharmacol.*, 14, 13.

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126P VASCULAR REACTIVITY IN DPSPX (1,3-DIPROPYL-8-SULFOPHENILXANTHINE)-INDUCED HYPERTENSION

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The prolonged infusion of DPSPX (1,3-dipropyl-8-sulfophenylxanthine), a non-selective antagonist of adenosine receptors, causes an hypertensive state (Albino-Teixeira *et al*, 1991), accompanied by an increase in plasma renin activity (Albino-Teixeira & Osswald, 1994). This suggests an involvement of the renin-angiotensin system in this experimental model of hypertension.

Hypertension is associated with changes in vascular reactivity. The aim of this study was to evaluate the effects of angiotensin II (AII), noradrenaline (NA) and potassium chloride (KCl), in the mesenteric vasculature of DPSPX-hypertensive rats.

Male Wistar-Kyoto rats weighing 250-300 g were used. Control rats (group A) received an infusion of saline i.p.. DPSPX (90 $\mu g.kg^{-1}.h^{-1}$) was infused i.p. during 3 (group B - killed on day 3) or 7 (group C - killed on day 15) days. Alzet 2ML1 minipumps were used for continuous i.p. infusions. Blood pressure was determined by the tail-cuff method. Animals were killed and segments of the rat mesenteric artery and vein were dissected free from conective and adipose tissues. Then, small rings were mounted in isolated organ baths, containing aerated (95% O_2 + 5% CO_2) Krebs-Henseleit solution at 37°C, and stretched to a resting tension of about 4.9 mN. After a stabilisation period of 90 min, concentration-response curves were obtained by adding increasing non-cumulative concentrations of AII (0,3-81 nM) or cumulative concentrations of NA (0.01-10 μM) or KCl (25-200 mM) to the organ bath. Mechanical isometric responses were recorded on a polygraph. Statistical analysis was done by ANOVA, Newman-Keuls test.

Systolic blood pressure was higher in DPSPX-treated rats ((mmHg) A: 115±1; B: 138±1*; C: 146±1$^{\#}$; * *vs* A, $^{\#}$ *vs* A and B; $p<0.05$; n=6). Maximum contractile effect of AII was lower in both groups of DPSPX-hypertensive rats ((mN/mg) Artery: A-0.78±0.11; B-0.26±0.07*; C-0.55±0.02$^{\#}$. Vein: A-5.70±0.07; B-2.93±0.02*; C-2.36±0.40*; * *vs* A, $^{\#}$ *vs* A and B; $p<0.05$; n=12-20). NA induced larger contractions in vessels from DPSPX-hypertensive rats, except in arteries from group B ((mN/mg) Artery: A-2.29±0.03; B-2.37±0.11; C-2.58±0.09*. Vein: A-1.02±0.03; B-2.12±0.05*; C-2.01±0.12*; * *vs* A; $p<0.05$; n=12-20). KCl effects were lower in arteries but higher in veins from DPSPX-hypertensive rats ((mN/mg) Artery: A-2.38±0.04; B-2.19±0.01*; C-2.20±0.03*. Vein: A-3.80±0.02; B-7.63±0.08*; C-4.65±0.02$^{\#}$. * *vs* A; $^{\#}$ *vs* A and B; $p<0.05$; n=12-20).

We can conclude that DPSPX-induced hypertensive state is associated with: (1) lower efficacy of AII; (2) increased efficacy of NA, except in the early phase of the hypertension in mesenteric artery; (3) lower efficacy of KCl in mesenteric artery but higher efficacy in mesenteric vein.

Albino-Teixeira, A., Matias, A., Polónia, J., *et al*, (1991) *J. Hypertens.*, 9 (suppl 6), S196-S197.
Albino-Teixeira, A. and Osswald, W. (1994) *J. Autonom. Pharmacol.*, 14, 13.

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127P XANTHINE OXIDASE INHIBITION BY DPSPX (1,3-DIPROPYL-8-SULFOPHENILXANTHINE)

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Xanthine oxidase (XO), an enzyme involved in purine metabolism is released into the circulation from organs rich in this enzyme (*i.e.* liver and intestine) following periods of metabolic stress. Since the enzyme reaction that transfers electrons from hypoxanthine to uric acid is coupled with a reduction of molecular oxygen into $O_2^{\cdot-}$ and H_2O_2, XO has been considered to play a crucial role in the pathogenesis of oxidant induced microvascular changes and tissue injury (Suzuki et al, 1998). There is also evidence that $O_2^{\cdot-}$ derived from XO might alter $NO^{\cdot}$ bioavailability in spontaneously hypertensive rats (SHR) (Cai and Harrison, 2000).

DPSPX, an adenosine receptor antagonist induces hypertension and alterations in the rat cardiovascular morphology (Albino-Teixeira *et al*, 1991). Since DPSPX is a xanthine, the aim of this study was to evaluate the interaction between DPSPX with XO in order to ascertain the putative contribution of XO in DPSPX model of hypertension.

The possible production of $O_2^{\cdot-}$ during the putative DPSPX metabolism by XO, was evaluated in a 96 well plate reader by monitoring the reduction of cytochrome c by $O_2^{\cdot-}$ at 560 nm (Valentão *et al*, in press).

The effect of DPSPX on XO activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Valentão *et al*, in press).

DPSPX did not behave as a XO substrate since no $O_2^{\cdot-}$ formation was observed on its addition. However, DPSPX (10, 20, 30, 40, 50 and 75 μM) was shown to have potent concentration-dependent XO inhibitor activity (percent inhibition: 8.19 ± 1.15, 19.36 ± 2.99, 26.64 ± 1.44, 40.76 ± 1.91, 50.88 ± 1.98 and 70.81 ± 0.64, respectively; mean ± s.e.m., n = 4). The IC50 was 51.68 ± 1.09 μM (mean ± s.e.m., n = 4). The results expressed as a Lineweaver-Burk plot demonstrate that DPSPX is a mixed noncompetitive inhibitor with respect to the xanthine substrate.

We can conclude that: (i) DPSPX is not a substrate for XO; (ii) DPSPX is a noncompetitive inhibitor of XO. This last effect might be involved in the development of DPSPX-induced hypertension.

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128P HUMAN PHOSPHODIESTERASE 4D - GENOMIC ORGANISATION AND IDENTIFICATION OF A PUTATIVE PROMOTER FOR SPLICE VARIANT FIVE

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Phosphodiesterase 4 (PDE4) is a distinct family of 3',5'-cyclic nucleotide phosphodiesterases with a high affinity for cAMP which play a pivotal role in regulation of airway smooth muscle relaxation and anti-inflammatory effects in many immune cells. There are 4 known subfamilies (A-D) which show a high degree of homology in amino acid and coding sequences. PDE4D lies on chromosome 5q12 and has been shown by molecular cloning to have five distinct 5' splice-variants (1-5) which are differentially expressed according to cell type, and have varying intracellular distribution (Bolger *et al.*, 1997). In the present study we aimed to define the genomic organisation of the PDE4D gene and to identify the promoter for PDE4D5.

In silico comparison of published cDNAs from the five isolated splice-variants of PDE4D with databases of the unfinished human genome using NCBI's BLAST facility, revealed a complex arrangement of 15 exons spanning a region of 1MB. Exons 6 to 15 encoding the catalytic region of the enzyme and an important regulatory element, upstream conserved region 2 (UCR2), were clustered within 19 KB of genomic DNA. They were separated from a second cluster of exons (2 to 5) encoding UCR1, which is present only in the long isoforms of PDE4D. The most 5' exons, numerically denoted 1(3), 1(5) and 1(4), are isoform-specific and have a putative role in intracellular localisation.

The lengths of exons 2 to 14 (except for a small hypervariable region in 8) were exactly comparable both between sub-families of PDE4 in *Homo sapiens* where sequences showed >85% homology at the encoded amino-acid level, and with the PDE4D gene in *Rattus norvegicus* where there was >90% homology in corresponding sub-families.

The region immediately upstream from the transcription start site of PDE4D5 was analysed using three independent public-access promoter predicting programs which, on the basis of transcription factor binding sites, indicated that it was a putative promoter. Short and long constructs (621 and 1544 bp. respectively) of this region were ligated into the PGL3-enhancer luciferase reporter vector (PGL3E). BEAS-2B cells were transiently transfected with either the long or short PDE4D5 promoter construct, or an appropriate control: SV-40 renilla (ratio 1:20) was used to correct for transfection efficiency. The short construct produced a 10.8 +/- 0.7 (n=4) fold increase in renilla-corrected luciferase activity over the empty PGL3E vector; the long construct produced a 27.4 +/- 2.0 (n=4) fold increase. These data compared with 11.3 +/- 0.7 (n=4) with PGL3-control, a positive control vector supplied by Promega with a strong SV-40 promoter ligated into the PGL3E vector frame. Statistical analysis of the raw data showed highly significant differences in induction of luciferase expression both between the two constructs and when compared to the empty PGL3E vector ($p<0.001$; ANOVA).

The study describes the complex PDE4D gene arrangement and provides data to suggest a strong intronic promoter for one of the splice-variants encoding the long isoform PDE4D5.

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Bolger G.B. *et al.* (1997) Biochem. J. 328, 539-48

129P ISOLATED ARTERIES FROM TESTICULAR FEMINISED MICE HAVE MAINTAINED DILATOR RESPONSES TO TESTOSTERONE BUT REDUCED VASCULAR REACTIVITY TO ACETYLCHOLINE

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Testosterone acts as a vasodilator in a variety of vascular beds (English *et al.*, 2001). Men with coronary artery disease have reduced testosterone levels (English *et al.*, 2000a) and testosterone-induced coronary vasodilation is postulated to account for the improvement in myocardial ischaemia associated with testosterone replacement therapy in these individuals (English *et al.*, 2000b). Testicular feminised (Tfm) mice lack a functional androgen receptor and as a consequence are androgen resistant and exhibit a lower circulating androgen profile compared to XY controls. The aim of the present study was to determine whether alterations in testosterone-induced vasodilation or vascular reactivity occur in these animals.

2mm lengths of femoral artery (n=48, diameter = 322±9 μm) were dissected from adult male Tfm (n=8, 21-27g) and age-matched XY littermate (n=8, 19-31g) mice, mounted in a wire myograph and loaded to a tension equivalent to 100mmHg. Vessels were contracted with potassium chloride (KCl, 80mM) to confirm viability prior to concentration- response curves being constructed to noradrenaline (NA, 1nM-100μM) (from baseline) and acetylcholine (ACh, 0.1-10μM) and testosterone (T, 1-100μM) (after preconstriction with 10μM NA). Plasma total T levels from age-matched Tfm and XY mice (n=46) were analysed via radioimmunoassay.

Vessels from Tfm mice had maintained vasodilation to T but significantly reduced vasodilation to ACh. Vasoconstriction to KCl was also significantly attenuated (Table 1 below).

	XY	Tfm
Total T (nM)	15.1 (2.9)	1.8 (0.1)**
E_{max} KCl (mN/mm)	4.67 (0.40)	3.37 (0.24)*
E_{max} NA (mN/mm)	5.61 (0.47)	4.71 (0.33)
E_{max} ACh (% relaxation)	49.9 (3.7)	36.3 (3.4)*
E_{max} T (% relaxation)	101.5 (1.9)	95.3 (3.6)
E_{max} V (% relaxation)	3.3 (5.4)	-3.1 (3.5)

(Results as mean (sem). * = P<0.01 ** = P<0.0001 via Students unpaired t test).

This data demonstrates that i) testosterone-mediated vasodilation is independent of the classical androgen receptor and ii) reduced circulating testosterone levels are associated with adverse alterations in vascular reactivity.

English KM, *et al.* (2001) Horm.Metabol.Res. *In Press*

English KM, *et al.* (2000a) Eur.Heart.J. 21, 890-894

English KM, *et al.* (2000b) Circulation. 102, 1906-1911

130P TESTOSTERONE INHIBITS AGONIST-INDUCED INCREASES IN INTRACELLULAR CALCIUM IN RAT AORTIC SMOOTH MUSCLE CELLS

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Testosterone acts as a coronary vasodilator (Webb *et al.*, 1999), a property proposed to underlie the improvement in myocardial ischaemia associated with testosterone replacement therapy in men with coronary artery disease (English *et al.*, 2000). The dilatory action of testosterone has been partially characterised in animal models and been shown to be independent of the classical androgen receptor and also of the release of endothelial-derived vasodilators (Yue *et al.*, 1995). The aim of the present study was to determine whether testosterone has a calcium antagonistic action.

A7R5 rat aortic smooth muscle cells were grown in culture until confluent. The cells were then trypsinized and re-suspended in media containing 5μM indo-1-AM for 30 min, to allow loading into the cells. The cells were then washed, re-suspended in physiological saline and analysed by flow cytometry pre- and post-exposure to either: A) 10μM prostaglandin F2α (PGF2α) (n=12), B) 10μM PGF2α preceded by 2min incubation with 1μM testosterone (n=7) or C) 10μM PGF2α preceded by 2min incubation with equivalent volume of ethanol vehicle (n=7). Cellular fluorescence indicative of the level of intracellular calcium is shown in Table 1 below.

Group	Control	Testosterone	Vehicle
Fluorescence Pre PGF2α	8.1(0.3)	8.8(0.4)	8.3(0.3)
Fluorescence Post PGF2α	17.1(0.9)	12.4(0.6)*	15.8(0.8)
Change in Fluorescence	9.0(1.1)	3.6(0.6)*	7.6(1.0)

(Results as mean (sem) in arbitrary units. * = P<0.005 from vehicle and control via students unpaired t test).

Incubation with 1μM testosterone had no significant effect upon the resting cell calcium concentration, but significantly attenuated the increase in intracellular calcium associated with exposure to 10μM PGF2α which occurs via extracellular calcium entry, possibly though voltage-gated calcium channels. This data therefore supports a calcium antagonistic action of testosterone potentially at the level of the voltage-gated calcium channels.

English KM, Steeds RP, Jones TH *et al.* (2000) Circulation; 102, 1906-1911

Webb CM, McNeill JG, Hayward CS *et al.* (1999) Circulation; 100, 1690-1696

Yue P, Chatterjee K, Beale C *et al.* (1995) Circulation; 91, 1154-1160

131P TESTOSTERONE SUPPRESSES CYTOKINE PRODUCTION IN WHOLE BLOOD FROM MEN WITH HEART FAILURE

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Chronic heart failure is characterised by metabolic disturbance, with excessive activity of catabolic hormones and pro-inflammatory cytokines, in particular tumour necrosis factor (TNF). (Anker *et al.*, 1997) Anabolic factors, including androgens, may be suppressed. (Tappler & Katz, 1979) Testosterone has anti-inflammatory as well as anabolic properties and may be an important endogenous counter to both the production and actions of TNF. We sought to determine whether testosterone inhibits the production of TNF in heart failure patients in-vitro.

Twenty seven male patients with chronic stable heart failure (>6 months) were studied. Mean ± SD age was 60.9 ± 9.3 years. All had moderate-severe impairment of left ventricular function (mean ejection fraction 37 ± 4%). Blood was collected between 0800 and 0900 hours into polypropylene tubes containing an equal volume of phosphate buffered solution, plus heparin in a final concentration of 10IU/ml. Samples were incubated at 37°C either alone, with vehicle control (cyclodextrin) or with testosterone in a concentration of 10^{-8}M, 10^{-6}M or 10^{-4}M (physiological range 1 - 4 x 10^{-8}M). Lipopolysaccharide was added to each container after 60 min (1μg/ml) and incubation continued for 3 hours. Samples were centrifuged at 3000g for 15 minutes. Levels of TNF in the supernatant were assayed by ELISA (Diaclone, France).

LPS induced marked production of TNF, which was not affected by vehicle control (12,729 ± 7342ng/ml v 12,558 ± 6747ng/ml, p=0.9). Pre-treatment with testosterone resulted in a concentration-dependent reduction in TNF production compared with control (Table 1).

Table 1. LPS-stimulated TNF production in whole blood incubated with testosterone

	Control	Testosterone 10^{-8}M	Testosterone 10^{-6}M	Testosterone 10^{-4}M	p#
TNF (ng/ml)	12557 ± 6747	11158 ± 5811	10285 ± 5795*	9166 ± 5668**	0.04

Data as mean ± SD #by ANOVA *p=0.08 v control **p=0.008 v control (unpaired t tests)

These results indicate that testosterone suppresses LPS-induced TNF production in whole blood from men with heart failure. Low androgen levels in men with heart failure may contribute to the cytokine activation and catabolic excess of this condition.

Anker SD, Chua TP, Ponikowski P *et al.* (1997) Circulation; 96: 526-534.

Tappler B, Katz M. (1979) Clinical Endocrinology; 10: 219-226

132P THE EFFECT OF AGE ON OESTROGEN-INDUCED RELAXATION OF RAT AORTA

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Age-related vascular changes may involve endothelial dysfunction including altered nitric oxide (NO) production or vascular responses to released NO. Some studies show reduced nitric oxide synthase (eNOS) activity and NO release in aged arteries (Barton et al.,1997). However, Tschudi & Lüscher (1995) observed no change in endothelium-dependent relaxation or response to sodium nitroprusside (NP), a direct NO donor, and Küng & Lüscher (1995) reported enhanced relaxation to NP in aged rat aorta. In this study we compared relaxant actions of 17β oestradiol (EST) which is reported to release NO from the endothelium (Binko & Majewski 1998) and NP in aorta from young and old rats.

Endothelium-intact or denuded aortic rings, 3-5mm wide, prepared from male Hooded Lister rats (250-350g) aged 3 months or old rats aged 24 months (350-400g) were placed in Krebs' solution containing 10μM indomethacin (37°C, 95% O_2, 5% CO_2) under 2g tension. The presence of endothelium was confirmed by relaxant responses (>30%) to acetylcholine (1μM) following contraction of aorta by KCl (60mM). Non-cumulative concentration-response curves to KCl (5-80mM) were constructed in intact and denuded rings from young or aged rats and repeated after incubation with either EST (10μM) or NP (1μM). No vehicle effects were observed.

Both EST (10μM) and NP (1μM) caused rightward shifts in the KCl concentration-response curve: reduction in Emax by EST or NP was greater in aged than young rats ($p<0.05$).To examine the effects on EST or NP-induced relaxation of modifying NO production and cGMP concentration, concentration-response curves to KCl were constructed in the absence or presence of EST (10μM) or NP (1μM): following inhibition of nitric oxide synthase or nitric oxide-sensitive guanylyl cyclase by L-NAME (100μM) or oxadiazolo-quinoxalin-1-one (ODQ, 1μM) respectively, the effect of either EST (10μM) or NP (1μM) on responses to KCl were re-tested. L-NAME (100μM) reduced relaxation induced by EST in intact, but not in denuded aorta in both young and aged rats. Inhibition was greater in aged than young rats ($p<0.01$) and was reversed by L-arginine (1mM) (n=6). In young rats, ODQ (1μM) inhibited EST-induced relaxation in intact but not in denuded rings: in aged rats inhibition was marked in both. ODQ (1μM) also inhibited NP-induced relaxation in both intact and denuded rings from young and aged rats, inhibition being greater in the aged rats ($p<0.001$) (Table 1), N=6.

Table 1. Relaxation (% reversal of contraction) of KCl responses (Emax, 80mM) by EST (10μM) or NP (1μM) alone or + ODQ (1μM) in intact or denuded aorta. **p<0.01, ***p<0.001. Student's paired t test.

			Intact	Denuded
Young rats:	KCl	EST	43.2±6.7	22.6±5.2
		NP	40.0±9.7	42.2±9.0
		EST+ODQ	No relaxation	8.6±7.8(NS)
		NP+ODQ	9.6±8.2***	17.4±8.1**
Aged rats:	KCl	EST	65.9±7.6	41.4±7.4
		NP	75.0±8.3	74.5±6.4
		EST+ODQ	No relaxation	12.9±7.2**
		NP+ODQ	12.5±7.8***	14.2±8.1***

Results show that in the aged animal, neither EST-induced relaxation or the direct relaxation by NP of vascular muscle was reduced: both appear to be increased in intact aorta. Both L-NAME and ODQ inhibited EST-induced relaxation in intact aorta of young and old rats, suggesting that the endothelium-dependent component of relaxation by oestradiol, which involves the NO-cGMP pathway, is not suppressed by age in the rat aorta.

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Küng C & Lücher T, (1995) *Hypertension* , 25 194-200.

Tschudi M & Lüscher T, (1995) *Circulation*, 91 2415-2422.

133P DIRECT SCAVENGING OF NITRIC OXIDE BY DPSPX (1,3-DIPROPYL-8-SULFOPHENILXANTHINE)

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DPSPX, a non selective adenosine receptor antagonist, induces hypertension, cardiovascular hypertrophy and hyperplasia in the rat (Albino-Teixeira *et al,* 1991). It was also demonstrated that DPSPX-hypertensive rats present an impaired endothelium-dependent vasodilation (MQ Paiva *et al*, 1997).

Nitric oxide, the endothelium derived relaxing factor, plays an important role in the regulation of the vasomotor tone. Alterations in nitric oxide release or action have importance on a number of major clinical situations such as hypertension.

Our study aimed at evaluating if DPSPX has a direct scavenging effect on nitric oxide (NO) levels.

NO was generated using sodium nitroprusside in aqueous solution. NO interacts with oxygen to produce nitrite ions which can be measured using a microplate assay method based on the Griess reaction (Green *et al*, 1982). Scavengers of NO compete with oxygen leading to reduced production of nitrites. Sodium nitroprusside (5mM) (1 ml) in phosphate buffered saline (PBS) was added in test tubes to DPSPX solutions (1, 30, 300 and 1000 μM in PBS) (1 ml) and the test tubes incubated at 25°C for 240 min

Every 60 min, the incubation solutions (100 μl) were removed, diluted with Griess reagent (100 μl) and the absorbance immediately read at 550 nm.

DPSPX demonstrated a scavenging activity of NO in a concentration dependent manner as shown in *Table 1*:

[DPSPX]	Nitrite production (% of control)
1 μM	85.51±2.05
30 μM	76.24±4.94
300 μM	63.43±3.50
1 mM	46.53±7.78

Table 1. Nitrite production (% of control) in the presence of DPSPX, incubation time 240 min. Results are expressed as mean ±s.e.m, n=4-7.

We can conclude that DPSPX is a potent scavenger of nitric oxide. These findings may also help to explain, at least in part, the alterations in the endothelium–dependent vasodilation in DPSPX model of hypertension.

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M. Q. Paiva, M. J. Santos, A. Albino-Teixeira (1997). *J Pharm. Pharmacol.* 49: 74-77.

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134P PINACIDIL, BUT NOT CROMAKALIM, -INDUCED Rb EFFLUX FROM RAT ISOLATED AORTA IS ATTENUATED BY N^G-NITRO-L-ARGININE METHYL ESTER (L-NAME).

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The chemical diversity of ATP-sensitive potassium channel openers (K_{ATP}COs) suggests the involvement of multiple sites of action on the target membrane (Lawson, 1996). L-arginine analogues discriminate between the vasorelaxant responses to the cyanoguanidine K_{ATP}CO pinacidil and the benzopyran K_{ATP}CO cromakalim in rat isolated aorta (Carr & Lawson, 1999). To gain a greater understanding of the mechanisms involved the effects of N^G-nitro-L-arginine methyl ester (L-NAME) on K_{ATP}CO-induced rubidium (Rb) ion efflux from rat isolated aorta was studied. The Rb efflux assay was modified from Terstappen (1999).

Aortic segments from Wistar rats (200-250g) were incubated in Kreb's bicarbonate solution containing RbCl (5.4 mM) for 4 hrs at 37°C, to which L-NAME (100μM), L-N^5-(1-iminoethyl)ornithine (L-NIO: 100μM) or vehicle (control) was added for the final 30 mins. Tissues were then washed 3 times in Kreb's bicarbonate solution containing KCl in place of RbCl and the relevant pretreatment. Pinacidil (PIN: 0.8μM) or cromakalim (CROM: 1.2μM) was then added (0.5ml) to the aortic segments for 60 sec, after which the supernatant (0.5ml) was removed and added to 4.5ml of distilled water. The tissue was then exposed to SDS (10%, 0.5ml) for 18hr, after which the lysate (0.5 ml) was added to 4.5 ml of distilled water. The Rb content of the supernatant and lysate was determined by atomic emission spectroscopy (780nm) from which % Rb efflux mean±s.e.m. values were calculated. For vasorelaxation studies, rat aortic rings, devoid of endothelium, were suspended under 2g resting tension in Kreb's bicarbonate solution (gassed with 95% O_2/5% CO_2 at 37° C). Cumulative concentration-relaxation curves (CRC) to PIN (0.05-25.6μM) and CROM (0.05-12.8 μM) were constructed 30 mins after incubation with L-NAME (100μM), L-NIO (100μM) or vehicle (control) in phenylephrine-contracted tissues. EC_{50} values (mean±s.e.m., n=7) and concentration ratios with 95% confidence limits CR(cl) were determined from paired preparations.

Rb efflux relative to control was increased by PIN (control 7.46±0.69%, PIN 10.95±1.53%, n=16, $P<0.05$) and CROM (control 6.01±1.13%, CROM 10.94±1.96%, n=9, $P<0.05$). In the presence of L-NAME, Rb efflux due to PIN and CROM was 6.64±0.96%, (n=16, $P<0.05$ vs PIN alone) and 10.90±2.44% (n=6), respectively. Whilst Rb efflux due to PIN in the presence of L-NIO was not different from that to PIN alone. L-NAME and L-NIO alone did not modify Rb efflux relative to controls. CRCs to PIN (EC_{50} 0.43±0.08μM) were displaced to the right of controls by L-NAME (CR(cl) 5.05(3.56-6.54), but not L-NIO (EC_{50} 0.73±0.23μM). In contrast, the relaxations to CROM (EC_{50} 0.40±0.24μM) were not modified by L-NAME (EC_{50} 1.3±0.90μM).

In conclusion, L-NAME selectively modified both the vasorelaxation and Rb efflux in rat aorta due to pinacidil, an effect that appears to be independent of nitric oxide synthase. This study suggests that the sensitivity of the pinacidil responses to L-NAME may be due to a direct effect at the level of the K_{ATP} channel. These findings support the hypothesis that pinacidil and cromakalim do not possess a common pharmacophore for the 'K_{ATP}CO receptor' on the K_{ATP} channel.

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Terstappen, G.C. (1999) *Anal. Biochem.*, **272**, 149-155.

135P MODULATION OF SARCOPLASMIC RETICULUM CALCIUM RELEASE IN RABBIT AORTA BY SODIUM NITROPRUSSIDE

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Nitric oxide (NO) reduces the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle. This is proposed to result in part from the modulation of Ca^{2+} handling by the sarcoplasmic reticulum (SR). To investigate this further, this study investigated the influence of NO donors on transient increases in $[Ca^{2+}]_i$ activated by application of caffeine or noradrenaline (NA) to aorta smooth muscle cells.

Thoracic aortae were removed from male New Zealand rabbits (2-2.5 kg) killed by lethal injection of sodium pentobarbitone (30 mg kg^{-1} i.v.) and smooth muscle cells isolated by enzymatic digestion. Cells were superfused at room temperature with physiological salt solution and caffeine (20 mM) or NA (10 μM) applied by pressure ejection from a micropipette. The $[Ca^{2+}]_i$ under the membrane was detected by measuring Ca^{2+}-activated K^+ current (I_{KCa}) at 0 mV, using the whole-cell, voltage-clamp technique. The bulk $[Ca^{2+}]_i$ was measured by fura-2 fluorescence in cells loaded with fura-2-AM (5 μM). Results are expressed as mean ± s.e. mean. Statistical comparisons used a Student's t-test or ANOVA with $P < 0.05$ indicating a significant difference.

Sodium nitroprusside (SNP) was found to induce a 77 ± 7 % (n = 5) increase in the amplitude of I_{KCa} activated by caffeine when applied at 1 μM, but inhibited it by 40 ± 8 % at 10 μM (n = 8). These effects resulted from changes in the $[Ca^{2+}]_i$ transient rather than direct channel modulation, because the current activated by depolarising steps applied from a holding potential of –80 mV was similar in the absence (250 ± 45 pA; n = 15) or presence (258 ± 53 pA) of SNP. In contrast, 1 μM SNP had no significant effect on the caffeine- or NA-induced $[Ca^{2+}]_i$ transients detected by fura-2 fluorescence (n = 8), suggesting that it may differentially influence the sub-membrane and bulk $[Ca^{2+}]_i$. At 10 μM, SNP reduced the caffeine-activated $[Ca^{2+}]_i$ transient by 43 ± 1 % (n = 9), consistent with its effects on I_{KCa}. This effect was blocked by ODQ and mimicked by the phosphodiesterase inhibitors zaprinast (200 μM) and IBMX (100 μM), which reduced the caffeine-activated $[Ca^{2+}]_i$ transient by 53 ± 3 % (n = 6) and 55 ± 2 % (n = 5), respectively. Although these results suggest that reduction of the $[Ca^{2+}]_i$ transient was mediated by cGMP, 1 mM 8-bromo-cGMP failed to mimic it.

Following the peak of the caffeine-activated $[Ca^{2+}]_i$ transient, the $[Ca^{2+}]_i$ declined with a time constant of 18 ± 3 s (n = 85), as Ca^{2+} was removed from the cytosol. This was unaffected by 10 μM SNP (20 ± 6 s) or 1 mM 8-bromo-cGMP, although it was increased by 397 ± 27 % (n = 4) in the presence of the SR Ca^{2+}-ATPase inhibitor, cyclopiazonic acid. This implies that inhibition of the $[Ca^{2+}]_i$ transient by SNP did not reflect an enhanced rate of SR Ca^{2+} uptake, but was more likely due to inhibition of the Ca^{2+} release process. The apparent increase in sub-membrane $[Ca^{2+}]$ seen at 1 μM SNP suggests that it may be able to stimulate SR Ca^{2+} uptake at low concentrations, but only in sub-membrane regions.

136P REGIONAL HAEMODYNAMIC EFFECTS OF THE NITRIC OXIDE SYNTHASE INHIBITOR S-METHYL L-THIOCITRULLINE IN CONSCIOUS SPRAGUE DAWLEY RATS

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S-methyl-L-thiocitrulline (SMTC) has been reported to be a relatively specific inhibitor of the neuronal isoform of nitric oxide synthase (nNOS) *in vitro* (Furfine *et al.*, 1994). Pressor effects of SMTC have been described in the anaesthetised rat (Narayanan *et al.*, 1995) possibly indicating that nNOS may be involved in the control of blood pressure (Komers *et al.*, 2000). We have compared the effects of SMTC with those of the non-selective NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), on regional haemodynamics in conscious, male, Sprague-Dawley rats (350-450g).

Under anaesthesia (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.), animals were chronically implanted with renal (R), mesenteric (M), and hindquarters (H) Doppler blood flow probes, and, at least 14 days later, with intravascular catheters for recording blood pressure (BP) and heart rate (HR), and for i.v bolus drug administrations. On the day after catheterisation (day 1) animals (n=7) received either saline vehicle (0.1ml), and 0.3 and 3 mg kg^{-1} of SMTC, or 0.1, 1 and 10 mg kg^{-1} of SMTC. On day 3, the dose regimen was switched to ensure that each animal received all the doses. This protocol was repeated for L-NAME in a different group of rats (n=8).

There were no significant effects of vehicle in either group. At a dose of 0.3 mg kg^{-1}, the only significant (Friedman's test) cardiovascular effects of L-NAME were bradycardia (area over curve 0-30 min (AOC); -713 ± 176 beats (mean ± s.e. mean) and mesenteric vasoconstriction (AOC; -477 ± 77 % min). At this dose, SMTC caused significantly (Mann-Whitney U-test) smaller falls in HR (-302 ± 117 beats) and MVC (-154 ± 39 % min) than L-NAME, in spite of having a significant pressor effect (area under curve 0-30min (AUC); +152 ± 27 mmHg min). Unlike L-NAME, SMTC (0.3 mg kg^{-1}) caused a fall in RVC (-174 ± 40 % min). At higher doses, the patterns of effect of L-NAME and SMTC appeared more similar, although, at the highest dose tested, some differences persisted. Thus, L-NAME caused a significantly greater bradycardia (-1330 ± 118 vs 941 ± 165 beats) and fall in MVC (-1478 ± 104 vs −1145 ± 140 % min) than SMTC. Although the integrated rises in BP (+814 ± 94 vs +974 ± 156 mmHg min) and falls in RVC (-1280 ± 89 vs −966 ± 202 % min) and HVC (-991 ± 104 vs −1071 ± 121 % min) were not different with L-NAME and SMTC, it was notable that the initial rise in BP following SMTC (+52 ± 7 mm Hg at 4 min) was significantly greater than the response to L-NAME at that juncture (+ 24 ± 2 mm Hg). The fall in RVC seen with the lower dose of SMTC but not L-NAME supports the suggestion that nNOS may be involved in the control of renal haemodynamics in normal rats (Komers *et al.*, 2000). The qualitatively similar effects of higher doses of SMTC and L-NAME are consistent with both compounds causing non-selective NOS inhibition. It is possible that the different time courses of effect of the two compounds relates to differential metabolism.

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137P INVESTIGATION OF THE FUNCTIONAL ROLE OF A NOVEL ANGIOTENSIN-CONVERTING ENZYME (ACE2) IN HUMAN INTESTINE

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A novel form of angiotensin-converting enzyme, ACE2, has recently been identified, showing predominant expression (investigated by northern analysis) in human heart, testis and kidney, with limited expression elsewhere (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000). ACE2 mediates the breakdown of angiotensin I and II, leading to the formation of angiotensin fragments (1-5), (1-7) and (1-9). In the present study, we have applied a more sensitive and quantitative technique (QRT-PCR) to measure the expression of mRNA for ACE2 in a range of 72 human tissues. Having identified unexpectedly high levels of ACE2 mRNA within the human gastrointestinal tract, we subsequently investigated a role of ACE2-derived angiotensin fragments in controlling intestinal motility.

All tissue samples were obtained through medically qualified intermediaries with the informed consent of the donor, and with the approval of the local ethics committee. Total RNA was isolated from 72 different human tissues from all the major organ systems, each tissue from three donors. Using a primer/probe set designed to be specific for ACE2, the expression of mRNA for ACE2 was determined by quantitative real-time PCR, using the ABI Prism 7700 sequence detection system (Applied Biosystems). In isolated tissue studies, the effects of angiotensin II and fragments (1-5), (1-7) and (1-9) were investigated on both motility and secretion in human ileum and colon. Smooth muscle strips were mounted in organ baths, bathed by gassed (95% O_2:5% CO_2) Krebs solution at 37°C, and were electrically stimulated to measure effects on neurally-mediated contractions., whilst sections of mucosa were mounted in Ussing chambers, bathed by gassed (95% O_2:5% CO_2) Krebs solution at 37°C, and were clamped at zero potential for measurement of short-circuit current. In all studies, concentration-*effect curves* were obtained to angiotensin II and fragments (1-5), (1-7) and (1-9), in tissues from the same donor. Statistical analysis used Student's t test, with $p<0.05$ being taken to indicate significance.

In accordance with Donoghue *et al*, high levels of ACE2 were found in tissues from the testis, heart and kidney. However, in addition and in contrast to the previous study, high levels of expression of mRNA for ACE2 were found in tissues from human intestine (Table 1). In isolated tissue experiments, angiotensin II caused potentiation of electrically-induced contractions in both ileum and colon, with a pEC_{50} of 7.6±0.2 and 7.8±0.1 respectively (n=5). At a concentration of 1μM, angiotensin (1-9) also potentiated electrically-induced contractions in ileum and colon, with a response of 48.0±15.0% and 63.9±20.3% of the maximum response to angiotensin II (n=3 and 5 respectively). In ileum from two further donors, however, angiotensin (1-9) was without significant effect. The fragments (1-7) and (1-5) were without significant effect in ileum or colon. In Ussing chamber studies, angiotensin II or its fragments (up to 1μM) had no significant effect on short-circuit current in either tissue (n=3).

Heart		Kidney		Intestine	
L. Atria	3.7±0.4	Cortex	4.6±0.4	Jejunum	5.1±0.4
L. Ventricle	4.1±0.2	Medulla	4.5±0.1	Ileum	5.4±0.1
Cor. Artery	3.9±0.4	Pelvis	2.8±0.1	Colon	4.4±0.3

Table 1. Expression of ACE2 in tissues from heart, kidney and intestine. Data are log copy numbers (per 100ng of total RNA), and are expressed as mean±s.e.mean in tissues from 3 donors.

In summary, we have shown for the first time that mRNA for ACE2 is highly expressed in tissues from the human gastrointestinal tract. Thus far, no functional effect of the known angiotensin-related products of ACE2 has been demonstrated on intestinal motility.

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138P THE PEROXISOME-PROLIFERATOR ACTIVATOR RECEPTOR-γ LIGAND PIOGLITAZONE REDUCES INFARCT SIZE CAUSED BY MYOCARDIAL ISCHAEMIA AND REPERFUSION IN THE RAT

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We have recently discovered that the endogenous prostaglandin D_2 metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) reduces myocardial infarct size in the anaesthetized rat (Wayman & Thiemermann, 2001). The mechanism(s) of the cardioprotective effect of 15d-PGJ_2 are unclear. Although 15d-PGJ_2 is a ligand for the peroxisome-proliferator activator receptor-γ (PPAR -γ) (Ricote *et al.*, 1998), not all of the effects of 15d-PGJ_2 are secondary to the activation of PPAR-γ (Petrova *et al.*, 1999). This study investigates the effects of the antidiabetic thiazolidinedione (TZD) pioglitazone, a ligand of PPAR-γ, on the infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetised rat.

Twenty-nine male Wistar rats (230-290 g) were anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.). All animals were tracheotomised and ventilated (inspiratory oxygen concentration: 30%; tidal volume: 8-10 ml kg^{-1}; respiration rate: 70 strokes min^{-1}). The carotid artery was cannulated to measure mean arterial blood pressure (MAP) and heart rate (HR), the jugular vein for the administration of drugs. The chest was opened (left sided thoracotomy) and a 6-0 silk thread placed around the left anterior descending coronary artery (LAD). The animals were allowed to recover for 15 min. Pioglitazone (0.3 mg kg^{-1}, n=5; or 1 mg kg^{-1}, n=5) or its vehicle (10% v v^{-1} dimethyl sulphoxide, DMSO, 1 ml kg^{-1}, n=11) was then administered as an i.v. bolus injection. Thirty minutes later, the LAD was occluded for 25 min and then reperfused for 2 h. At the end of the experiment, the LAD was re-occluded, and 1 ml of Evans Blue dye (2% w v^{-1}) was injected into the jugular vein to determine the non-perfused area at risk (AR). Infarct size (IS) was determined by incubation of small pieces of the AR with nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1} at 37°C for 40 min). All data are expressed as mean±s.e.mean. Statistical differences between groups were analysed by ANOVA followed by a Bonferroni's test. A probability of <0.05 was considered to indicate significance.

When compared to rats which had been subjected to thoracotomy, but not LAD-occlusion (sham-operation, n=8, IS: 1±1%,), occlusion and reperfusion of the LAD of vehicle-treated rats resulted in an infarct size of 47±3% of the AR. Pre-treatment of rats with the PPAR-γ ligand pioglitazone caused a reduction in infarct size from 47±3% (vehicle-control) to 28±3% (0.3 mg kg^{-1}) and 26±4 (1 mg kg^{-1}) ($p<0.05$). The AR was similar in all of the groups studied (sham: 51±1%, vehicle-control: 51±2%, pioglitazone (0.3 mg kg^{-1}): 48±3%, pioglitazone (1 mg kg^{-1}): 49±3%; ($p>0.05$). There were no significant differences for MAP or heart rate in any of the experimental groups studied.

Thus, the PPAR-γ ligand pioglitazone causes a significant reduction in the IS caused by regional myocardial ischaemia and reperfusion in the anaesthetised rat. The mechanism(s) by which pioglitazone protects the heart against ischaemia-reperfusion injury warrants further investigation.

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139P PROTECTION BY AN ADENOSINE A_3 AGONIST FROM MYOCARDIAL STUNNING INDUCED BY SIMULATED ISCHAEMIA OF GUINEA-PIG LEFT ATRIA

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Braunwald & Kloner (1982) first defined reversible contractile dysfunction of myocardium following acute ischaemia and reperfusion as myocardial stunning. The recovery of contractile function can be mediated by adenosine, protecting against the damaging effects of inadequate blood flow and oxygen supply (Ely & Berne, 1992). This study examined the effect of an adenosine A_3 agonist, IB-MECA (N^6-(3-iodobenzyl) adenosine-5'-N-methylcarboxamide), on the recovery of contractility after simulated ischaemia.

Left atria from male Dunkin-Hartley guinea-pigs (250-300g) were arranged in 50ml organ baths containing Krebs bicarbonate solution at 37±0.5°C gassed with 5% CO_2 in oxygen. Atria were paced throughout at 2Hz with threshold voltage+50%. Resting tensions of 0.5-1g were applied and isometric tension recorded. Simulated ischaemia was induced after 30min equilibration by gassing with 5% CO_2 in nitrogen and substituting the glucose with choline chloride (7mM). After 30min simulated ischaemia, atria were reoxygenated and glucose was reintroduced. IB-MECA (3×10^{-7}M) was added either at the onset of simulated ischaemia, at reoxygenation or 5min into reoxygenation. Mean developed tension±SEM ($n\geq4$) was expressed as a percentage of the pre-ischaemia developed tension. Comparisons were made by paired Student's t-tests, $P<0.05$ indicating a significant difference.

Simulated ischaemia decreased developed tension to 17.5±2.0% of the pre-ischaemic level. Contracture also occurred as an increase in diastolic tension to 48.4±14.1% at the end of ischaemia. After 10min of reoxygenation, the developed tension returned to 35.6±1.8% of pre-ischaemic value. This was a significant reduction indicating myocardial stunning.

IB-MECA (3×10^{-7}M) added at the onset of simulated ischaemia did not significantly affect the recovery of contractility. The developed tension after 10min reoxygenation recovered to a value of 36.7±3.2%. However, when IB-MECA was added at reoxygenation, the developed tension after 10min significantly improved to 61.2±3.7% of the pre-ischaemic level. Furthermore the rate of relaxation of diastolic tension during reoxygenation was significantly enhanced by IB-MECA. When IB-MECA was added 5min into reoxygenation, the developed tension did not significantly improve, being 42.1±5.2% of the pre-ischaemic value after 10min reoxygenation. This indicates that the improved recovery by IB-MECA is not due to positive inotropy.

In conclusion, the adenosine A_3 agonist, IB-MECA protects atria from myocardial stunning induced by simulated ischaemia when added immediately at reoxygenation.

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140P THE EFFECT OF HO-1 INDUCTION ON THE POST-ISCHAEMIC RECOVERY OF THE ISOLATED RAT HEART

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HO-1, which can be induced by hemin (*Maines, M.D.,1997),* is reported to be involved in a number of situations where oxidative stress occurs, and has been demonstrated to be up-regulated upon cardiac reperfusion (*Maulik et al.,1996*). HO-1 is involved in the breakdown of haem resulting in the formation of bilirubin, which has antioxidant properties and CO, which is a potent vasodilator. Bilirubin has been suggested as the agent responsible for the protective role of HO-1 in ischaemia-reperfusion injury (*Clark et al.,2000).* In the present study, we have compared the effect of hemin pre-treatment on the recovery of cardiac function in the isolated rat heart under conditions of ischaemia-reperfusion using a constant flow perfusion sytem. Tin protoporphyrin, a known inhibitor of HO-1 (*Maines, MD 1981*) was also used to investigate the effect of HO-1 inhibition in this model.

Male Wistar rats (270-320g) were pre-treated with 75μmol/kg Hemin (24 hours) or 40μmol/kg tin protoporphyrin (1 hour) intraperitoneally, and anaesthetised with Euthatal (120mg/kg). Hearts were removed and perfused using the Langendorff technique with Krebs-Henseleit solution (118mM NaCl, 1.25mM $CaCl_2$, 1.2mM KH_2PO4, 2.8mM KCl, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, 11.1mM glucose) gassed with 95% O_2/5% CO_2 at a constant flow rate of 10ml/min. After a period of 30 minutes stabilisation, a 20 minute period of zero flow global ischaemia was introduced; this was followed by 30 minutes of reperfusion. Measurements recorded included left ventricular developed tension (LVDT), heart rate and coronary perfusion pressure (CPP). Tissue was then prepared for immunoblot analysis, which was carried out using a mouse anti-rat HO-1 antibody. Data are presented as mean±SEM; significant differences were calculated using ANOVA.

Immunoblot analysis determined that the dose of hemin used increased the expression of HO-1. Under pre-ischaemic conditions, a significant decrease in coronary perfusion pressure was observed in the hemin-treated rats compared to control (116±9 mmHg with hemin compared to 141±3 mmHg for control; $p<0.05$, n=5), indicating a vasodilatory component. This effect was abrogated using the inhibitor tin protoporphyrin, where hemin and inhibitor increased the CPP to 131 ± 10mmHg. After ischaemic injury, hemin pre-treatment significantly increased recovery of contractility (recovery to 45±8% of initial levels in hemin-treated rats, compared to 14 ± 4% for control, where $p<0.05$ n=5) and a significant reduction in CPP compared to control from 2-30 minutes post-ischaemia ($p<0.05$, n=5). The addition of tin protoporphyrin partially reduced the recovery of contractility to 27±13% (compared to 7±3% in rats treated with inhibitor alone), and attenuated the post-ischaemic effect of hemin on CPP.

These data suggest that hemin pre-treatment results in an increased recovery from ischaemic injury. Further data obtained using a HO-1 inhibitor, and also immunoblot analysis, indicate that these effects may be due to the induction of HO-1 by hemin. The effects of hemin on CPP suggest a vasodilatory component, possibly via CO release.

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141P A HIGHLY INTERACTIVE COMPUTER ASSISTED LEARNING (CAL) PROGRAM TO TEACH BETTER EXPERIMENTAL DESIGN

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In excess of 50 million animals are used in biomedical research in the world each year (Festing, 1996). For both ethical and economic reasons it is important that research scientists use experimental animals efficiently and in the minimum numbers consistent with achieving the scientific objectives of the study. More effective experimental design could help to achieve a significant reduction in the number of animals used and, by improving the repeatability of animal experiments, could make alternative methods easier to validate. Here we describe a CAL program designed to teach scientists how to estimate the number of animals needed, the importance of uniformity, how to deal with variability and how to increase efficiency and therefore cost effectiveness of their experiments. It is aimed at all research scientists using experimental animals, but the principles of experimental design are applicable to most areas of biological and medical research. Briefly the content covers:

Introduction - highlights poor design in a significant proportion of animal experiments and consequent unnecessary use of animals. The section engages the user with data from a simple experiment to highlight design flaws.

Choice of Animal Model - explores the use different strains (inbred and outbred stock) and covers the various types of animal model (predictive, explanatory, exploratory).

The Experimental Unit - the importance and critical nature are explained by using interactive examples.

Eliminating Bias – uses interactive examples to explain the techniques which may be employed to remove systematic differences between treatment groups and reduce bias.

Applying Valid Statistics - covers the application of valid statistical tests to your data, the use of statistical tests to compare two groups, parametric versus non-parametric tests, assumptions underlying parametric tests, and the way in which they can be examined. Explores the definition of hypotheses, choices of statistical tests, interpretation of P. Introduction to the analysis of variance (ANOVA).

Improving Precision - making experiments more precise so that we can detect treatment differences. Ways of achieving this - ensuring uniformity, use of randomised blocking as a way of minimising heterogeneity, using power analysis and the resource equation method.

Increasing the Range of Applicability - using resources effectively to enable interpretation of findings over a wider range e.g. different treatments, different strains, sexes, sizes. Use of multi-factorial design.

Planning and Organising - key issues in designing and analysing effective (simple) experiments.

Self-Assessment Activity - series of case studies and true/false questions with feedback to self-assess your understanding.

Software Tools & References – other information resources.

The program requires a PC with a minimum specification of 166 MHz Intel Pentium II processor, Windows 95/98/NT4, 32 Mb RAM, 16 bit colour graphics.

Festing (1996) *Statistics in Toxicology* (Morgan B. J. T., ed), pp. 3-11. Clarendon Press, Oxford, ISBN 0 19 8523297.