305P NON-SELECTIVE EFFECTS OF THE PUTATIVE PHOSPHOLIPASE C INHIBITOR, U73122, ON ADENOSINE A_1-RECEPTOR-MEDIATED SIGNAL TRANSDUCTION EVENTS IN CHO-K1 CELLS

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It has been suggested that the aminosteroid U73122 acts as an inhibitor of phospholipase C (PLC, Bleasdale *et al.*, 1990). In this study we have compared the ability of U73122 to inhibit adenosine A_1-receptor mediated phosphatidylinositide (PI) hydrolysis and inhibition of adenylyl cyclase in CHO-K1 (CHO-A1) cells expressing the human A_1-receptor. The sensitivity of the histamine H_1-receptor stimulated PI response, to U73122, in CHO-K1 (CHO-H1) cells, was also determined for comparison.

Accumulation of [^{3}H]-cyclic AMP and total [^{3}H]-inositol phosphates ([^{3}H]-IP) in cell monolayers, and agonist stimulated [^{35}S]-GTPγS binding to cell membranes were measured essentially as previously described (Megson *et al.*, 1995, Clark and Hill 1996). Data, presented as the mean ± standard error of triplicate values from ≥3 experiments, were analysed by two-way ANOVA and then by post-hoc Newman-Keuls tests.

U73122 (30 min pre-treatment), but not its inactive analogue U73343, inhibited both basal and N^6-cyclopentyladenosine (CPA, 1μM) stimulated levels of total [^{3}H]-IP accumulation in CHO-A1 cells (Table 1). The same pattern was observed for inhibition of histamine (300nM) stimulated PI responses in CHO-H1 cells. However, U73122, also reversed the CPA- (1μM) inhibition of forskolin (FSK, 3μM) stimulated cyclic AMP accumulation in CHO-A1 cells, over the same concentration range, despite raising the FSK response alone (Table 1). U73122 also reduced both basal and agonist stimulated [^{35}S]-GTPγS binding in this cell-line (Table 1). The inactive analogue U73343 also decreased [^{35}S]-GTPγS binding slightly. (For example, at 15μM U77343, CPA (10μM) stimulated [^{35}S]-GTPγS binding was reduced from 148 ± 3 to 134 ± 2% of control basal levels, $p<0.05$, *cf* Table 1).

Overall, it is clear that U73122 is not acting as a selective inhibitor of PLC and some of its effects might be mediated at the level of G-proteins or receptor-ligand interactions.

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Table 1 Effect of U73122 on Adenosine A_1 Receptor Mediated Signal Transduction Events

	PI Response (% Cont. Basal)		Cyclic AMP Response (% Control FSK)		[^{35}S]GTPγS Binding (% Cont. Basal)	
U73122 (μM)	Basal	1μM CPA	3μM FSK	3μM FSK+1μM CPA	Basal	10 μM CPA
0.0	100	173 ± 8	100	20 ± 1	100	148 ± 3
1.0	96 ± 6	155 ± 6*	97 ± 3	27 ± 3	81 ± 2*	92 ± 2*
4.0	72 ± 3*	86 ± 3*	126 ± 4*	51 ± 4*	74 ± 2*	84 ± 2*
10.0	58 ± 4*	59 ± 4*	160 ± 5*	125 ± 5*	72 ± 2*	65 ± 2*
15.0	54 ± 3*	54 ± 3*	151 ± 3*	100 ± 3*	68 ± 2*	70 ± 2*

* $p<0.05$ with respect to control (in absence of U73122); 2-way ANOVA (Newman Keuls).

306P POTENTIATION OF SODIUM NITROPRUSSIDE-STIMULATED CYCLIC GMP FORMATION IN NG108-15 CELLS IN THE PRESENCE OF CYCLIC AMP STIMULI

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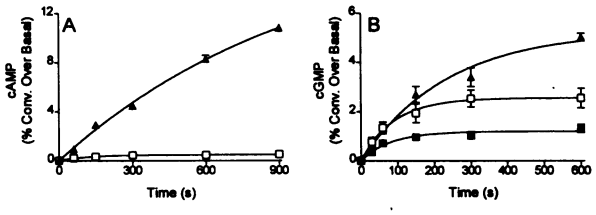
We observed previously in the guinea-pig cerebellum, that agents which couple to elevation of cAMP (forskolin or the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine, NECA) enhance basal and sodium nitroprusside (SNP)-elevated cGMP levels (Hernández *et al.*, 1994). However, this effect was not observed with dibutyryl-cAMP, suggesting the lack of involvement of protein kinase A in this phenomenon. In the present study, we have investigated the effects of forskolin, NECA, and dibutyryl cyclic AMP on cGMP accumulation in the neuroblastoma-glioma hybrid cell line, NG108-15.

NG108-15 cells were cultured in 24 well cluster dishes (DMEM + 2 mM glutamine, 6% fetal calf serum, 100 μM hypoxanthine, 1 μM aminopterin and 16 μM thimidine). Cells were incubated with [^{3}H]-guanosine or [^{3}H]-adenine for 2 hours, washed and the accumulation of [^{3}H]-cGMP or [^{3}H]-cAMP was carried out at 37°C in the presence of adenosine deaminase (1 unit mL^{-1}) as previously described (Neil *et al.*, 1996). Results were expressed as a percentage conversion from the total [^{3}H]-guanine or [^{3}H]-adenine nucleotides with basal levels subtracted, from experiments carried out on at least 3 separate occasions.

Basal accumulations of [^{3}H]-cAMP and [^{3}H]-cGMP were 0.38 ± 0.03 and 0.29 ± 0.03 % conversion, respectively. cAMP accumulation was stimulated in the presence of either 30 μM forskolin or 10 μM NECA (Figure 1A). The half times of accumulation were 680 and 82 s, respectively, with maximal responses of 17.9 ± 2.1 and 0.48 ± 0.02 % conversion, respectively). cGMP accumulation was stimulated in the presence of 1 mM SNP, and with combinations of SNP plus forskolin or NECA with estimated half-times of 56, 160 and 60s, respectively, and maximal responses of 1.20 ± 0.08, 5.27 ± 0.43 and 2.55 ± 0.07 % conversion, respectively. At 600 s, the presence of forskolin or NECA significantly enhanced the SNP cGMP response (1.32 ± 0.17, 4.36 ± 0.45, 2.38 ± 0.37 % conversion, $P<0.01$). Forskolin had no effect on cGMP accumulation in the absence of SNP. The response to SNP was not significantly altered in the presence of 100 μM dibutyryl-cAMP (estimated half-time 42 s; maximal response 1.10 ± 0.08 % conversion ($p>0.05$ after 600s)).

Figure 1. **A.** Time course for cAMP production in the presence of forskolin (▲) and NECA (□). **B.** Time course for cGMP production in the presence of SNP (■), forskolin (▲) and NECA(□). Curves were fitted to a one phase exponential association model (GraphPad Prism 2.01).



These results suggest a causal linkage between cAMP generation and the enhanced accumulation of cGMP. The lack of effect of dibutyryl-cAMP infers that cAMP but not cAMP-dependent protein kinase activity mediates this cross-talk.

KEN is a BBSRC Glaxo Wellcome CASE Student.

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307P SIGNAL TRANSDUCTION CHARACTERISTICS OF CULTURED SMOOTH MUSCLE CELLS OF THE HUMAN UTERINE ARTERY MAINTAINED IN A LOW (12.5%) OXYGEN ENVIRONMENT

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The majority of studies investigating the characteristics of cultured smooth muscle cells taken from the human uterine artery (Lynn & Gillespie, 1995; Schoeffter *et al.*, 1996) have been performed in oxygen concentrations in excess of that to which they would normally be exposed in situ (pO_2 circa 95mmHg; Williams, 1989). Changes in the oxygen level surrounding cells has been shown to induce a number of changes in the characteristics of those cells, including changes in membrane-dependant functions (Bhat & Block, 1992). This study therefore evaluates the phosphoinositide responses of human cultured uterine artery smooth muscle cells established and maintained in an atmosphere which mimics that in situ (12.5% O_2) compared to those grown in ambient oxygen.

Human uterine artery smooth muscle cells (HUTASM) were obtained from tissue taken at the time of abdominal hysterectomy. Briefly the tissue was chopped into $1mm^3$ pieces and agitated in 2mg/ml Type II collagenase at 37°C for 3 hours. The digested material was titruated then passed through a 120µm filter, centrifuged and the cells resuspended in D-val DMEM containing 25mM HEPES, 10% foetal calf serum, 2mM and antibiotics. Cells were then grown in either a 21% O_2 (HUTASM21) or a 12.5% O_2 (HUTASM12.5) environment maintained at a humidified 37°C with 5% CO_2. All experiments using HUTASM12.5 were performed in an air-tight glove box providing an atmosphere of 12.5% O_2 by displacement with nitrogen gas. Media for HUTASM12.5 was pre-gassed in a 12.5%O_2 environment for several hours before use. Accumulation of ^{3}H-inositol phosphates was measured on confluent cell monolayers as described previously in detail (Hawley *et al.*, 1995). Identity of the smooth muscle cells was confirmed by immunocytochemical analysis using an antibody to alpha smooth muscle actin and myosin.

Both HUTASM12.5 and HUTASM21 exhibited the typical 'hill and valley' morphology of smooth muscle cells for at least 10 passages. Phosphoinositide responses to 1mM carbachol, 1mM 5-hydroxytryptamine and 1µM bradykinin were of a similar magnitude in both HUTASM12.5 and HUTASM21. The response to 1mM histamine was found to be 6.2±0.9 fold over basal levels in HUTASM12.5 (n=6) which was significantly different to the response in HUTASM21 (13.1±2.4; n=6; $p<0.05$). Further investigation revealed that the EC_{50} values for histamine obtained in HUTASM12.5 and HUTASM21 were similar (5.25±0.04 and 5.17±0.05 respectively). In addition, apparent K_D values for a number of histamine H_1-receptor antagonists were found to be similar in both cell types. However the time course of the response to 50µM histamine was much more rapid in HUTASM21 when compared to the time course of the response in HUTASM12.5.

The results of this study demonstrate that the smooth muscle cells of the human uterine artery can be grown in culture at an oxygen tension which mimics that *in situ*. Furthermore these cells retain their ability to increase phosphoinositide turnover in response to several agonists but show some differences in the size and the time course of the response.

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308P INVOLVEMENT OF PROTEIN KINASE C ISOFORMS IN AGONIST-MEDIATED INOSITOL PHOSPHATE ACCUMULATION IN CHO-K1 CELLS EXPRESSING RECOMBINANT HUMAN SOMATOSTATIN sst_5 RECEPTORS

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Numerous studies have implicated protein kinase C (PKC) in the modulation of phospholipase C (PLC)-linked responses but little is known about the individual PKC isoforms involved or which are stimulated by agonist-induced receptor activation (e.g., see Willars *et al.*, 1996). We have examined the ability of PKC to modulate PLC activity stimulated by somatostatin (SRIF) in CHO-K1 cells, stably expressing recombinant human SRIF sst_5 receptors ($CHOsst_5$ cells), and compare this with activation by UTP, which acts at endogenous $P2Y_2$ purinoceptors (Wilkinson *et al.*, 1996).

Total [^{3}H]-inositol phosphate ([^{3}H]InsPx) accumulation was measured in $CHOsst_5$ cells, grown to confluence in 24-well multiplates (approx. 100µg protein $well^{-1}$), which were loaded for 24h with [^{3}H]*myo*-inositol, as an index of PLC activity. PKC was activated with phorbol, 12-myristate, 13-acetate (PMA) or inhibited by the non-selective inhibitor, Ro31-8220, (Davies *et al*, 1989) or the Ca^{2+}-sensitive PKC isoform selective inhibitor, Go6976 (Martiny-Baron *et al*, 1993). PKC isoforms were detected by Western blotting using specific antisera. Translocation of PKC isoforms to the membrane, which is indicative of their activation, was determined by Western blotting of cytosolic and particulate fractions, separated by centrifugation (17,000 x *g* for 10min).

Western blots of $CHOsst_5$ cell lysates, under basal conditions, indicated the presence of PKC γ (Ca^{2+}-sensitive), δ, ε, ι, γ and ζ (all Ca^{2+}-insensitive) (see, Nishizuka, 1995). PKC γ was largely associated with the particulate fraction, whereas the others were cytosolic. PMA (1µM, 10min), resulted in the translocation of PKC δ, ε and µ, whilst SRIF (1µM) and UTP (100µM) caused translocation of PKC δ and ε.

Following a 5min incubation, SRIF (1µM) and UTP (100µM) increased [^{3}H]InsPx accumulation from basal values of 1160 ± 68 and 1190 ± 155 to 4001 ± 183 and 3696 ± 148, respectively, (values are in dpm and are the mean ± s.e.mean, n = 4). PMA (1µM,10min pretreatment), decreased SRIF- and UTP-induced increases in [^{3}H]InsPx accumulation by 42 ± 8% (n = 6) and 72 ± 5% (n = 4), respectively, whilst Ro 31-8220 (10µM, 10min pretreatment) induced a small, but significant ($p < 0.05$) augmentation of responses to SRIF (30 ± 13%, n = 6), but not UTP (16 ± 9.5%, n = 4). Go6976 (10µM, 10min pretreatment) had no effect on responses to either SRIF or UTP. Preincubation with PMA resulted in a small, but significant, reduction in basal [^{3}H]InsPx accumulation of 16.2+5.2% ($p < 0.05$, n = 6), whereas neither Ro31-8220 or G06976 had any effect. The ability of PMA to inhibit SRIF- and UTP-induced [^{3}H]InsPx accumulation could be abolished by co-incubation with Ro 31-8220, but not with Go6976.

The ability of Ro31-8220 but not Go6976, to augment SRIF-induced increases in [^{3}H]InsPx accumulation, suggests the presence of a tonically active PKC-dependent inhibitory feedback loop on the sst_5 receptor, when expressed in CHO-K1 cells, which is mediated by Ca^{2+}-insensitive PKC-isoforms. Activation of PKC with PMA, mimics this putative inhibitory feedback loop, similarly involving stimulation of Ca^{2+}-insensitive isoforms. These conclusions are supported by Western blotting which shows the translocation of Ca^{2+}-insensitive PKC isoforms by both agonist and PMA.

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309P CHARACTERISATION OF THE SOMATOSTATIN RECEPTORS ON HUMAN PARIETAL DERIVED HGT-1 CELLS

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In rat isolated mucosa, the somatostatin (SRIF) receptor mediating inhibition of acid secretion, at the level of the parietal cell, is similar to the human recombinant sst_2 receptor (Wyatt *et al.*, 1995). The aim of this study was to pharmacologically characterise the sst receptor previously reported in the human parietal cell line HGT-1 and which is negatively coupled to adenylyl cyclase (Reyl-Desmars *et al.*, 1986).

HGT-1 cell membranes were prepared in buffer containing; 5mM Hepes, 5mM Hepes Na^+, 0.1mM EDTA, 5mM $MgCl_2$, 0.2mg ml^{-1} bacitracin and 1μg ml^{-1} soya bean trypsin inhibitor at pH 7.4. Membranes were also prepared after 24hr incubation with 100ng ml^{-1} pertussis toxin. HGT-1 cell membranes (20μg protein) were incubated with either [^{125}I]-Tyr^{11}-SRIF (0.03nM) for 2hrs or [^{125}I]-BIM-23027 (0.1nM) for 1hr at 37°C. Non-specific binding was defined by 1μM SRIF. Samples were filtered over GF/C filters pre-soaked with 1% polyethyleneimine and washed with ice-cold water. Data from competition studies using a range of SRIF analogues (see Raynor *et al.*, 1993) were best fitted to a one site competition curve (GraphPad Prism). All values are mean ± s.e.mean of at least 3 experiments.

[^{125}I]-Tyr^{11}-SRIF binding reach steady state within 2hrs. The estimated B_{max} was 41.6±12.5 fmol mg^{-1} protein, with a K_d of 0.06±0.01 nM. The selective sst_2 agonist, BIM-23027, inhibited specific [^{125}I]-Tyr^{11}-SRIF binding by 89.9±1.1%. These BIM-23027 sensitive SRIF-binding sites were further characterized using [^{125}I]-BIM-23027 (McKeen *et al.*, 1996). SRIF and a range of SRIF analogues (Raynor *et al.*, 1993) inhibited specific [^{125}I]-BIM-23027 binding (Table 1). At the highest concentrations studied all ligands abolished binding.

Table 1: Inhibition of [^{125}I]-BIM-23027 binding in HGT-1 cells by a range of SRIF ligands

Ligand	pIC_{50}	n_H
SRIF	9.61 ± 0.02	1.14 ± 0.14
SRIF-28	9.79 ± 0.04	1.16 ± 0.06
BIM-23027	9.97 ± 0.03	1.09 ± 0.06
MK678	9.80 ± 0.04	1.07 ± 0.05
Octreotide	9.45 ± 0.09	1.03 ± 0.06
BIM-23055	7.06 ± 0.02	1.14 ± 0.09
BIM-23056	6.76 ± 0.03	0.92 ± 0.05
L362-855	9.11 ± 0.05	0.97 ± 0.07

Specific [^{125}I]-BIM-23027 binding could also be inhibited by 120mM Na^+ (45.3±1.5%), 10μM GTPγS (51.3±2.6%), 120mM Na^+ and 10μM GTPγS (85.6±0.8%) and 100ng ml^{-1} pertussis toxin (95.4±0.8).

The affinities of the ligands at inhibiting specific [^{125}I]-BIM-23027 binding is similar to that seen when human recombinant sst_2 receptors are expressed in Ltk$^-$ cells (McKeen *et al.*, 1996). These findings, together with the ability of Na^+, GTPγS and pertussis toxin to inhibit specific [^{125}I]-BIM-23027 binding, suggest that the majority of SRIF binding sites in the human cell line HGT-1 are similar to the sst_2 receptor. The identity of the small proportion of BIM-23027-insensitive SRIF binding sites in HGT-1 cells and their functional significance remains to be determined.

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310P A SPECIES DIFFERENCE IN THE FUNCTIONAL ANTAGONISM OF THE NEUROMEDIN B-PREFERRING (BB_1) RECEPTOR WITH PD165929

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The study of the functional role(s) of the BB_1 receptor has been hampered by the lack of selective, high affinity antagonists. However, recent reports have described BIM23127, a cyclo-somatostatin octapeptide analogue, as a BB_1-antagonist, with an affinity of 21nM in the rat (Orbuch et al. 1993; Ladenheim et al. 1994). Therefore, in the present study we have compared the functional antagonism of BIM23127 with that of the novel compound PD165929 (2-[3-(2,6-Diisopropyl-phenyl)-ureido]-3-(1H-indol-3-yl)-2-methyl-N-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide) at both the human and rat BB_1 receptor.

Activity at the human BB_1 receptor was assessed using CHO cells expressing the cloned human BB_1 receptor (CHO-BB_1) in a Cytosensor microphysiometer. The cells were seeded into Cytosensor cups (~$0.6x10^6$ cells per cup) and perfused at 120μl min^{-1} with bicarbonate-free Hams F-12 (pH 7.4), with the acidification rate being measured every 2min. Neuromedin B (NMB, 1pM-1μM) was serially added to the perfusate in the presence or absence of BIM23127 (10nM-3μM) or PD165929 (0.1-1μM). The peak responses were normalised to that evoked by 3μM UTP, which activates constitutive P_{2U} receptors.

Activity at the rat BB_1 receptor was assessed using a rat oesophagus preparation. The muscularis mucosa was dissected from Wistar rats, halved longitudinally and placed into separate organ baths containing Krebs solution. Contraction of the preparation was measured using an isometric transducer. One half of the oesophagus was exposed to increasing concentrations of NMB (10pM-1μM) in the presence or absence of BIM23127 (0.3-3μM) or PD165929 (3-10μM), whilst the other half was exposed to NMB alone. All data are presented as mean±s.e.mean unless otherwise stated.

NMB caused a concentration-dependent increase in the acidification rate of CHO-BB_1 cells, with an EC_{50} of 0.41nM (range 0.28-0.55nM, n=8). This response was antagonised by PD165929 (0.1-1μM) in a concentration-related, surmountable manner, with an apparent pK_B of 7.83±0.11 (n=3). Low concentrations (10 or 100nM) of BIM23127 had no effect on the NMB-induced acidification response (n=2), but higher concentrations (1 or 3μM) antagonised the response in a surmountable manner, with an apparent pK_B of 6.27±0.18 (n=4).

NMB also caused a concentration-dependent contaction of the rat oesophagus, with an EC_{50} of 3.8nM (range 2.8-4.0nM, n=5). This response was antagonised by BIM23127 in a surmountable manner, with an apparent pK_B of 5.8±0.25 (n=3). However, PD165929, even at doses of 10μM, failed to antagonise the NMB-induced response in the rat (n=3).

These data clearly demonstrate that the novel compound PD165929 is a high affinity competitive antagonist at the human BB_1 receptor. Furthermore, this compound compares very favourably with the reported BB_1 antagonist, BIM23127 (Orbuch et al. 1993, Ladenheim et al. 1994), having ~50-fold higher functional activity. However, PD165929 was inactive at the rat BB_1 receptor, suggesting that the functional antagonism of the BB_1 receptor with this compound is species dependent. Indeed, BIM23127 also had a slightly lower functional affinity at the rat BB_1 receptor. Moreover, it is worth noting that the pK_B for functional antagonism of BIM23127 was ~100 fold less than the previously reported K_i for binding at the rat receptor (Ladenheim et al. 1994), implying BIM23127 has a high affinity for, but low functional antagonism at, the BB_1 receptor.

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311P ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE BY TUMOUR NECROSIS FACTOR-α AND HYDROGEN PEROXIDE IN ENDOTHELIAL CELLS

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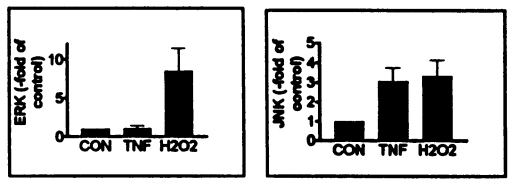
The inflammatory cytokine, tumour necrosis factor α (TNFα), and reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), have been implicated in the pathogenesis of atherosclerosis (Collins, 1993). The mitogen-activated protein kinase (MAPK) family are known to mediate intracellular signalling that control many cell functions, such as proliferation and differentiation (Cano and Mahadevan, 1995). TNFα and H_2O_2 have been shown to activate members of the MAPK family (Westwick et al., 1994, Abe et al., 1994). We have examined the activation of extracellular-signal regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK, also called stress activated protein kinase, SAPK), members of the MAPK family, following stimulation of Ea.hy 926 cells (a human endothelial hybridoma cell line) with TNFα and H_2O_2. The ERK and JNK activation following H_2O_2 stimulation was further investigated.

Confluent Ea.hy 926 cells were kept in serum-free medium over night prior to experiment. Cells were exposed to agonists, and subsequently lysed. Where indicated, cells were preincubated with sodium benzoate (40 mM), mannitol (40 mM), catalase (160 U/ml), or N-acetyl cysteine (NAC, 40 mM) for 60 min prior to addition of H_2O_2. Cell extracts were analysed for kinase activity by the in-gel kinase assay (Kameshita and Fujisawa, 1989), using myelin basic protein (MBP) for the ERK assay, and recombinant GST-c-Jun (aminoacids 1-135) as a substrate for the JNK assay. Autoradiographs of the dried gels were analysed by optical densitometry of the appropriate bands (in ERK assay, p44 ERK1 and p42 ERK2, and in JNK assay, p54 JNK and p46 JNK).

It was found that H_2O_2 activates both ERK and JNK after 30 min. exposure, with optimal activation at 4 mM. In contrast, TNFα (10 ng/ml, 30 min.), did not activate the ERKs, but potently activated the JNKs (See Fig.1. Data are presented relative to control values, mean±s.e. mean, $n=3$).

Fig.1(a) ERK-activity Fig.1(b) JNK-activity



Treatment of cells with antioxidants, such as sodium benzoate or mannitol, followed by exposure of cells to H_2O_2, did not inhibit ERK or JNK activation. However, exposure of cells to catalase or NAC inhibited H_2O_2-induced activation of both JNK (catalase: 98.1%, NAC: 84.5%, $n=2$) and ERK (catalase: 100%, NAC: 75.2%, $n=2$). None of these agonists affected ERK or JNK activity in the absence of H_2O_2.

Our data indicate that TNFα and H_2O_2 may differentially activate members of the MAPK family. ERK and JNK activation following exposure of cells to H_2O_2 was not sensitive to scavengers of hydroxyl radical. Rather, kinase activation was inhibited by agonists which preferentially act on H_2O_2 itself, indicating that H_2O_2 rather than subsequently formed free radicals, such as hydroxyl radical, activate ERK and JNK in these cells.

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312P UPREGULATION OF MONOCYTE-ENDOTHELIAL CELL ADHESION IN RESPONSE TO OXIDISED LDL IS INDEPENDENT OF NF-κB

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Oxidatively modified low density lipoprotein (oxLDL) appears to be an important mediator in the pathogenesis of atherosclerosis. For example oxLDL preferentially accumulates at sites of the vascular wall predisposed to atherosclerosis. Additionally oxLDL exhibits a number of pro-atherogenic properties *in-vitro* (Steinberg *et al.*,1989). It is chemotactic to monocytes, can inhibit endothelium dependent relaxation, and induces smooth muscle cell proliferation. However, the molecular mechanism(s) of increased monocyte adhesion in response to oxLDL remains unclear. In the present study we have investigated the role of the stress response transcription factor NF-κB in the oxLDL-mediated regulation of endothelial-monocyte interactions in the human endothelial cell line EA.hy 926. NF-κB resides in the cytosol bound to an inhibitor protein, IκB. Following activation, NF-κB dissociates from IκB (which is degraded) and is translocated to the nucleus. To study monocyte adhesion, we used a myeloperoxidase based 96 well plate assay (Dwivedi *et al.*,1996). Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression was measured by ELISA. Degradation of IκB was determined by western blotting using specific anti-IκB antibody. LDL was prepared by ultracentrifugation of human plasma and was oxidised by incubating LDL (1mg/ml protein) in phosphate buffered saline at 37°C, with 50μM $CuSO_4$ for 18-20h. Oxidation of LDL was measured by the ferrous oxidation/xylenol orange method (FOX) assay.

In response to oxLDL (100μg/ml protein), monocyte adhesion to EA.hy 926 cells increased 1.55±0.08 fold and 1.7±0.02 fold after 6 and 24h respectively (n=6). Normal LDL (100μg/ml protein) had no effect on monocyte adhesion after 6 and 24h (n=6). Adhesion of monocytes in response to oxLDL increases in a dose (0-100μg/ml) (fig.1a; n=6) and time-dependent manner up to 24h (fig.1b; n=3). Immunoblotting of a cytosolic extract, prepared after 15 min incubation with tumor necrosis factor α (TNFα, 10ng/ml), a well characterised inducer of adhesion and NF-κB, or oxLDL (50μg/ml), indicated degradation of IκB by TNFα, but not by oxLDL (fig.2; n=3). oxLDL had no effect on the TNFα induced IκB degradation. Incubation of EA.hy 926 cells with oxLDL for 6 and 24h had no effect on the expression of ICAM-1 and VCAM-1 (n=6).

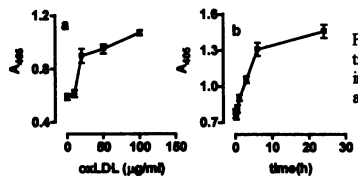

Figure 1. (a) Dose-response and (b) time-dependent adhesion of monocytes in response to oxLDL. Data is expressed as mean ± s.e.mean.

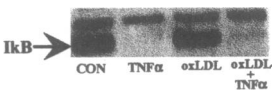

Figure 2. Degradation of IkB following treatment with oxLDL or TNFα.

Our data suggest that increased monocyte adhesion in response to oxLDL may occur independently of NF-κB activation. Since, ICAM-1 and VCAM-1 are not upregulated in response to oxLDL, we suggest that other cell surface adhesion receptor(s) may be responsible for monocyte adhesion under these conditions.

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313P EFFECT OF ONO-1505, A NOVEL PHOSPHODIESTERASE TYPE V INHIBITOR, ON VASCULAR SMOOTH MUSCLE CELL PROLIFERATION *IN VITRO*

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Vascular smooth muscle cell (VSMC) dysfunction, characterised by impaired vasodilator responses, hyperplasia and uncontrolled production of extracellular matrix, is a key feature of atherosclerosis. Phosphodiesterase (PDE) activity plays an important role in regulating intracellular concentrations of cyclic AMP and cyclic GMP. PDE V is specific for cyclic GMP and is predominantly located in VSMC and platelets. Thus inhibitors of PDE V are able to relax smooth muscle and inhibit platelet aggregation by enhancing intracellular cyclic GMP levels. In the current study we examined the effect of the novel PDE V inhibitor, 4-[2-(2-hydroxyethoxy)ethylamino]-2-(1*H*-imidazol-1-yl)-6-methoxyquinazoline (ONO-1505) on the proliferation of primary rat aortic smooth muscle cells (RASMC) *in vitro*.

RASMC (passage 1 to 4) were seeded at $2x10^4$ cells/cm^2 in 96 well plates in RPMI 1640 medium containing 10% new born calf serum (NBCS). Sub-confluent cells were growth-arrested in serum-free medium for 48h, and then incubated for 16h in medium containing ONO-1505 (3-100μM) or vehicle. Alternatively, cells were pre-treated for 24h with ONO-1505 and then 16h in medium. Cells were then labelled with [^{3}H]thymidine (18.5Bq/well, 74GBq/mmol) for 4h. After this time medium was removed and the cell layer washed with ice cold 5% trichloroacetic acid (TCA). The TCA-precipitable material was solubilised in 0.4ml 1M NaOH, and thymidine incorporation measured.

Figure 1 shows a dose-dependent inhibition of proliferation in RASMC by ONO-1505. In addition, pre-treatment for 24h followed by incubation with medium containing 2% NBCS also caused a marked inhibition of proliferation. ONO-1505 had no effect on cell viability as measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay which measures mitochondrial dehydrogenase activity in living cells.

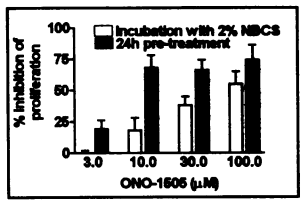

Figure 1. Inhibition of RASMC proliferaion by ONO-1505. The data are mean±s.e. mean (n=5). Drug effects are expressed as % inhibition of proliferaion, with 0% being control untreated cells.

These data indicate that ONO-1505 has anti-mitogenic properties. Pre-treatment with the drug was also effective, suggesting a build up from very low levels of cyclic GMP to a functionally effective concentration where inhibitory effects are apparent. Our data are consistant with a role for nitric oxide (NO), via guanylyl cyclase, produced in the vasculature, in maintaining VSMC in a non-proliferative phenotype, thus contributing towards vessel wall homeostasis. Other studies have shown that increased cyclic GMP inhibits the proliferative response to mitogenic stimulation (Assender *et al.*, 1992). Indeed Dubey *et al.* have suggested that this is the mechanism by which NO inhibits proliferation in these cells. In summary therefore inhibitors of PDE V may possess anti-proliferative properties by virtue of their ability to maintain high cyclic GMP levels (Marcoz *et al.*, 1993).

This work was funded by ONO Pharmaceutical Co., Ltd., Osaka Japan.

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314P POTENTIATION OF NO-DEPENDENT VASORELAXATION AND INHIBITION OF PLATELET AGGREGATION BY THE NOVEL PHOSPHODIESTERASE TYPE V INHIBITOR, ONO-1505, *IN VITRO*

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Phosphodiesterase (PDE) V inhibitors potentiate nitric oxide (NO)-dependent vasorelaxation and oppose platelet aggregation by enhancing intracellular levels of cyclic guanosine monophosphate (cGMP) (Mellion *et al.*, 1981; Harris *et al.*, 1989; Laight *et al.*, 1996). We assessed the effect of the novel PDE V inhibitor, 4-[2-(2-hydroxyethoxy)ethylamino]-2-(1*H*-imidazol-1-yl)-6-methoxyquinazoline (ONO-1505), on both sodium nitroprusside (SNP)-mediated vasorelaxation and inhibition of platelet aggregation *in vitro*.

Male Wistar rats (300-350 g) were anaesthetised with pentobarbitone sodium (60 mg/kg i.p.) and the carotid artery cannulated for exanguination. 10 ml blood was collected in sodium citrate (3.4 %) at a 9:1 ratio and centrifuged to provide platelet-rich plasma (PRP). PRP was stirred at 37 °C for 30 s. ONO-1505 (10 μM) and SNP (1-100 μM) were then added 2 and 1 min, respectively, before the aggregating agent, adenosine diphosphate (1 μM). Aggregation was assessed by the maximal fall in light transmission in a Born aggregometer. Rat thoracic aortic rings were prepared *post mortem* and mounted under isometric conditions in Krebs-Henseleit solution (composition in mM: NaCl 133; KCl 4.7; NaH_2PO_4 16.3; $MgSO_4$ 0.61; $CaCl_2$ 2.52; glucose 7.8) gassed with carbogen and warmed to 37 °C. Rings were treated with N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM) and ONO-1505 (0.3-10 μM) 20 and 10 min, respectively, before precontraction with noradrenaline (100 nM) and SNP (1-300 nM) added in a cumulative manner. Data are mean±s.e. mean. Comparisons were made using repeated measures ANOVA.

Vasorelaxation to SNP was potentiated by ONO-1505 (Table 1). In addition, ONO-1505 (10 μM) augmented established anti-aggregatory effects of SNP (10-100 μM) (Figure 1). Aggregation in the presence of ONO-1505 (10 μM) alone was 98.3±3.9 % of the control response (P>0.05, n=6).

Table 1. Effect of ONO-1505 on vasorelaxation to SNP in rat aorta. N=4.

ONO-1505 (μM)	SNP (pD_2)
Control	7.84±0.09
0.3	7.60±0.16
1	7.95±0.06
3	8.16±0.13*
10	8.63±0.13**

*P<0.05; **P<0.01 with respect to control

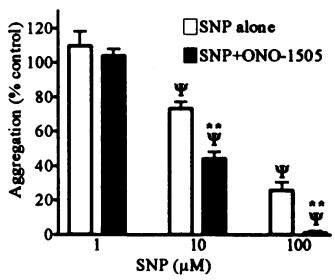

Figure 1. Effect of ONO-1505 (10 μM) on responses to SNP in aggregating rat platelets. Ψ P<0.01 with respect to control aggregation (≒100 %); ** P<0.01 with respect to SNP alone. N=6.

In conclusion, the ONO-1505-mediated potentiation of vasodilator and anti-aggregatory effects of SNP is consistent with an enhanced intracellular accumulation of cGMP in response to exogenous NO.

This work was supported by ONO Pharmaceutical Company, Ltd., Osaka, Japan.

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315P VASODEPRESSOR EFFECT OF THE NOVEL PHOSPHODIESTERASE TYPE V INHIBITOR, ONO-1505, IN THE RAT *IN VIVO*

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The novel phosphodiesterase (PDE) V inhibitor, 4-[2-(2-hydroxyethoxy)ethylamino]-2-(1*H*-imidazol-1-yl)-6-methoxyquinazoline (ONO-1505), relaxes precontracted vascular smooth muscle *in vitro* in an endothelium- and nitric oxide (NO)-dependent manner (Laight *et al.*, 1996). We have now characterised ONO-1505 as a vasodepresser agent *in vivo*.

Male Wistar rats (250-300 g) were anaesthetised with thiopentone sodium (100 mg/kg i.p.) and a carotid artery cannulated for the measurement of mean arterial pressure (MAP). A jugular and femoral vein were cannulated for the administration of drugs. Cumulative bolus doses of ONO-1505 (1-100 mg/kg i.v.) were administered following 30 min stabilisation and after an additional 30-40 min infusion with either sodium nitroprusside (SNP, 25 µg/kg/min i.v.) or phenylephrine (PEP, 0.5 µg/kg/min i.v.) to facilitate vasodepression to ONO-1505 (Merkel *et al.*, 1992). Vasodepression is expressed as the area over which MAP was depressed, representing both absolute falls in MAP (mm Hg) and the time (min) for which MAP was lowered (Merkel *et al.*, 1992). Data are mean±s.e. mean.

SNP (25 µg/kg/min i.v.) reduced basal MAP (138.5±7.9 mm Hg, n=9) by 60.2±2.3 mm Hg after 30 min; while PEP (0.5 µg/kg/min i.v.) raised basal MAP (142±10.2 mm Hg, n=7) by 47.0±5.6 mm Hg after 40 min. An early refractoriness to the transient vasodepressor effect of low cumulative doses of ONO-1505 was apparent during infusion with either SNP or PEP (Figure 1); but this was surmounted by higher cumulative doses such that ONO-1505 elicited graded and reversible vasodepression.

In conclusion, the vasodepressor activity of ONO-1505 is consistent with the accumulation of cyclic guanosine monophosphate within the vascular smooth muscle of resistance vessels, leading to vasodilation (Dundore *et al.*, 1993; Trapani *et al.*, 1991). This activity is promoted by stimulating guanylyl cyclase activity with an NO donor; or by inducing vasoconstrictor tone with an α_1-adrenoceptor agonist.

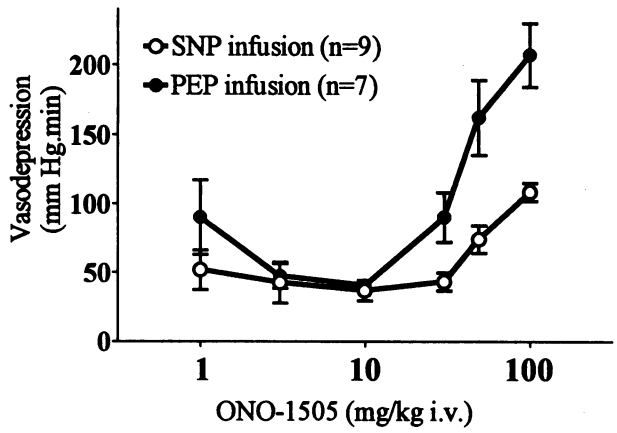

Figure 1. Vasodepressor effects of ONO-1505 in the anaesthetised rat during continuous infusion with either SNP or PEP.

This work was supported by ONO Pharmaceutical Company, Ltd., Osaka, Japan.

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316P PROTEASOME INHIBITORS BLOCK TUMOR NECROSIS FACTOR-α AND TRANSFORMING GROWTH FACTOR-β-STIMULATED ENDOTHELIN-1 SYNTHESIS IN BOVINE AORTIC ENDOTHELIAL CELLS

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The response of the endothelium to cytokines plays a pivotal role in co-ordinating inflammatory processes. Many of these events can be attenuated by inhibitors of proteasome-dependent signalling pathways (Read *et al.*, 1995). *In vitro* and *in vivo*, tumor necrosis factor-α (TNFα) stimulates the synthesis and secretion of endothelin-1 (ET-1) (Corder *et al.*, 1995; Klemm *et al.*, 1995). This has implications for a number of vascular pathologies and inflammatory conditions. Here we have tested the effects of proteasome inhibitors, calpain inhibitor 1 (CI1) and MG115 (Read *et al.*, 1995), on TNFα and transforming growth factor-ß (TGFß) stimulated ET-1 synthesis by cultured bovine aortic endothelial cells (BAEC).

The effects of CI1 (10 - 100 µM) and MG115 (3 - 100 µM) alone or in combination with TNFα (10 ng/ml) or TGFß (1 ng/ml) were studied in serum free DMEM using confluent cultures of BAEC (12 x 22 mm well plates). After 6 h incubation with inhibitor or cytokine the medium was collected and ET-1 release was measured by specific immunoassay (Corder *et al.*, 1995). Data as mean ± s.e. mean were compared by ANOVA with Fisher's LSD test (n = 12 - 20).

Mean basal release of ET-1 was 113 ± 4 fmol/well/6 h (n = 20). Both CI1 and MG115 significantly inhibited the ET-1 responses to TNFα and TGFß (Table 1).

These results provide the first evidence that ET-1 synthesis in endothelial cells is regulated by the proteasome pathway. Moreover, although TNFα-induced gene expression in many systems is blocked by CI1 or MG115, the effects of TGFß have not been previously linked to proteasome-dependent signalling pathways.

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Table 1 Effect of proteasome inhibitors on basal and TNFα or TGFß stimulated ET-1 release from BAEC
(values are % of control basal release, * P <0.01 compared to corresponding conditions without inhibitor present)

	basal	+ TNFα	basal	+ TGFß
control	100 ± 2.4	177.9 ± 5.5	100 ± 2.8	161.1 ± 9.4
10 µM CI1	71.2 ± 4.3*	127.7 ± 5.2*	106.8 ± 6.3	151.7 ± 9.4
30 µM CI1	65.6 ± 3.9*	100.8 ± 4.6*	95.1 ± 7.7	138.6 ± 9.6
100 µM CI1	56.1 ± 2.5*	68.1 ± 3.0*	82.9 ± 6.2	119.7 ± 9.6*
3µM MG115	75.6 ± 7.3*	98.4 ± 6.9*	92.9 ± 6.8	120.1 ± 5.6*
10µM MG115	67.8 ± 6.4*	84.4 ± 6.0*	84.3 ± 4.8	115.9 ± 13.4*
30µM MG115	64.4 ± 3.3*	71.8 ± 4.0*	76.1 ± 3.5*	105.8 ± 5.9*
100µM MG115	49.6 ± 4.4*	53.6 ± 3.1*	49.9 ± 3.5*	95.1 ± 9.5*

317P FORSKOLIN AND TUMOR NECROSIS FACTOR-α INCREASE ENDOTHELIN-2 SECRETION FROM HUMAN RENAL ADENOCARCINOMA (ACHN) CELLS

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Studies of the vasoconstrictor peptide endothelin-1 have shown its synthesis to be induced by proinflammatory cytokines such as tumor necrosis factor-α (TNFα) (Klemm *et al.*, 1995; Marsden & Brenner 1992). In comparison, the factors regulating the expression and release of endothelin-2 (ET-2) have yet to be identified. Here, we have investigated the effects of forskolin, an activator of adenylate cyclase, and TNFα on the ET-2-secreting human renal adenocarcinoma cell line (ACHN) (Ohkubo *et al.*, 1990). In addition, the roles of the two distinct TNF receptors, TNFR-p55 and TNFR-p75, have been evaluated using receptor selective TNF mutants.

ACHN cells were grown to confluence on 6 x 35 mm well plates in DMEM containing 10% foetal calf serum. TNFα (0.1 - 100 ng/ml), forskolin (0.1 - 30 μM) or the combination were incubated in serum free DMEM for 6 h. In a separate study, cells were stimulated with the TNFR-p55 selective mutant, R32W-S86T (0.6 - 200 ng/ml), the TNFR-p75 selective mutant, D143N-A145R (0.6 - 200 ng/ml) or both for 6 h (Loetscher *et al.*, 1993). Phosphoramidon (0.3 μ M) was included in the medium to inhibit proteolytic degradation of ET-2 immunoreactivity. Medium was acidified with acetic acid and extracted using disposable octadecasilylsilica columns. ET-2 was measured by radioimmunoassay using a rabbit antiserum raised against $ET_{[16-21]}$ (Corder *et al.*, 1993), with ET-2 as standard. RP-HPLC confirmed the secreted peptide as ET-2. Data are mean ± s.e. mean for triplicate evaluations in 3-5 separate experiments and were analysed by ANOVA with Fisher's LSD test.

The mean basal release of ET-2 was 21.8 ± 1.2 fmol/35 mm well/6 h. Forskolin and TNFα caused concentration-dependent increases in ET-2 release. At the highest concentrations tested, forskolin (30μM) and TNFα (100 ng/ml) increased ET-2 release by 202.2 ± 11.1% and 80.2 ± 11.6% compared to control release ($P<0.05$). Combination of forskolin with TNFα had a significantly greater effect than either alone (297.4 ± 18.3% increase compared to control, $P<0.05$). The TNFR-p55 selective mutant, R32W-S86T, caused a concentration-dependent increase in ET-2 secretion (20 ng/ml, 188.5 ± 14.0% of control, $P<0.05$), which was not significantly different when D143N-A145R was also present. D143N-A145R alone had no significant effect on ET-2 levels at any concentrations tested (200 ng/ml; 95.3 ± 7.9% of control release).

These data show that forskolin and TNFα cause a marked increase in ET-2 secretion from ACHN cells. The significantly greater response to the combination of these two agents suggests that there is an upregulation of two separate signalling pathways. The increased secretion of ET-2 after incubation with R32W-S86T demonstrates that the effects of TNFα are mediated by the TNF-p55 receptor in these cells.

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318P 3-AMINOBENZAMIDE REDUCES INFARCT SIZE IN A RABBIT MODEL OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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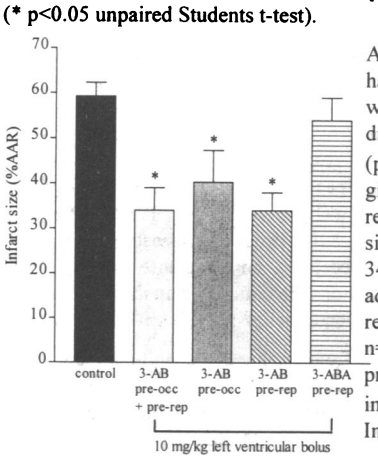
There is good evidence that reactive oxygen species contribute to reperfusion injury of the previously ischaemic myocardium (Bolli *et al.*, 1988). In cultured cells, radicals cause DNA strand breakage which triggers an energy-consuming repair process by activating the nuclear enzyme poly (ADP-ribose) synthetase (PARS). PARS transfers ADP-ribose from NAD resulting in a fall in the intracellular levels of NAD, and subsequently of ATP, which ultimately causes cell death (Schraufstatter *et al.*, 1986). PARS inhibitors, such as 3-aminobenzamide (3-AB) and nicotinamide, reverse these detrimental effects (Thies and Autor, 1991). We hypothesised that inhibition of PARS activity *in vivo* may improve cellular energy homeostasis in pathological conditions associated with oxidant stress. Here we investigate the effects of 3-AB on infarct size resulting from regional myocardial ischaemia and reperfusion in the anaesthetised rabbit.

Male New Zealand white rabbits (2.5-3.5 kg) were premedicated with Hypnorm (0.1 $ml \cdot kg^{-1}$, i.m.). General anaesthesia was induced (20 $mg \cdot kg^{-1}$, i.v.) and maintained with sodium pentobarbitone. The animals were ventilated with room air. A left intercostal thoracotomy was performed, and a ligature was placed around the first antero-lateral branch of the left coronary artery (LAL). Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded. The LAL was occluded for 45 min and reperfused for 2 h. 3-AB (10 $mg \cdot kg^{-1}$), 3-aminobenzoic acid (3-ABA, 10 $mg \cdot kg^{-1}$), or vehicle (saline) were injected into the left ventricle (i) 1 min prior to occlusion and 1 min prior to reperfusion, (ii) before occlusion only, or (iii) before reperfusion only. At the end of the experiment the LAL was reoccluded and Evans blue dye (2%w/v) was injected into the left ventricle for determination of area at risk (AAR). Infarct size was determined by incubation of the AAR with nitro-blue tetrazolium (0.5 $mg \cdot ml^{-1}$ for 20 min.). Values are expressed as mean ± s.e.mean (* $p<0.05$ unpaired Students t-test).



AAR (approx. 38%) and haemodynamic parameters were not significantly different between groups ($p>0.05$). 3-AB (10 $mg \cdot kg^{-1}$) given prior to occlusion and reperfusion reduced infarct size (from 59±3%, n=9; to 34±5%*, n=9), as did administration prior to reperfusion only (34±4%*, n=7), (see figure). 3-AB given prior to occlusion reduced infarct size to 40±7%* (n=5). In contrast, 3-ABA, which does not inhibit PARS activity, failed to reduce infarct size.

Thus, 3-aminobenzamide, but not 3-aminobenzoic acid, causes a reduction in infarct size in the anaesthetised rabbit, even when given prior to reperfusion of the ischaemic myocardium. The precise mechanism of the cardioprotective effect is unclear, but may involve inhibition of PARS activity and preservation of intracellular levels of ATP during reperfusion.

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319P 3-AMINOBENZAMIDE REDUCES INFARCT SIZE AND ATTENUATES DYSFUNCTION IN THE ISOLATED, PERFUSED HEART OF THE RABBIT

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Poly (ADP-ribose) synthetase (PARS) is a nuclear enzyme which is activated when strand breaks are introduced into DNA following oxidant stress. Activation of PARS stimulates an energy-consuming repair process which can result in cell death (Schraufstatter *et al.*, 1986). We have shown at this meeting that 3-aminobenzamide (3-AB), a PARS inhibitor, reduces the infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetised rabbit (Bowes and Thiemermann). Here we investigate the effects of 3-AB on infarct size and myocardial dysfunction caused by global ischaemia and reperfusion in the isolated, perfused heart of the rabbit.

Male New Zealand White rabbits (2.5-3.0 kg) were premedicated with Hypnorm (0.1 ml·kg^{-1}, i.m.) and anaesthetised with sodium pentobarbitone (20 mg·kg^{-1}, i.v.). After heparinisation (1000 U·kg^{-1}), the heart was rapidly excised and perfused (according to Langendorff) at constant flow (40 ml·min^{-1}) with oxygenated (95%O_2/5%CO_2) Krebs' solution (37°C) and paced via the right atrium (180 beats·min^{-1}). Coronary perfusion pressure (CPP), left ventricular end diastolic pressure (LVEDP) and left ventricular developed pressure (LVDP) were continuously measured. After 25 min equilibration, the hearts were subjected to no-flow global ischaemia (30 min) and reperfusion (R, 120 min). 3-AB (100 μM) or vehicle (Krebs' solution) were infused for the first 60 min of reperfusion. At the end of the experiment infarct size was determined by incubating slices of the left ventricle with nitro-blue tetrazolium (0.5 mg·ml^{-1} for 20 min.). Values are expressed as mean ± s.e.mean (* $p<0.05$, unpaired Students t-test).

		LVDP (mmHg)	LVEDP (mmHg)	CPP (mmHg)
Base	control (n=7)	77±3	6±1	44±1
	3-AB (n=6)	80±3	6±1	50±2
15 min R	control (n=7)	19±4	54±8	62±4
	3-AB (n=6)	32±6	23±5*	59±2
30 min R	control (n=7)	24±6	61±10	73±6
	3-AB (n=6)	45±8	28±7*	76±8
60 min R	control (n=7)	29±6	66±12	87±9
	3-AB (n=6)	54±8*	27±7*	96±13
120 min R	control (n=7)	31±6	79±18	112±12
	3-AB (n=6)	56±8*	29±11*	121±17

The baseline recordings for LVDP, LVEDP and CPP were not significantly different between groups ($p>0.05$). Global ischaemia and reperfusion resulted in a substantial rise in CPP, LVEDP and a 60% impairment in LVDP. Reperfusion of the heart with buffer containing 3-AB attenuated the rise in LVEDP and enhanced the recovery of LVDP during reperfusion of the heart (see table). In addition, 3-AB reduced infarct size from 48±9% (n=7) to 22±7%* (n=6)(of the left ventricle).

Thus, 3-AB reduces infarct size and contractile dysfunction caused by global ischaemia and reperfusion of the isolated, buffer-perfused heart of the rabbit. The cardioprotective effect of 3-AB is independent of alterations in myocardial blood flow or of the inhibition of the function of platelets or neutrophils.

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320P INHIBITION BY E-TYPE PROSTAGLANDINS OF THE FORMATION OF ENDOTHELIN-1 BY BOVINE ENDOTHELIAL CELLS

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Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor peptide produced by endothelial cells yet identified. E-type prostaglandins (PGE_0, PGE_1 and PGE_2) are potent vasodilators and inhibit platelet aggregation and neutrophil activation (Schrör et al., 1988). On the basis of their responses to various agonists and antagonists, E-type prostanoid receptors have been divided into four subtypes: EP_1, EP_2, EP_3 and EP_4 (Eglen &Whiting, 1989). Here, we investigate the effects of PGE_0, PGE_1, PGE_2 or sulprostone (selective agonist of prostanoid EP_1/EP_3 receptors) on the release of ET-1 from (i) basal, (ii) stretch-stimulated or (iii) tumour necrosis factor alpha (TNFα)-activated bovine aortic endothelial cells (BAEC).

Endothelial cells were isolated from bovine aortas and maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% foetal calf serum. Cells were then seeded onto either 6-well plates (stretch-induced ET-1 release) with flexible bottoms or 24-well plates (TNFα-induced ET-release) and grown to confluence. Twenty-four h prior to experiments, cells were serum-deprived. The stretching apparatus was located in an incubator without CO_2 gassing. The Flexcell strain unit consists of a baseplate connected to a computer controlled vacuum. To induce ET-1 release by stretching, wells of the culture plates were deformed with a pressure of -8 kPa (corresponding to 10% elongation) at a frequency of 1 Hz for 6 h, or in control experiments left unstretched. In addition, ET-1 release was also stimulated with TNFα (10 U ml^{-1}). To assess the effects of various prostaglandins on the release of ET-1 from BAEC stimulated with TNFα or by stretching, vehicle (saline), PGE_0, PGE_1, PGE_2 or sulprostone (all 1 and 100 nM, n=6) were added to the cells 15 min before start of the experiments. At 6 h, medium from individual wells was collected and the amounts of ET-1 determined by a specific radioimmunoassay (RIA). A Student's unpaired *t*-test was used. *$p<0.05$ indicates statistical significance.

Stretching of BAEC resulted within 6 h in a 2-fold increase in the production of ET-1 from 105±10 fmol ml^{-1} (unstretched control) to 211±24 fmol ml^{-1} (stretched). Pretreatment of the cells with PGE_0, PGE_1, PGE_2 or sulprostone caused a concentration-dependent inhibition of the stretched-induced increase in the production of ET-1 from BAEC. Interestingly, already the lowest concentration of the PGs used (1 nM), abolished the stretch-induced increase in the production of ET-1 (PGE_0: 120±21 fmol ml^{-1}; PGE_1: 99±21 fmol ml^{-1}: PGE_2:118±30 fmol ml^{-1}; sulprostone: 109±19 fmol ml^{-1}; $P<0.05$). Incubation of BAEC for 6 h with TNFα also resulted in a sinificant increase in the production of ET-1 from 44±3 fmol ml^{-1} to 231±21 fmol ml^{-1}, which was inhibited by PGs and sulprostone in a concentration-dependent manner (PGE_0: 163±13 and 149±17 fmol ml^{-1}; PGE_1: 170±10 and 146± fmol ml^{-1}; PGE_2: 158±16 and 137±11 fmol ml^{-1}; sulprostone: 165±14 and 149±11 fmol ml^{-1}; at 1 nM and 100 nM, respectively; $P<0.05$).

Thus, prostaglandins inhibit the release of ET-1 from BAEC by activation of EP_1/EP_3 prostanoid receptors. These results suggests that vasodilator PGs may play a critical role in regulating vascular tone and growth by reducing the production of ET-1.

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321P DIFFERENTIAL METABOLISM OF γ-LINOLENIC ACID AND THE COMPONENTS OF EVENING PRIMROSE OIL BY MCF7 HUMAN BREAST EPITHELIAL CELLS *IN VITRO*

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Evening primrose oil (EPO), which typically contains approximately 65-80% cis-linoleic acid (LA) and 10% γ-linolenic acid (GLA) (Gunstone 1992), is licensed for the treatment of cyclical and non-cyclical mastalgia. The active component is believed to be GLA which is known to be a precursor of arachidonic acid and ultimately prostaglandins (Horrobin 1993), although as yet there is no evidence to suggest that these actions are involved in the treatment of mastalgia. The aim of this study was to elucidate the metabolism of GLA and LA in breast cells.

Human breast epithelial carcinoma cells, MCF7, were cultured in DMEM-F12 1:1 mixture containing 10% FCS until just pre-confluent. The medium was then changed to phenol red-free DMEM containing 5% dextran charcoal stripped FCS for 48 h. to initiate differentiation after which the medium was supplemented with either EPO or GLA 3μl/100ml. After 12, 24, 48 and 72h. the fatty acid components of the cell monolayer and the supernatant medium were derivatised to fatty acid methyl esters using the method of Metcalfe and Schmitz (1961) and assayed by GLC. All procedures were performed on three separate occasions and results are expressed as mean ±s.e.mean.

Analysis of the fatty acid content of the cell culture medium after 72h. showed that supplementation with GLA resulted in up to a five-fold increase of GLA content. That medium with added EPO showed up to a four-fold increase in LA but no change in GLA content. Supplementation of the medium with either EPO or GLA lead to the appearance, with time, of two GLC peaks within the supernatant, one with a retention time less than the GLA and LA derivatives and one with a greater retention time.

Extraction of the fatty acids from the cell monolayer showed that treatment with EPO resulted in a significant increase, with time, in the cell content of both GLA and LA compared to control ($P<0.025$ & 0.01 respectively, ANOVA): GLA and LA concentrations reaching 164±29 and 127±5% of control respectively. Treatment with GLA however, resulted in a 41±6% decrease in the cellular content of GLA at 12h. rising to control values by 72h. ($P<0.025$, ANOVA). Supplementation with GLA also caused an increase in cellular content of LA, the LA content rising to 139±11% of control ($P<0.01$, ANOVA). With time, treatment with GLA resulted in the appearance of one, and EPO the appearance of two, additional GLC peaks, all with retention times less than those for GLA and LA.

The findings of increased GLA in those cells supplemented with EPO is not unexpected as LA is known normally to be metabolised to GLA by δ-6-saturase. The finding that administration of GLA caused an increase in the cellular content of LA, however, was unexpected. This suggests that the raised concentrations of GLA shifts the equilibrium such that GLA is converted to LA. With time, as the concentrations of LA increase, the equilibrium is restored and the concentrations of GLA return to control values. Both GLA and LA caused the appearance, in the supernatant, of products giving unidentified chromatographic peaks which may be long chain fatty acids. These results suggest that MCF7 human breast epithelial cells are able to utilise GLA and LA in the synthesis of longer chain metabolites but that the kinetics of the fatty acid metabolic cascade are such that the use of a mixture of fatty acids, for example in the form of EPO may be more likely to result in raised concentrations of GLA than the use of GLA alone.

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322P ANTIBODIES TO PDGF-BB INHIBIT FATTY STREAK DEVELOPMENT, BUT NOT NEOINTIMAL FORMATION AFTER BALLOON INJURY TO THE CAROTID ARTERY, IN CHOLESTEROL-FED RABBITS

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The importance of growth factors in the pathogenesis of atherosclerosis has been demonstrated by several studies. Some of these factors such as platelet derived growth factor (PDGF) are contained within platelets, and are also expressed by macrophages and cells of the vessel wall (Ross *et al.*, 1990; Rutherford *et al.*, 1996). In the current study we examined the role of the PDGF-BB isoform, using neutralising endogenous antibodies in; (a) the development of atherosclerotic lesion formation in aortae, and (b) neointimal development in the carotid artery following balloon injury, in cholesterol-fed rabbits.

3 month old male New Zealand white rabbits (approximately 2.5kg) were immunised with platelet cytosol (PLT), PDGF-BB, human serum albumin (HSA) or injected with Freund's adjuvant (non-immune (NI) control). Lesion formation was initiated by cholesterol feeding (2% w/w in the diet) for 3 months. Two weeks prior to killing the carotid artery was injured with a 2F Fogarty balloon catheter. Rabbits were killed by anaesthetic overdose and their arteries perfusion fixed with 4% paraformaldehyde. Sections of aorta and carotid artery were stained with haematoxylin and eosin and intimal and medial thickness determined by computerised morphometry. All data are expressed as mean±s.e. mean.

Cholesterol levels did not differ significantly between groups (range: 36.9±1.2-47.6±7.9; $p>0.05$). The antibodies raised were specific and neutralising as judged by western blotting, ELISA, chemotaxis and mitogenesis assays. Immunisation with PDGF-BB or PLT was associated with a significant inhibition in the percentage of aorta affected, compared to NI animals, as measured by the ratio of intimal:medial thickness (aortic I:M ratio) at the level of the first intercostal branch point (Fig.1a; NI n=5, HSA n=4, PDGF n=5, PLT n=7). Analysis of intimal:medial thickness ratio in the carotid artery (carotid I:M ratio) revealed no significant difference between all four groups (Fig.1b; NI n=6, HSA n=7, PDGF n=6, PLT n=8).

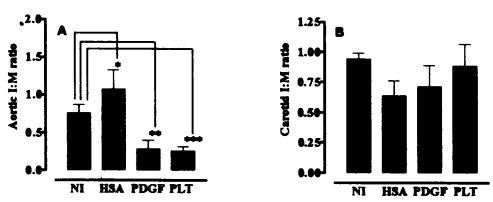

Figure 1. The effects of endogenous antibody formation in the cholesterol-fed rabbit, as measured by intimal:medial thickness ratio. (A) aorta; (B) carotid artery. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to NI using a Bonferroni test with a selected pairs correction.

In conclusion our data suggests that PDGF-BB is a major contributor to fatty streak development in the aorta, but not the neointimal response in the carotid artery following balloon injury in the cholesterol-fed rabbit, indicating that growth factors other than PDGF may be important in the formation of a neointima in the ballooned cholesterol-fed rabbit model.

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323P THE APICAL L-DOPA UPTAKE SYSTEM IN OK CELLS HAS THE CHARACTERISTICS OF AN ORGANIC CATION POTENTIAL-DEPENDENT AND PROTON-INDEPENDENT TRANSPORTER

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Renal epithelial cells of rat and human proximal tubules take up L-DOPA and decarboxylate it to dopamine, a local natriuretic hormone. The uptake of L-DOPA in rat renal proximal tubules has been demonstrated to occur through a saturable process (Soares-da-Silva et al., 1994) sensitive to inhibition by other neutral amino acids (Soares-da-Silva & Pinto-do-Ó, 1996) and to organic cation inhibitors, but not to organic anion inhibitors (Soares-da-Silva, 1995). The present work was aimed to study the kinetics and nature of the apical (luminal) L-DOPA transporter in Opossum kidney (OK) cells. 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 without essential amino acids supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B and 100 µg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 µg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, OK cells were preincubated (30 min) with Hanks medium with added pargyline (100 µM), tolcapone (1 µM) and benserazide (50 µM). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. Saturation experiments were performed in OK cells incubated for 6 min with increasing concentrations of L-DOPA (1 to 2500 µM); non-linear analysis of the saturation curve revealed for L-DOPA a K_m of 129 µM (114, 145) and a V_{max} of 30.0±0.4 nmol mg $protein^{-1}$ 6 min^{-1}, respectively. The uptake of L-DOPA (250 µM) was inhibited in concentration-dependent manner by cyanine 863, an organic cation inhibitor, with a K_i value of 638 (430, 947) µM; the organic anion inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), was devoid of effect upon the uptake of L-DOPA. To determine whether the L-DOPA transporter behaves similarly to the potential-dependent organic cation transport over the basolateral membrane or the luminal H^+-organic cation antiport (Gründemann et al., 1994), the dependence of L-DOPA uptake on membrane potential and pH gradients was tested. The uptake of L-DOPA (250 µM) was significantly ($P<0.02$) decreased (25% reduction) when cells were incubated in the presence of 137 mM K^+, 5 mM Na^+, when compared with the control condition (137 mM Na^+, 5 mM K^+); substitution of NaCl by choline chloride (137 mM) did not affect L-DOPA uptake. Similarly, inwardly or outwardly directed proton gradients of 0.5 pH units (7.9, 7.4, 6.9, 6.4 and 5.9) were found not to change L-DOPA uptake. In conclusion, the L-DOPA system in OK cells in the apical (luminal) border has virtually the characteristics of an organic cation potential-dependent and proton-independent transporter, as measured over the basolateral membrane of renal proximal tubules.

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324P EFFECTS OF GLUCOSE AND INSULIN ON ISOPRENALINE-INDUCED RELAXATION OF RAT MESENTERIC MICROVESSELS

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Diabetes is associated with numerous cardiovascular abnormalities which may partially result from direct effects of high glucose concentrations or altered insulin concentrations on the blood vessels. Thus, insulin can sensitize ß-adrenoceptor-mediated relaxation of large blood vessels, e.g. rat aorta (Gros et al. 1994). Since data from conductance vessels may not be representative for the resistance vasculature, we have examined the effects of insulin and glucose on isoprenaline-induced relaxation of rat mesenteric microvessels (200-300 µm diameter).

Experiments were performed in a Mulvany-Halpern myograph as recently described (Chen et al. 1996). Preparations were pre-incubated with the indicated glucose and insulin concentrations for 45 min prior measurements of contraction and relaxation. Data are shown as mean ± s.e. mean. Statistical significance of differences was analyzed by repeated measures ANOVA followed by Dunnett's test with a $P < 0.05$ considered significant.

Contractions were elicited by 100 µM methoxamine (13.8 ± 1.4 mN; n = 8); methoxamine-induced contraction was not significantly altered by repeated administration or by glucose or insulin preincubation. Isoprenaline caused concentration-dependent relaxation (pEC_{50} 6.69 ± 0.12; maximum effect 96 ± 1%; n = 8), and this could be repeated for at least 4 times without signs of desensitization. In all further experiments the first isoprenaline curve was used as control and compared to subsequent curves obtained in the presence of the indicated concentrations of glucose or insulin.

Preincubation of the microvessels with 10, 30, 100 and 300 pM insulin reduced the sensitivity to isoprenaline (pEC_{50} 6.11 ± 0.19) by 0.64, 0.68, 0.82 and 0.94 log units, respectively (n = 7 each; all $P < 0.05$ vs. control). This was accompanied by reduction of maximal relaxation from 82 ± 3% to 73 ± 5%, 67 ± 6%, 63 ± 4% and 65 ± 7%, respectively (all $P < 0.05$ vs. control). Preincubation of the microvessels with 10, 20 and 40 mM glucose did not significantly affect maximal isoprenaline induced relaxation (84 ± 1%; n = 8) but caused slight reductions of isoprenaline potency (pEC_{50} 6.46 ± 0.07) by 0.16, 0.20 and 0.21 log units, respectively ($P < 0.05$ vs. control for 20 and 40 mM glucose).

We conclude that short-term exposure to increased glucose concentrations corresponding to high pathophysiological values has no major effect on isoprenaline-induced relaxation of rat mesenteric microvessels. In contrast short-term exposure to insulin desensitizes isoprenaline-induced relaxation of rat mesenteric microvessels with regard to agonist potency and maximal effects. While the reasons for the discrepancy to a reported sensitization e.g. in rat aorta, remain to be determined, it is interesting to note that insulin can desensitize isoprenaline-induced cAMP formation in cultured rabbit lung cells (Davis et al. 1992).

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325P CHARACTERIZATION OF THE HISTAMINE RECEPTOR SUB-TYPE COUPLED TO ACTIVATION OF PHOSPHOLIPASE C IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Histamine is an important contractile agonist for airway smooth muscle and is believed to initiate the contractile response in this tissue by activation of phospholipase C. Cultured human airway smooth muscle (HASM) cells provide a useful model system to study receptor coupling in the airways (Hall & Kotlikoff, 1995). In order to characterise the histamine receptor present in these cells, we used total [^{3}H]-inositol phosphate formation as an index of receptor activation. Primary cultures of HASM were prepared from explants of human trachealis muscle obtained at post mortem. For experiments, cells were plated in 24 (×1 ml) well plates. Formation of [^{3}H] -inositol phosphates was measured as previously described (Daykin et al., 1993).

Histamine (1nM-1mM) induced concentration dependent [^{3}H]-inositol phosphate formation in HASM cells (EC_{50}= 4.8 ± 0.6 μM, n = 20). The rate of accumulation of inositol phosphates in response to stimulation by 0.1 mM histamine was essentially linear up to 45 mins. We studied the ability of a number of selective antagonists to inhibit the inositol phosphate response to a near maximal concentration of histamine (100μM) in these cells. The K_d values for the agents studied are shown in table 1: these data suggest that the inositol phosphate response to histamine in HASM cells is mediated through the H_1 receptor subtype. To confirm this observation, we studied the effects of H_2- and H_3 - (tiotidine, 1μM; thioperamide, 1μM) selective antagonists on the response to histamine (0.1μM-1mM). Neither agent had significant effects upon histamine induced inositol phosphate formation in these cells (n=3). We conclude that cultured HASM cells express a classical H_1 receptor.

Antagonist	Kd(nM)	n
Doxepin	0.03±0.01	7
Promethazine	0.18±0.04	4
Chlorcyclizine	1.8 ± 0.3	5
Chlorpromazine	15.0± 1.5	6
Mepyramine	1.0 ± 0.3	5
(+)Chlorpheniramine	5.1 ± 1.3	4

Table 1: K_d values for inhibition of histamine induced inositol phosphate formation by selective antagonists in HASM cells. Data represent the mean ±s.e mean of data obtained in individual experiments.

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326P EFFECTS OF HISTAMINE AND SUBSTANCE P *IN VITRO* ON EQUINE EOSINOPHIL MIGRATION AND ADHERENCE

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Eosinophils are the predominant inflammatory cell found in lesions of sweet itch, a pruritic, allergic skin disease of horses. Histamine (H) release has been detected in affected horses and this mediator induces eosinophil accumulation in equine skin (Riek, 1954). The first aim of this study was to examine the effects of H and substance P (SP), which may be released by H from cutaneous sensory nerve endings, on equine eosinophil migration *in vitro*. The second aim was to establish the effects of these mediators on eosinophil adherence. H and SP induce free radical production by eosinophils (Foster & Cunningham, 1996) and adherent cells may have enhanced responses.

Eosinophil migration through nitro-cellulose filters was determined using 48-well microchemotaxis chambers as previously described (Foster *et al.*, 1992). H (10^{-8}-10^{-3}M), SP ($3x10^{-5}$-$3x10^{-4}$M), the positive control leukotriene (LT) B_4 (10^{-7} M) or vehicle were added to the lower wells (3 replicates for each concentration). Eosinophils ($2.5x10^5$ cells) purified from normal horses were added to the upper compartments, with or without the histamine receptor antagonists mepyramine (mep) 10^{-6}M, chlorpheniramine (chlor) 10^{-6}M, cimetidine (cim) 10^{-6}M or thioperamide (thio) 10^{-6}M. After incubation for 3h at 37°C, filters were processed and the number of cells that had migrated through the filter quantified. For eosinophil adherence assays 96-well microtitre plates were sequentially coated with 10% autologous serum and blocked with 2% bovine serum albumin, each for 1h at 37°C. H (10^{-7}-10^{-3}M), SP (10^{-7}-$3x10^{-4}$M), the positive control phorbol myristate acetate (PMA; 10^{-11}-10^{-7}M) or vehicle were added to the test wells (6 replicates for each concentration). Eosinophils ($1x10^5$ cells) purified from normal horses were added with or without the histamine receptor antagonists and incubated for 25 min at 37°C. Adherence was quantified as described previously (Foster *et al.*, 1994). Analysis of variance and Dunnett's test were used to analyse the data.

H (10^{-3}M) induced a significant increase in eosinophil migration (P<0.01 n=4; 75±24 v 6±1 cells 0.3mm^{-2} for histamine & vehicle, respectively). SP ($3x10^{-4}$M) also caused a small but significant response (P<0.05 n=8; 45±20 v 7±2 cells 0.3mm^{-2} for SP & vehicle, respectively). LTB_4 induced migration of 109±26 & 71±10 cells 0.3mm^{-2} in H and SP studies, respectively. H and SP caused significant dose-dependent increases in eosinophil adherence (P<0.01, n=4), H (10^{-3}M) and SP ($3x10^{-4}$M) causing 13±3% and 10±2% adherence, respectively. PMA caused maximal adherence (19±4%) at 10^{-8} M. Both H-induced migration (10^{-3}M) and adherence (10^{-4}M) were inhibited by H_1, but not H_2 or H_3, receptor antagonists (Table 1).

Table 1. Effect of H receptor antagonists on eosinophil migration and adherence.

Migration	H	+mep	+chlor	+cim	+thio
(cells 0.3mm^{-2})	84±16	5±2**	11±5**	79±12	70±29
Adherence (%)	7±2	1±0**	1±0**	5±2	5±2

**=P<0.01 vs H alone; n=4.

These findings suggest that H and SP, if released after antigen challenge, could induce the migration and adherence of equine eosinophils in the skin of sweet itch horses.

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327P DOSE-RELATED EFFECT OF DEXAMETHASONE ON LEUCOCYTE EXTRAVASATION

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The inhibition of leucocyte recruitment by corticosteroids, such as dexamethasone (DEX), is one of the major features of this class of anti-inflammatory drugs. Here, we have investigated the effect of DEX treatment on the leucocyte-endothelial interaction within the rat mesenteric bed following intraperitoneal (i.p.) injection of interleukin-1ß (IL-1ß) (Nourshargh *et al.*, 1995) or superfusion with platelet-activating factor (PAF) (Zimmerman *et al.*, 1994).
Male Sprague Dawley rats (175-200 g body weight) were fasted overnight and injected i.p. with either 1 ml sterile saline or with 20 ng rat recombinant IL-1ß. Animals received different doses of DEX (0.01-1mg kg^{-1} s.c.) 1 h prior challenge with IL-1ß. The mesenteric microvascular bed was mounted as described [2] onto a microscope (Zeiss Axioskop "FS") equipped with a x40 water immersion objective lens. Images were projected onto a Sony Triniton colour video monitor by a video camera and recorded for subsequent off-line analysis. At 2 h post-IL-1ß injection, the number of adherent cells was determined in 3-5 randomly selected vessels of at least 100 µm in length and emigrated leucocytes were monitored at 0-50 and 50-100 µm away from the vessel wall. Leucocyte activation was also obtained by superfusion of the rat mesentery with 0.1 µM PAF for 60 min, recording at 20-30 and 50-60 min intervals for subsequent off-line analysis. In few cases we measured the time required by the adherent leucocyte to complete the emigration process. Statistical differences between experimental groups were determined by ANOVA followed by the Bonferroni test.
IL-1ß treatment resulted in an increase in the number of adherent as well as emigrated leucocytes DEX produced a dose-dependent inhibition of both parameters, with approximate ED_{50}s of >1 $mgkg^{-1}$ and 52 μgkg^{-1}, respectively (see Table 1).

Table 1. DEX effect on leucocyte adhesion and emigration

Experimental group	No. of adherent cells	No. of emigrated cells 0-50 µm	50-100 µm
Saline	6.0 ± 0.8	2.1 ± 0.8	0.8 ± 0.3
IL-1ß	23.2 ± 2.3	28.9 ± 1.7	15.8 ± 0.9
+ DEX 10 µg kg^{-1}	26.3 ± 1.2	31.5 ± 9.3	15.4 ± 0.7
30 µg kg^{-1}	18.5 ± 1.5	14.9 ± 1.4 *	8.1 ± 1.2 *
100 µg kg^{-1}	15.1 ± 1.2 *	5.4 ± 0.7 *	2.1 ± 0.3 *
1 mg kg^{-1}	13.5 ± 1.4 *	3.2 ± 0.4 *	1.5 ± 0.3 *

(Mean± s.e.mean of 5-8 rats per group) *P <0.01 *vs.* IL-1ß.

PAF superfusion of the rat mesentery resulted in a time-dependent increase in cell adhesion (maximal at 30 min, 6.3 ± 1.8 cells, n=6 rats) and emigration (maximal at 60 min, 17.7 ± 4.5 cells). DEX administration (1 $mgkg^{-1}$) did not alter leucocyte adhesion but significantly modified emigration (-50%, n=8 rats ;P <0.05). Whereas in control rats the leucocyte emigration process was completed within 225 ± 17.9 s (n=30 cells), 560 ± 83.4 s (n=21 cells) were required in DEX-treated rats (P < 0.01).
In conclusion, direct observation of the initial steps of the leucocyte extravsation process allowed us to identify the emigration through the endothelium as the one preferentially affected by DEX. These data promote the concept that different doses of corticosteroids exert different effects through mechanisms of action depending upon the stimulus used.

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328P EFFECT OF SINGLE POINT MUTATIONS OF THE HUMAN TACHYKININ NK_1 RECEPTOR ON ANTAGONIST AFFINITY

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Different regions of the tachykinin NK_1 receptor are important for substance P (SP) or non-peptide antagonist binding (Gether *et al*, 1993). In this study we have used the Semliki Forrest virus (SFV) system to express the wild type (WT) and a series of single point mutations of the human tachykinin NK_1 receptors in Chinese hamster ovary (CHO) cells to determine the regions involved in ligand binding.

Mutants were selected from our model based on sequence alignments to bacteriorhodopsin. The cDNAs for the coding region of the wild type and mutant receptors were introduced into the pSFV1 vector and recombinant SFV particles generated (Lundstrom *et al*, 1994) Membranes were prepared from SFV infected CHO cells 16 hours post infection, and binding experiments performed as previously described (Beattie *et al*, 1995).

Saturation analysis indicated that [^{3}H]-SP bound in a saturable manner with a similar affinity for all preparations. There was a significantly ($p<0.01$) lower number of binding sites (B_{max}, pmol/mg protein) at mutant receptors compared to wild type. Table 1.

Table 1. Estimates of K_D and B_{max} (pmol/mg protein) (n=3 ±sem)

	WT	Mutant Q165A	T170A	H197A	F268A
pK_D	9.6±0.15	9.22±0.11	9.45±0.15	9.41±0.19	9.4±
B_{max}	61±6	3.8±1.0	15±3	26±3	2.0±0.8

Competition studies indicate that SP and the peptide antagonist GR82334 (see Hawcock *et al*, 1995) had a similar affinity for the mutants and WT receptors. There was a significant reduction ($p<0.01$) in affinity of GR203040, and CP-99,994 (see Beattie *et al*, 1995) for Q165A indicating the importance of hydrogen bonding. There was a significant reduction ($p<0.05$) in affinity of GR203040 and CP-99,994 for F268A, demonstrating an aromatic interaction. RP67580 (see Hawcock *et al*, 1995) was unaffected by these substitutions Table 2.

Table 2. Ligand affinity estimates (pK_i ±SEM n=3-6 except #, where n=2.) * $p<0.05$, ** $p<0.01$ (Dunnett's test)

	WT	Mutant Q165A	T170A	H197A	F268A
SP	8.7±0.2	8.6(8.4-8.8)#	8.7±0.1	8.7±0.1	8.8±0.1
GR203040	9.4±0.2	8.2±0.3**	9.7±0.1	9.4±0.2	8.7±0.1*
CP-99,994	9.0±0.2	7.2±0.1**	9.3±0.3	8.6±0.2	8.4±0.1*
RP67580	7.4±0.2	7.4±0.4	6.9±0.2	7.2±0.3	7.7±0.2
GR82334	7.0±0.2	6.6±0.2	7.2±0.4	6.7±0.3	6.7±0.3

The affinity of [^{3}H]-SP and SP for the WT in this study is lower than observed in stable transfections, possibly due to an increase in the proportion of uncoupled receptors. This could also explain the reduced affinity of GR203040, and CP-99,994 for the WT. These results indicate that RP67580 and the peptide antagonist GR82334 interact at the receptor with a dissimilar mechanism to the other antagonists examined in this study

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329P ALTERATIONS IN STRIATAL PREPROTACHYKININ AND NIGRAL TYROSINE HYDROXYLASE mRNA IN TETRAHYDROBIOPTERIN-DEFICIENT MICE

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Tetrahydrobiopterin (BH4) acts as cofactor for the intracellular hydroxylation of phenylalanine to tyrosine and tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) (Leeming et al.,1981). Impaired BH4 biosynthesis in brain results in the occurrence of L-DOPA-responsive dystonia in humans (Segawa et al., 1971). Tyrosine hydroxylase (TH) activity is reduced in the brain of BH4 deficient mice (hph-1) (Hyland et al.,1996) and this may function as a model of the human disorder. The brain concentration of BH4 is decreased by approximately 50% in the hph-1 mouse (Brand et al., 1995). We now report on gene expression of preprotachykinin (PPT), preproenkephaline (PPE) in the striatum and TH in the substantia nigra of hph-1 and normal control C57BL mice.

Six hph-1 and six normal C57BL mice (30 days of age, weight 10-12g) were killed by cervical dislocation. Subsequently, cryostat sections (20μm) through the medial striatum (AP 0.2mm from bregma) and substantia nigra (3rd cranial nerve roots level) were incubated with ^{35}S-labelled oligodeoxyribonucleotide probes hybridizing with PPT, PPE and TH mRNAs. Quantitative evaluation of autoradiograms was undertaken by computerized densitometry and results were analysed by paired Student's *t*-test.

The intensity of TH mRNA labelling in the substantia nigra of hph-1 mice was significantly higher than that of normal C57BL mice (Table 1). In the striatum, the level of PPT mRNA but not PPE mRNA, was elevated in hph-1 mice, compared to C57BL mice.

The increased TH mRNA in the substantia nigra in BH4 deficient mice may represent a compensatory mechanism for the reduction in striatal TH protein activity (Rajput et al., 1994). The selective elevation in striatal PPT implies that alteration may occur in the D1 receptor mediated direct output pathway from striatum and this has been implied the genesis of dystonia.

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Table 1. Gene expression of striatal PPE and PPT and nigral TH in hph-1 and C57BL mice brain (mean±sem)

	PPE mRNA		PPT mRNA		TH mRNA	
	left side	right side	left side	right side	left side	right side
hph-1 mice	117.8±5.8	121.8±5.8	37.3±2.5*	37.1±2.2#	66.1±3.1†	64.82±1.9§
C57BL mice	122.4±4.4	123.4±5.0	29.7±1.6	28.6±1.1	57.2±2.1	58.93±1.5

*P < 0.04, #P <0.01, †P < 0.05 and §P < 0.04 vs C57BL mice, paired Student *t*-test.

330P STAGE-SPECIFIC TUMOUR-PROMOTING PHORBOL ESTERS AS MARKERS OF MACROPHAGE ACTIVATION STATUS IN AN *IN VITRO* MODEL OF METAL OXIDE PARTICLE TOXICITY

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Reactive oxygen intermediates have been implicated in the development of airway hyper-responsiveness (Liberman *et al.*, 1995). Alveolar macrophages perform intracellular killing of potential pathogens by generating toxic oxygen species following superoxide release, and this operates under stringent intracellular control. Metal oxides are important environmental contaminants and there may be intense occupational exposure which may predispose individuals to a greater risk of respiratory disease. It would therefore be useful to assess alterations in macrophage activation status induced as a result of particle exposure. Phorbol esters stimulate superoxide release. Parallels have been identified between restricted tumour promoting ability, selective protein kinase C activation and the sensitivity of differing cell populations to stimulation by these compounds (Evans *et al.*, 1990). Here, we report alteration of the superoxide generating capacity of guinea pig lung - derived mononuclear cell cultures by metal oxide exposure and stimulation by the second stage tumour promoter, sapintoxin D (12-O-[2-methylaminobenzoyl]-phorbol-13-acetate).

Cells were harvested from male Dunkin-Hartley guinea pigs (350-800g) by a combination of bronchiolar lavage and agitation of sliced lung parenchyma, using Hank's balanced salt solution (HBSS) at 4°C. Mononuclear cells were separated by density centrifugation on Histopaque 1077 (Sigma, Poole, UK), washed and resuspended in HBSS containing 160μM cytochrome C and 1% bovine serum albumin at 10^6 ml^{-1}. Superoxide dismutase inhibitable reduction of cytochrome C was measured as increase in absorbance at 550 nm over 45 min at 37°C.

Table 1. Sapintoxin D stimulated superoxide release by lung derived mononuclear cells exposed to metal oxide.

Sapintoxin D (nM)	0	50	100	200
Control	5.96 (±0.01)	11.27 (±1.28)	12.99 (±1.14)	17.39 (±1.29)
+Nickel oxide (1mg ml^{-1})	4.46 (±1.04)	6.39* (±1.49)	7.59* (±0.15)	7.24* (±0.71)
+Aluminium oxide (1mg ml^{-1})	5.77 (±0.71)	15.06* (±0.43)	15.33* (±0.43)	17.27* (±0.71)

n = 3-4. Data presented as nmoles superoxide produced per 45 min incubation per 10^6cells (±SEM). ($p < 0.05$ nickel oxide vs alumina by unpaired Student's t test).

Exposure to nickel or aluminium oxides was found to have opposing effects on the ability of cells to respond to sapintoxin D with nickel treated cells reaching only 43% of the maximal response attained following alumina treatment. These effects may be due to a direct interaction with protein kinase expression or activity, or a synergistic action on other signalling pathways. Measurement of macrophage responsiveness to selective tumour promoters could reflect activation status, and the findings may indicate a mechanism for the chronic toxicity of metal oxide.

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331P ASSESSMENT OF ANTI-OXIDANT ACTIVITY USING THE SIN-1 DEGRADATION ASSAY

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The balance between nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot-}$) may be important for vascular reactivity under normal physiological conditions. $O_2^{\cdot-}$ may also be involved in accelerating $NO^{\cdot}$ oxidation under pathological conditions where oxidative stress is a factor, such as atherosclerosis. In addition, $NO^{\cdot}$ and $O_2^{\cdot-}$ can result in the formation of toxic peroxynitrite. We have previously described how the use of 3-morpholinosydnonimine (SIN-1), which generates $NO^{\cdot}$ and $O_2^{\cdot-}$ spontaneously, can be used to evaluate the interaction of these two radical species (Quine *et al.*, 1996). In this study we measured the production of nitrite from SIN-1 in the presence of various compounds in order to assess their potential as anti-oxidants. Nitrite (NO_2^-) was released from SIN-1 (10mM; pH 5.5) in a time dependent manner. Data is expressed as mean±s.e. mean of 3 determinations. In all experiments SIN-1 was incubated in the presence of anti-oxidant for 4h at 37°C, samples were then removed for nitrite analysis. Nitrite was assessed by addition of Griess reagent (0.5% sulphanilamide, 0.05% N-naphthylethylene diamine dichloride and 2.5% phosphoric acid). The compounds investigated included ebselen and sodium selenite which have similar properties to glutathione peroxidase; plant derived flavanoids (genistein, flavone, chrysin, and caffeic acid) that have chain breaking anti-oxidant activity. In addition, ONO-DC-560 (2-[4-[2-[3,5-di(tert-butyl)-4-hydroxyphenylthio]ethyl]-1,3-thiazol-2-yl]guanidine hydrochloride) and ONO-DC-582 (2-[5-[4-(6 hydroxy-2,2,5,8-tetramethylchroman-7-yl)butyl]-1,3- thiazol-2-yl]guanidine hydrochloride), chain breaking anti-oxidants combined with the inducible nitric oxide synthase inhibitor aminoguanidine, were also investigated (table 1). We suggest that the compounds that increased SIN-1 derived NO_2^- may catalyse the destruction of $O_2^{\cdot-}$ resulting in a possible decrease in peroxynitrite formation. In contrast, anti-oxidants that decrease NO_2^- may act by preventing the oxidation of $NO^{\cdot}$ to $NO_2^{\cdot}$.

The degradation of SIN-1 provides a model system for analysis of $NO^{\cdot}$ oxidation and the interaction of $NO^{\cdot}$ with $O_2^{\cdot-}$, processes increasingly important in the regulation of $NO^{\cdot}$ dependent vasodilation. This assay may be useful in assessing the actions of compounds with anti-oxidant properties which are able to modulate the interaction of $O_2^{\cdot-}$ and $NO^{\cdot}$ by either scavenging $O_2^{\cdot-}$ or by blocking $NO^{\cdot}$ to NO_2^-.

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Quine, L., Carrier, M., Änggård, E. (1996). *Br. J. Pharmacol.* In press.

Table 1: Nitrite release from SIN-1 (10mM) after incubation for 4h at 37°C. * $p<0.0005$ compared to SIN-1 only (unpaired Student's t test).

Incubation	Nitrite Release (μM)	Incubation	Nitrite Release (μM)	Incubation	Nitrite Release (μM)	Incubation	Nitrite Release (μM)
SIN-1 (10mM)	10.2±0.2*	+ONO-DC-560	28.9±4.9*	+ genistein (10mM)	22.8±1.8*	+ caffeic acid (1mM)	5.1±0.4*
+ebselen (1mM)	14.7±0.4*	+bilirubin (5mM)	14.9±0.7*	+ flavone (10mM)	47.7±5.2*	ONO-DC-582 (1mM)	5.9±0.7*
+sodium selenite (100μM)	20.0±0.5*	+aminosalicylate (1mM)	3.7±0.4*	+chrysin (1mM)	40.3±1.5*	+Nacetylhydroxy-tryptamine(1mM)	1.78±0.2*

332P INSULIN-LIKE GROWTH FACTOR I (IGF-I) INCREASES COLLAGEN IV IN THE RAT KIDNEY *IN VIVO*: POTENTIATION IN THE GLOMERULUS BY THE NOS INHIBITOR, l-NAME

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The ability of insulin-like growth factor I (IGF-I) to stimulate renal function is mediated by nitric oxide (NO) (Haylor *et al.*, 1991), possibly through activation of cNOS in the vascular endothelium (Tsukahara *et al.*, 1994). IGF-I, a potent renal mitogen, is well known to stimulate matrix protein production in many cell types including, bone and fibroblasts. However, the direct effect of IGF-I on matrix proteins in the kidney has not been well established and a role for IGF-I in the development of chronic renal failure remains therefore a controversial issue. Since NO itself has recently been demonstrated to inhibit matrix protein formation (Tratchman *et al.*, 1995), experiments have been performed to establish whether NO may influence the effect of IGF-I on renal collagen accumulation in the rat.

A renal tissue cannulae, attached to a subcutaneous osmotic minipump was implanted into the left kidney of male Wistar rats (240-350g) under ether anaesthesia (Matjeka & Jennische 1992). Primed 7-day pumps (200μl) contained either recombinant human IGF-I (rhIGF-I) 50μg or saline vehicle. After 7 days, the left kidney was removed under ether anaesthesia, fixed, sliced and subjected to immunohistochemical staining for type IV collagen and quantified by point counting.

RhIGF-I 50μg/week produced a significant increase (n=6 per group) in collagen IV staining in both the glomerulus (44 ± 5 % vs 50 ± 3 %, $P<0.005$) and the tubular interstitium (34 ± 1 % vs 43 ± 2 %, $P<0.001$) compared to saline-infused controls. In a second experiment, rats received l-nitroarginine methyl ester (l-NAME), a NOS inhibitor, 50μg/ml in the drinking water. Following l-NAME administration, a significant increase in collagen IV was also observed in rats infused with rhIGF-I 50μg/week (n=6 per group) for both the glomerulus (41 ± 3 % vs 58 ± 4 %, $P<0.001$) and for the tubular interstitium (38 ± 1 % vs 46 ± 1 %, $P<0.01$) compared to saline-infused controls. However, l-NAME administration produced a significant potentiation ($P<0.01$) of the effect of rhIGF-I on collagen IV in the glomerulus by approximately 2-fold, no potentiation being seen in the tubular interstitium. In non-cannulated rats, staining for type IV collagen was significantly greater ($P<0.05$, n=6 per group) in both glomerular and tubulointerstitial compartments following l-NAME administration.

The results suggest that rhIGF-I may increase kidney collagen IV, an effect inhibited in the glomerulus by the generation of nitric oxide.

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333P ODQ PREVENTS THE INCREASE IN [^{3}H] THYMIDINE AND [^{14}C] LEUCINE UPTAKE INDUCED BY NITROPRUSSIDE IN RAT KIDNEY SLICES

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In glomerular mesangial cells, nitric oxide (NO) has been demonstrated to inhibit both protein synthesis and cell proliferation (Garg & Hassid 1989). However in other cell types, including fibroblasts, proliferative effects of nitrovasodilators have been described (O'Connor et al 1991). In the present experiments, organ slices have been used to investigate the influence of nitroprusside (SNP) on protein synthesis and cell proliferation in the kidney. The effect of SNP on [^{14}C] leucine and [^{3}H] thymidine uptake has been examined using rat kidney cortical slices in the presence and absence of ODQ, a selective inhibitor of the guanylate cyclase activated by NO (Garthwaite et al 1995).

Male Wistar rats were anaesthetised with thiopentone (100mg/kg i.p.), the kidneys removed, decapsulated, sliced and incubated with [^{3}H] thymidine and [^{14}C] leucine in Krebs-Henseleit solution for a 2-hour period. Isotope uptake was expressed as a percentage change from control slices derived from the same kidney incubated in the absence of SNP.

As shown in Table 1, SNP exerted a biphasic effect on both [^{3}H] thymidine and [^{14}C] leucine uptake. SNP 0.5μM produced a significant increase in both [^{3}H] thymidine and [^{14}C] leucine uptake while following incubation with SNP 50μM a significant decrease was observed. Incubation with ODQ 2μM, prevented the increase in both [^{3}H] thymidine uptake (-1 ± 12 %) and [^{14}C] leucine uptake (+9 ± 9%) produced in the kidney slice to SNP 0.5μM. However, in the presence of ODQ 2μM, the decrease in both [^{3}H] thymidine uptake (-47 ± 5% vs -49 ± 6%) and [^{14}C] leucine uptake (-49 ± 8% vs -63 ± 4%) produced by some 100-fold higher concentrations of SNP at 50μM remained unchanged (n=6 per group).

SNP (μM)	0.5	5	50	500
[^{14}C]Leucine Uptake (% change)	+33±8*	-1±12	-54±4**	-92 ±2**
[^{3}H]Thymidine Uptake (% change)	+27±3*	+13±9	-25±7*	-54±6**

*P<0.05, **P<0.01, n=6 per dose

Table 1. Concentration-response data for the effect of SNP on [^{14}C] leucine and [^{3}H] thymidine uptake in kidney slices

The results suggest that in the kidney, the cGMP activated by NO may help to mediate stimulation rather than inhibition of protein synthesis or cell proliferation induced by nitroprusside.

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334P INDUCIBLE ISOFORMS OF CYCLOOXYGENASE (COX-2), NITRIC OXIDE SYNTHASE (iNOS) AND HEME OXYGENASE (HO-1) IN RAT CNS/PNS IN ACUTE INFLAMMATION

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Induction of the enzymes COX-2 and iNOS is associated with pro-inflammatory activity, whilst elevated levels of HO-1 coincide with suppression of inflammation (Willis *et al.*, 1996). At the site of inflammation the respective products of COX, NOS and HO (prostaglandins; nitric oxide; carbon monoxide and bile pigments) have the capacity to modulate the activity of these enzymes. COX, NOS and HO are expressed in the CNS, thus the potential exists for interactions to occur not only at the site of inflammation but in neuronal tissues. Therefore, protein expression and immunolocalisation of inducible enzymes were investigated in sciatic nerve (SN), dorsal root ganglia (DRG) and spinal cord (SC), after the induction of inflammation, to elucidate potential targets for inflammatory modulation.
Inflammation was induced by unilateral intraplantar injections of 1% carrageenin in saline into the hind paw of male Wistar rats (n=6). Enzyme protein expression and immunolocalisation were assessed in SC and ipsilateral and contralateral SN and L4/L5 DRGs, at 3, 6, 12, 24h after injection.
Western blot analysis showed constitutive expression of COX-2, iNOS and HO-1 protein in SN, DRG and SC lumbar enlargement in control rats. In inflamed animals, levels of inducible enzyme protein were unchanged at all time points in comparison to controls. Furthermore, protein levels in ipsilateral and contralateral SN and DRGs were similar.
In peripheral inflammatory loci, HO-1 is detected as a 32kDa protein in macrophages by Western blotting.. However, in neuronal tissues, a 46kDa protein (HO-L) was detected. This observation confirms a previous report in the CNS and may represent a novel isoform of HO.
Inducible NOS was immunolocalised to the vascular smooth muscle (VSM) of SN, DRG and anterior spinal artery of the SC. COX-2 was observed in selected neurones of the DRG, SN axons and abundantly in SC central grey matter. HO-L was confined to SN and DRG VSM and occasional large diameter DRG neurones, VSM of the anterior spinal artery and SC neurones of lamina X. Immunolocalisation was unchanged 3h after the inflammatory event.
Although protein levels and cellular expression were unchanged after carrageenin-induced acute inflammation, this may not be true for a cell mediated chronic inflammation. A recent report, where Freund's complete adjuvant was substituted for carrageenin, shows a significant increase in COX-2 mRNA in the DRG and lumbar SC (Beiche *et al.*, 1996). Therefore, further investigations are underway, including selective inhibition of COX, NOS and HO to elucidate their roles in neurogenic inflammation.

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335P PURIFIED *STAPHYLOCOCCUS AUREUS* LIPOTEICHOIC ACID INDUCES NITRIC OXIDE SYNTHASE ACTIVITY IN MURINE MACROPHAGES

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Lipoteichoic acid (LTA) is an amphiphilic molecule of the cell wall of Gram-positive bacteria. LTA contains a hydrophilic poly(glycerophosphate) chain and two-three lipophilic acyl residues (see Fischer, 1988). *S.aureus* LTA, either alone or in synergy with a component of peptidoglycan, N-acetyl-D-glucosaminyl-β-[1→4]-N-acetylmuramyl-L-ala-D-isoglutamine (NAG-AP) induces nitric oxide synthase (iNOS) activity in murine macrophages (Kengatharan *et al.*, 1996). Commercially available LTA is extracted from whole cells using phenol (see Fischer, 1988). Separation of *S.aureus* LTA by hydrophobic interaction column chromatography (HIC) subsequent to the extraction using phenol, results in the generation of a trace constituent (and not LTA) which stimulates interluekin-6 formation in U373 astrocytoma cells (Kusunoki *et al*, 1995). Furthermore, there is some evidence that highly purified *S.aureus* LTA does not enhance nitrite formation in bone marrow-derived mononuclear phagocytes (Keller *et al.*, 1992). Here we investigate whether HIC purified *S.aureus* LTA induces nitrite in J774.2 macrophages either alone or in synergy with NAG-AP.

J774.2 cells were cultured in 96-well plates with culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4 mM) until confluence. LTA (Sigma, Dorset, UK) was separated by HIC and the phosphate levels (indicating the presence of LTA) in the subsequently eluted fractions was determined according to a method described by Kusunoki *et al.*, (1995). The fractions were added to the cells either alone or in combination with polymyxin B (0.5μg.ml^{-1}) or NAG-AP (1μg.ml^{-1}). Nitrite accumulation, an indicator of nitric oxide formation, was measured 24h later in the supernatant of J774.2 cells by the Griess method.

LTA (10μg.ml^{-1}) caused an increase in nitrite formation in the supernatant of the J774.2 cells from 2±1μM (baseline) to 38±3μM (P<0.05, n=12). Polymyxin B (0.5μg.ml^{-1}), an agent which binds and inactivates endotoxin did not affect the increase in nitrite caused by LTA, but this agent abolished the increase in nitrite caused by endotoxin (1μg.ml^{-1}). The eluted fractions (8-14) that corresponded to the active fractions in the study by Kusunoki *et al.*, (1995) did not elicit nitrite formation either alone or in combination with NAG-AP (Table 1). In contrast, fractions 39-43 which contained phosphate (and hence LTA) significantly increased the formation of nitrite. The nitrite formed by these fractions were not attenuated by polymyxin B. Furthermore, these fractions synergised with NAG-AP to induce nitrite formation. NAG-AP (1μg.ml^{-1}) did not cause nitrite formation. However, subsequently eluted fractions (52-76) did not elicit nitrite formation either alone or in combination with NAG-AP.

Table 1. Effect of HIC separated products of the crude extract of *S.aureus* LTA on nitrite formation in J774.2 cells.

Fraction No.	Phosphate (mM)	Nitrite (μM)		
		alone	+Polymyxin B	+NAG-AP
4	0	2.3±0.4	2.4±0.2	1.6±0.2
10	0.2	1.9±0.2	2.9±0.6	2.5±0.2
20	0	2.9±0.4	2.4±0.2	2.1±0.2
39	0.2	9±2.2*	5.5±1.8	12.7±0.6*
40	0.8	5±1.3	4±1.3	8.7±0.6*
42	1.3	25±4.9*	24.4±5.3*	42.2±6*#
43	0.7	21±5.4*	14.0±5.2*	50±5.6*#
56	0	3.7±0.6	4±1.8	11±3.9*
64	0	2.2±0.1	2.6±0.4	1.8±0.2
74	0	2.3±0.1	2.2±0.2	2±0.2

Values are given as mean ± s.e.mean (n=6-9); *P<0.05 *vs* baseline & #P<0.05 *vs* fractions alone by ANOVA (Bonferroni's test).

These results show that *S.aureus* LTA *per se* and not a trace constituent of the LTA extract is responsible for inducing nitrite formation on its own or synergy with NAG-AP in J774.2 macrophages.

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336P INCREASED NITRATE FORMATION IN LPS/γIFN-STIMULATED ASTROCYTES OF THE *hph-1* (TETRAHYDROBIOPTERIN DEFICIENT) MOUSE

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The *hph-1* mouse is 90 % deficient in GTP-cyclohydrolase activity (the rate limiting enzyme for tetrahydrobiopterin (BH_4) synthesis; McDonald *et al.*, 1988). Consequently, brain BH_4 levels of the *hph-1* are approximately half that of normal (C57BlxCBA) mice (Brand *et al.*, 1995). Since BH_4 is an essential cofactor for all isoforms of nitric oxide synthase (NOS) it has been postulated that activity of NOS in *hph-1* mice will be impaired. Indeed, when compared with normal mouse brain, constitutive NOS activity of *hph-1* brain is decreased (Brand *et al.*, 1995). However, little is known of how impaired BH_4 synthesis affects mechanisms involving the inducible form of NOS (iNOS). In view of this, we have studied astrocytes isolated from *hph-1* mice, stimulated with lipopolysaccharide/interferon-γ (LPS/γIFN; factors known to induce iNOS). Astrocytes, from either normal or *hph-1* mice, were isolated and cultured for 13 days. On day 13, cells were either stimulated with a combination of LPS (1μg/ml) and γIFN (100 U/ml), or remained untreated but receiving a vehicle control (unstimulated). After 24 hr cells were harvested and used for analyses, BH_4 by HPLC, iNOS protein by Western blot and total protein using a Biorad kit with BSA as a standard. Nitrite (NO_2^-) in the cell culture media was measured using the Greiss reagent, total NO_2^- and nitrates (NO_3^-) was measured by conversion of NO_3^- to NO_2^- by nitrate reductase. Student's t test was used for statistical analysis. BH_4 in unstimulated, normal, astrocytes was 4 times that of *hph-1* (17.6±5.4 and 3.3±0.42 pmole/mg protein respectively, p<0.05, n=4); following stimulation with LPS and γIFN BH_4 in both cell types was doubled (38.9±8.8 for control mice and 7.9±4.6 pmole/mg protein for *hph-1*). Western blot analysis showed that in both cell types iNOS protein was present after stimulation. Unstimulated NO_2^-+NO_3^- release was low (approx 20 μmole/mg) and not different between normal and *hph-1* cells. The total NO_2^-+NO_3^- and individual NO_2^- or NO_3^- measured in response to LPS/γIFN are shown in Table 1.

Table 1. LPS/γIFN stimulated NO_2^- & NO_3^- (μmole/mg protein)

	NO_2^-+NO_3^-	NO_2^-	NO_3^-
normal	74.3±8.5	47.6±7.4	27.5±4.7
hph-1	105.6±11.8*	51.3±5.5	55.0±10.6*

Data shown as mean±s.e.mean, n=6-7, *p<0.05, *hph-1* vs normal. It appears that *hph-1* astrocytes, both unstimulated and stimulated, are deficient in BH_4 by up to 80% compared with normal cells. However, the results suggest that the BH_4 deficiency enhances the production of NO_3^- following iNOS induction. Whether this is due to increased iNOS activity or expression remains unclear. Since, in conditions of suboptimal BH_4, NOS can form O_2^- in addition to NO (Mayer & Werner 1995), it is tempting to speculate that the increased NO_3^- derives from peroxynitrite (product of NO and O_2^-). Finally, the *hph-1* mouse may provide a useful model for studying iNOS mechanisms in conditions such as familial dopa responsive dystonia, where BH_4 levels are suboptimal.

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337P THE EFFECTS OF METABOLITES PRODUCED BY SEMICARBAZIDE-SENSITIVE AMINE OXIDASE ON GLUTATHIONE REDUCTASE ACTIVITY AND ON MOUSE CULTURED MESANGIAL CELLS

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Reduced glutathione is important in the defence against reactive oxygen species. It is maintained in this state by glutathione reductase (EC. 1.6.4.2; Reed, 1986). This enzyme is inhibited by thiol reactive compounds including aldehydes produced in the oxidative deamination of amines by enzymes, such as semicarbazide-sensitive amine oxidase (SSAO, EC. 1.4.3.6; see Callingham *et al.*, 1995). Hydrogen peroxide is generated at the same time by SSAO, which could increase aldehyde toxicity.

To test this possibility, glutathione reductase activity in homogenates of rat lung was assayed by the method of Carlberg & Mannervik (1985) in the presence and absence of the aldehydes, acrolein, formaldehyde or methylglyoxal with or without hydrogen peroxide (all at 100 μM) for 15 min.

Table 1. Effect of aldehydes with and without hydrogen peroxide on glutathione reductase activities of rat lung.

	glutathione reductase activity ($nmolmin^{-1}mg\ protein^{-1}$ ± s.e.mean)	
aldehyde	without H_2O_2	with H_2O_2
none	16.8 ± 0.78	15.4 ± 1.00
acrolein	13.4 ± 0.57†	7.60 ± 0.76*
methylglyoxal	15.2 ± 1.21	10.4 ± 0.93*
formaldehyde	14.2 ± 0.90†	8.80 ± 0.67*

* $P < 0.05$) vs aldehyde alone (Student's t-test).
† $P < 0.05$) vs control (Student's t-test). n = 4.

Allylamine and methylamine alone inhibited rat lung glutathione reductase ($P < 0.05$), while hydrogen peroxide produced no inhibition When aldehyde (acrolein, methylglyoxal or formaldehyde) and H_2O_2 were present, the inhibition of glutathione reductase was significantly enhanced ($P < 0.05$).

To examine the cellular toxicity of aldehydes and peroxide generated by SSAO, conditionally immortalised mouse renal glomerular mesangial cells (Kolatsi-Joannou et al, 1995), grown to confluence, were incubated in the presence of the parent amines, allylamine, methylamine and aminoacetone (all at 200 μM), with and without semicarbazide (1 mM, to inhibit SSAO) for 24 h. Cell damage was assessed by the reduction of 3-(3,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (MTT) method of Mossman (1983).

Table 2. The effect of inhibition of SSAO activity on the toxicity of aldehydes on mouse mesangial cells in culture.

	MTT reduction (per cent of control ± s.e.mean)	
Aldehyde	Control	with 1 mM semicarbazide
allylamine	28.0 ± 7.5***	94.7 ± 7.7
aminoacetone	61.4 ± 8.6***	94.9 ± 7.9
methylamine	39.3 ± 6.5***	92.0 ± 4.69

*** $P < 0.001$ (Student's t-test) n = 6 replicate wells from a single passage.

The increased inhibition of glutathione reductase by aldehydes in the presence of H_2O_2 and their simultaneous generation by SSAO suggest that toxicity could be increased in cells that contain this enzyme when amine levels of aminoacetone and methylamine are abnormally raised under conditions such as diabetes, or through ingestion of allylamine (see Callingham *et al.*, (1995).

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338P EFFECTS OF AH6809 ON PROSTANOID-INDUCED RELAXATION OF HUMAN MYOMETRIUM *IN VITRO*

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The prostanoid EP receptor that mediates inhibition of human myometrial contractility is thought to be of the EP_2 subtype (Senior *et al.*, 1991), with no evidence for EP_4 receptor involvement (Brown & Crankshaw, 1995). AH6809, though primarily a prostanoid EP_1 and DP receptor antagonist (Coleman *et al.*, 1990) has recently been shown to block the human recombinant EP_2 receptor when expressed in COS-7 cells (Woodward *et al.*, 1995). We have examined the effects of AH6809 on the relaxation of human myometrium induced by a variety of prostanoids.

Strips of human myometrium from non-pregnant, pre-menopausal donors were obtained and set-up for isometric recording of inhibitory responses as described by Fernandes & Crankshaw (1995). Concentration-effect curves to inhibitory prostanoids were obtained by cumulative addition to matched strips in the absence and in the presence of AH6809 (42 μM).

The putative EP_2 receptor agonists prostaglandin (PG)E_2, misoprostol, and 11-deoxy PGE_1 were antagonised by AH6809 with apparent pA_2 values of 5.85±0.08, 5.6±0.3 and 5.1±0.9 (mean ± s.d.mean, n=4) respectively. AH6809 did not affect the IP receptor agonist cicaprost (n=4) but did antagonise the DP receptor agonist BW245C (Coleman *et al.*, 1990) with an apparent pA_2 of 5.9±0.4. In similar experiments the DP receptor antagonist BW A868C (Giles *et al.*, 1989) at 50nM had no effect on responses to PGE_2 or misoprostol (n=4).

The inactivity of AH6809 against cicaprost argues against a non-specific effect of this compound on inhibitory responses in human myometrium. AH6809 probably antagonises BW245C by its action at DP receptors, since BW245C is also blocked by BW A868C (Fernandes & Crankshaw, 1995). Since BW A868C did not affect PGE_2 or misoprostol, the effect of AH6809 on the putative EP_2 receptor agonists must be at a site distinct from the DP receptor. Therefore, these data, using intact tissue support evidence obtained using recombinant receptors (Woodward *et al.*, 1995) suggesting that AH6809 is a human EP_2 receptor antagonist. Furthermore, AH6809 appears to have a similar affinity for human DP and EP_2 receptors. Thus, because it can block DP, EP_1, and EP_2 receptors, the use of AH6809 in the classification of human prostanoid receptors should be attempted with caution.

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339P CHARACTERISATION OF OESTROGEN-DEPENDENT OXYTOCIN RECEPTOR EXPRESSION IN AN IMMORTALISED HUMAN MYOMETRIAL SMOOTH MUSCLE CELL LINE

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Adachi & Oku (1995) described oestrogen-regulated oxytocin receptor expression in human myometrium monolayer primary culture. We have chararacterised binding of [^{3}H]oxytocin to immortalised human myometrial cells (Perez-Reyes *et al.*, 1992) cultured in the presence or absence of β-oestradiol.

Cells were cultured in DMEM/F12 mix (9% FCS). A crude membrane preparation was made by homogenisation (Ultra-Turrax, 3x10sec, full speed) followed by centrifugation (48,000g, 30min, 4°C). Membrane pellets were resuspended in binding assay buffer (50mM HEPES, 10mM $MgCl_2$, pH 7.4). Radioligand binding experiments were performed in 96-well plates using [^{3}H]oxytocin (90 min equilibration, 21°C). An excess (10μM) of unlabelled oxytocin was used to define non-specific binding. Bound and free [^{3}H]oxytocin were separated using filtration (glass fibre, Wallac) and radioactivity counted using solid scintillant (Wallac) and a 96 well beta counter (Wallac Microbeta).

Human myometrial cells cultured in the absence of β-oestradiol showed no significant specific binding of 1nM [^{3}H]oxytocin. However, when cultured for 5 days in the presence of 1μM β-oestradiol, there was significant specific binding of [^{3}H]oxytocin (78fmol/mg protein bound at 1nM [^{3}H]oxytocin).

Immortalised myometrial cells were cultured in the presence of 1μM β-oestradiol for 10 days, and binding of [^{3}H]oxytocin characterised by saturation and competition analysis. Results are shown in tables 1 and 2 (data analysed by non-linear curve fitting).

Oxytocin receptor expression levels were 10 fold higher than those decsribed by Adachi & Oku (1995) in primary culture. The affinity of the compounds correlated very well with binding on membranes prepared from pregnant human myometrial tissue (Jasper *et al.*, 1995; table 2) suggesting that [^{3}H]oxytocin is binding to a similar oxytocin receptor on the immortalised cells.

These results demonstrate that cultured immortalised myometrial cells express oxytocin receptors, but that expression is oestrogen-dependent. These cells should provide a useful model for studying control of oxytocin receptor expression in human myometrium.

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Table 1. Saturation analysis for [^{3}H]oxytocin binding. Results show mean ± s.e.mean (n=3).

pK_d	9.00±0.13
B_{max} (fmol/mg protein)	376±64
Hill slope	1.33±0.19

Table 2. Competition binding against 1nM [^{3}H]oxytocin. Results show mean ± s.e.mean limits (n=5).

	pK_i	pK_i human uterus (Jasper *et al.*, 1995)
Oxytocin	8.68±0.07	8.72
Arg-Vasopressin	8.07±0.08	8.15
Desmopressin	6.79±0.20	6.85
[Thr^4,Gly^7]oxytocin	7.84±0.06	7.66

340P CHARACTERISTICS OF DRUG UPTAKE INTO VESICLES CONTAINING THE MULTIDRUG RESISTANCE ASSOCIATED PROTEIN, MRP

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Multidrug resistance associated protein (MRP) is involved in effluxing cytotoxic drugs, e.g. anthracyclines and vinca alkaloids, but the mechanism by which this occurs is unclear. Reduced glutathione (GSH) appears to be essential for this efflux (Versantvoort et al., 1995). MRP is known to transport glutathione conjugates including 2,4-dinitrophenyl glutathione (DNP-SG) (Jedlitschky et al., 1996). However it is unclear whether MRP can transport daunorubicin or vincristine (VCR) directly, either unchanged or in conjugate form. We here examine uptake of DNP-SG and VCR into inside-out membrane vesicles prepared from resistant MRP-expressing human lung tumour cell line, COR-L23R (Barrand et al., 1994). We compare uptakes with those obtained from membrane vesicles prepared in the same manner from drug-sensitive COR-L23P parent cells and from the resistant human lung tumour cell line, H69/LX4, which expresses P-glycoprotein (Pgp), a direct transporter of daunorubicin and VCR.

Vesicles were prepared from cells lysed in 1mM TrisHCl (pH 7.4). Following centrifugation and resuspension in 250mM sucrose, 10mM TrisHCl (pH7.4), the membranes were centrifuged on a cushion of 38% sucrose, 10mM TrisHCl (pH 7.4). Protease inhibitors were present throughout the procedure. Vesicles were collected from the interface, washed and resuspended for uptake experiments. Following exposure to 3μM ^{3}H-DNP-SG or 200nM ^{3}H-VCR with 1mM ATP (plus regenerating system) at 37 °C, the amount of radiolabelled compound accumulated was measured following rapid filtration. The presence of the two transporter proteins in their respective vesicle preparations was confirmed by Western blot analysis. Sidedness of the vesicles was established by latency of acetylcholinesterase.

ATP-dependent and osmotically sensitive uptake of ^{3}H-DNP-SG (Neo et al, 1996) was observed into MRP-containing vesicles (348±44 pmole mg^{-1} min^{-1}, mean±s.e.m., n=13). Uptake was inhibited by the anion transport inhibitors, DIDS(95%,1mM) and probenecid (64%,1mM), by the sulphydryl reagent DTNB (85%,1mM), and by oxidized glutathione (90%,1mM). No ATP-dependent uptake of ^{3}H-DNP-SG was observed into Pgp-containing vesicles.

We could detect ATP-dependent uptake of ^{3}H-VCR into Pgp-containing vesicles (at 4min with 200nM VCR, ATP-sensitive uptake 21.5±3.9 pmole mg^{-1}, mean±s.e.m., n = 4). There was however no evidence of ATP-sensitive uptake of ^{3}H-VCR with or without 5mM GSH into our MRP-containing vesicles. This is in contrast to the results obtained by Paul et al (1996) and Loe et al (1996). Thus either the MRP in our vesicles cannot transport VCR or VCR leaks out of the vesicles too rapidly to allow nett accumulation. In Pgp-containing vesicles, back leak of VCR was rapid (half-time to steady state of less than 1 min) but uptake was nevertheless detectable. We suggest that VCR transport may depend not only on the presence of MRP but also on other cellular components which may or may not be retained during vesicle preparation. We are currently investigating this further.

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341P MEASUREMENT OF INTRACELLULAR pH (pH_i) IN TUMOUR CELLS CONTAINING THE MULTIDRUG RESISTANCE ASSOCIATED PROTEIN, MRP

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The ability of tumour cells to regulate their internal pH (pH_i) allows them to survive in the unfavourable acidic environment often present inside solid tumours. Therefore various strategies to alter pH have been tried to increase tumour cell kill (Boyer & Tannock, 1992). Many multidrug resistant tumour cell types have elevated pH_i (Thiebaut et al, 1990; Simon et al., 1994) a feature which would decrease their retention of lipophilic weakly basic anticancer drugs. It has been suggested that the drug transporter, P-glycoprotein that is present in these cells may play a role in modulation of pH_i regulation (Roepe, 1995). We have looked at pH_i in the multidrug resistant human lung tumour cell line, COR-L23R (Barrand et al., 1994) that overexpresses another transporter, multidrug resistance associated protein, MRP. The mechanism whereby basic drugs are expelled by this protein are poorly understood but are clearly different from that of P-glycoprotein (Loe et al., 1996). It can however transport anionic species directly.

pH_i was calculated by ratiometric analysis of dual-excitation fluorimetric measurements made at 37 °C using the pH sensitive dye biscarboxyethyl-5(6)carboxyfluorescein (BCECF). Cells grown to near confluence on plastic cover slips were loaded by exposure for 1hr to 5-15μM of the acetoxymethyl ester of BCECF in a solution containing (in mM) 140 NaCl, 5 KCl, 0.5 $MgCl_2$, 1.3 $CaCl_2$, 10 HEPES. When measured at external pHs of 7.0 and 7.4, pH_i reported by the dye inside the resistant cells expressing MRP remained nearer to that of the external solution. At external pH 7, pH_i inside the resistant cells was significantly lower than that inside the parental drug sensitive cells (6.93±0.03 and 7.33±0.04, mean±s.e.m n=7, $p<0.05$). Furthermore, the ability of the resistant cells to recover their pH_i following acidification by ammonium withdrawal was less than that seen with the parental cells. Such differences in the ability to adjust pH_i should mean that resistant cells are more susceptible to changes in external pH, and might be more readily destroyed by strategies that exploit the lower external pH.

It is already recognised that accumulation of BCECF and other fluorescent compounds is lower in cells expressing the MRP protein, but the ratiometric calculation of pH should be independent of the absolute amount of dye present. However when we examined the intracellular distribution of the fluorescent form of the drug using confocal microscopy, clear differences were evident between the resistant and sensitive cells. In the parent cells the dye was evenly spread throughout, but in the resistant cells it was concentrated into small intracellular vesicles. The clear difference in distribution of the pH monitoring dye between the two cell types precludes the simple use of this method to determine the difference in their resting cytoplasmic pH, and raises questions about the interpretation of the fluorescence changes seen following ammonium withdrawal. Distribution of the fluorescent dyes should be taken into account in using such fluorescent indicator dyes.

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342P A NOVEL ASSAY TO DETECT ACTIVATION OF G PROTEIN COUPLED RECEPTORS BY COEXPRESSION OF $G\alpha_{16}$ AND AEQUORIN

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Heterologous expression in mammalian cells of the G-protein alpha sub-unit $G\alpha_{16}$ permits coupling of a wide range of G-protein coupled receptors to phospholipase Cβ activity (Offermans et al,1995). The *Aequorea victoria* photoprotein aequorin emits a characteristic luminescence in the presence of calcium and has been expressed in mammalian cells in order to report agonist signalling through a number of $G\alpha_{q/11}$-coupled receptors that activate phospholipase Cβ (Button et al, 1993).

A CHO cell line stably expressing human $G\alpha_{16}$ was constucted. cDNAs encoding aequorin and receptors of interest were then transiently cotransfected into both the $G\alpha_{16}$ CHO and wild type CHO cells seeded in a 96 well plate. 48 hours post transfection the culture media was replaced with 100μl of fresh media containing 5μM coelentrazine and incubated for two hours. Agonists were added to each well using a microinjector system in 100μl volume of extracellular buffer (140mM NaCl, 20mM KCl, 20mM HEPES, 5mM glucose, 1mM $MgCl_2$, 2mM $CaCl_2$ and 1mg/ml BSA) at room temperature. Luminescence was detected with a conventional plate reader luminometer and was recorded for a ten second interval. An integrated signal, as relative light units in response to agonist or vehicle was used to calculate a fold increase in luminescence.

Application of NECA (1μM), isoprenaline (10μM) and melatonin (10μM) to adenosine A1and A2a, β2-adrenoceptor melatonin Mel_{1A} expressing cells respectively resulted in a large, rapid and transient increase in luminescence, seen only in the $G_{16}\alpha$ subunit expressing CHO cells. Responses to test agonists are plotted in Figure 1.

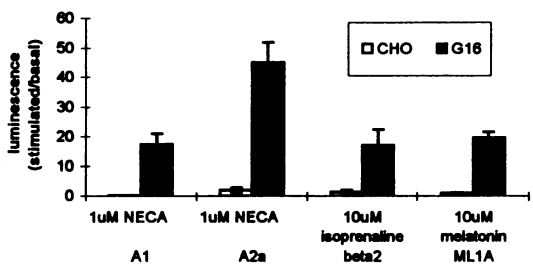

Figure 1. Luminescent Responses to a range of agonists in $G\alpha_{16}$ expressing cells. (n>3)

Through coexpression of $G\alpha_{16}$, aequorin and receptor of interest in mammalian cells we have designed a generic assay to detect agonist stimulation of G protein coupled receptors.

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343P EQUINE NEUTROPHIL ADHERENCE IN RESPONSE TO PAF, C5a, IL-8 AND PMA IS MEDIATED VIA A CD18-DEPENDENT MECHANISM

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Neutrophil recruitment to the airways is a characteristic feature of the equine allergic respiratory condition 'chronic obstructive pulmonary disease' (COPD). Neutrophil recruitment requires adhesion of the cell to the vascular endothelium and extracellular matrix and the CD18 adhesion molecule is considered important in these processes in other species (Zimmerman et al 1992). Equine neutrophils have previously been shown to adhere *in vitro* to fibronectin coated plates (Foster et al 1997) but the underlying mechanisms were not examined. In the present study, equine neutrophil adherence to serum and fibronectin coated plastic has been compared and the effect of pre-incubation with a monoclonal antibody against the equine CD18 molecule has been investigated.

PAF (10^{-11}-10^{-7}M), human recombinant (hr) C5a (10^{-11}-10^{-7}M) and IL-8 (10^{-11}-10^{-7}M) or PMA (10^{-7}M) were added to microtitre plates coated with either human fibronectin or autologous serum. Neutrophils isolated from 6 normal horses ($1x10^5$cells/well) were added 5min later. After a 30min incubation at 37°C non-adherent cells were removed and adherent cells lysed. The alkaline phosphatase content was measured colorimetrically and adherence calculated as a % of the total cells added to each well. Neutrophils were also pre-incubated with the anti-equine CD18 monoclonal antibody H20A (VMRD Inc., Washington, USA) or an isotypically matched control antibody for 30min. The effects on adherence caused by a single concentration of each agonist was tested.

Equine neutrophils adhered to both serum and fibronectin coated plastic in response to each of the agonists tested.

Table 1 Effect of preincubation with an anti-CD18 antibody on equine neutrophil adherence to serum and fibronectin coated plastic

Antibody(μg/ml)	Control Ab	1	10	50
Serum (n=3)		**% adherence**		
PAF (10^{-9}M)	15.8±5.2	11.3±4.9	7.7±2.9	*6.4±2.9
hIL-8 (10^{-9}M)	6.9±1.5	*1.7±0.2	*0.9±0.2	*0.1±0.2
hC5a(10^{-8}M)	7.0±2.0	*3.2±1.0	**2.1±1.1	*3.7±2.4
PMA ($2.5x10^{-9}$M)	8.7±2.9	10.0±2.9	*1.3±2.6	3.7±2.4
Fibronectin (n=3)				
PAF (10^{-9}M)	21.9±3.1	16.5±2.3	11.3±0.3	*6.5±1.6
hIL-8 (10^{-8}M)	17.0±3.6	9.2±1.5	6.1±0.4	*3.6±1.5
hC5a(10^{-8}M)	17.5±2.5	13.8±2.3	8.8±0.6	4.8±1.1
PMA ($2.5x10^{-9}$M)	17.1±5.1	12.6±5.0	8.2±1.9	4.6±1.8

*p<0.05 **p<0.01 vs control (2 way ANOVA and Tukeys HSD test)

Preincubation with the anti-CD18 antibody significantly reduced adherence to serum in response to all agonists (table). Similarly, agonist-induced adherence to fibronectin was reduced. However, this only reached statistical significance for PAF and IL-8.

These results demonstrate that adherence of equine neutrophils to both serum and fibronectin in response to PAF, C5a, IL-8 and PMA occurs via a CD18 dependent mechanism.

We thank the Home of Rest for Horses for financial support.

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344P THE HUMAN CRF1 RECEPTOR EXPRESSED IN HEK 293 CELLS STIMULATED [^{35}S]-GTPγS BINDING AS WELL AS cAMP ACCUMULATION

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As well as a role in the regulation of pituitary hormone secretion, corticotropin-releasing factor (CRF) is thought to act as a neurotransmitter in human brain, and dysfunction of CRF systems has been implicated in psychiatric disorders (Gold et al, 1995). Functional testing for the cloned human CRF1 receptor has used cAMP accumulation (e.g. Chen et al, 1993), but this technique is relatively slow, expensive and requires intact cells. We have therefore examined [^{35}S]-GTPγS binding to cell membranes as an alternative functional assay.

Table 1: Radioligand binding (pK_i) and functional (pEC_{50} or pK_b) potencies at cloned human CRF1 receptors.

Compound	Binding	[^{35}S]-GTPgS	cAMP
	pK_i	pEC_{50} / *pK_b	pEC_{50} / *pK_b
urocortin	8.4 ± 0.1 (4)	8.1 ± 0.2 (4)	8.8 ± 0.1 (3)
sauvagine	8.0 ± 0.1 (3)	6.8 ± 0.1 (3)	8.8 ± 0.1 (3)
h/rCRF	7.6 ± 0.1 (6)	7.3 ± 0.1 (4)	8.2 ± 0.1 (3)
oCRF	7.5 ± 0.1 (10)	6.9 ± 0.1 (3)	8.7 ± 0.2 (3)
VIP	<6	<5	<5
α-hel-CRF	6.5 ± 0.1 (3)	*6.8 ± 0.2 (4)	*6.5 ± 0.1 (3)

Values given are mean ± s.e.m. from (n) experiments.

Abbreviations: h/rCRF = human/rat CRF; oCRF = ovine CRF; α-hel-CRF = alpha helical CRF-(9-41)

Our studies used HEK 293 cells expressing the human CRF1 receptor. Radioligand binding and cAMP assays were carried out as described (Herdon et al, 1995; Chen et al, 1993) with the exception that cAMP accumulation was stopped with perchloric acid and neutralised with NaOH. [^{35}S]-GTPγS binding used the method of Thomas et al (1996) with a GDP concentration of 1 μM.

All the agonists had intrinsic activities similar to that of h/rCRF in both assays. In [^{35}S]-GTPγS binding assays, the rank order of potency of agonists was similar to that for radioligand binding affinity, with the exception of sauvagine (Table 1). In cAMP accumulation assays, all the agonists had similar potencies which were higher than in GTPγS binding. VIP was inactive in all assays. α-hel-CRF (2 μM) produced a parallel rightward shift of the h/rCRF concentration-response curve with no change in maximum in both assays and had functional potency which matched its radioligand binding affinity.

For the ligands tested, [^{35}S]-GTPγS binding appears to offer an alternative functional test for the human CRF1 receptor. The pharmacology observed in general resembles that seen in radioligand binding although data are needed for a larger number of ligands to confirm the relationship between binding and functional potencies. The higher agonist potencies seen in the cAMP assay compared with [^{35}S]-GTPγS binding suggests amplification of the transduction pathway downstream of G-protein activation. Further studies are needed to determine whether the two functional responses are mediated through the same G-protein.

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Thomas, D.R. et al. *J. Receptor & Signal Trans. Research* **15**(1-4) 199-211 (1995)

345P ANTI-SENSE AND IMMUNONEUTRALISATION STUDIES REVEAL A ROLE FOR LIPOCORTIN 1 IN THE CONTROL OF GROWTH HORMONE *IN VIVO* AND *IN VITRO*

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We have recently provided evidence for a role for lipocortin 1 (LC1) in the regulatory actions of glucocorticosteroids on growth hormone (ir-GH) release *in vitro* (Taylor *et al*, 1996). In the present study we have used *in vivo* and *in vitro* models to investigate further the effects of LC1 on the control of ir-GH release. *In vitro* studies exploited an antisense oligonucleotide to a unique sequence of cDNA which encodes the N-terminal to rat LC1. Anterior pituitary tissue from adult male rats was dispersed (collagenase and trituration) and the resultant cell suspension was plated out ($2.5x10^5$cells/well/ml) into 24 well tissue culture plates and incubated for 2.5h under controlled conditions at 37°C. The cells were then challenged (1h) with submaximal concentrations of forskolin (100nM), vasoactive intestinal peptide (VIP, 100nM) or the L-Ca^{++} channel agonist BAY K8644 (10nM); the medium was collected and assayed for ir-GH using enzyme-linked immunosorbant assay and the tissue was stored for LC1 measurement and histology. Results were analysed using Duncan's multiple range test for *in vitro* results (n=6). Where appropriate dexamethasone (Dex., 0.1μM) was included throughout all incubation periods and LC1 antisense, sense or a scrambled control (50nM) were added at times 0, 1 and 2.5h. Examination of the cells at the electron microscope level revealed that the ultrastructural morphology was well maintained after the dispersal procedure. In oligonucleotide-free groups forskolin, VIP and BAY K8644 induced significant ($P<0.01$) approximatly four fold increases in ir-GH release which were significantly ($P<0.01$), reversed by Dex. LC1 antisense had no effect on basal ir-GH release in either the presence or absence of Dex.; however, the antisense oligonucleotide reversed ($P<0.05$) the inhibitory actions of Dex. on the secretagogue-induced release of ir-GH (eg. forskolin alone 250±36 vs. forskolin + Dex. 87±14 ng/ml vs. forskolin + Dex. + antisense 268±11 ng/ml. By contrast, in all experiments LC1 sense and the scrambled oligonucleotide control were without effect. Western blot analysis demonstrated that in oligonucleotide-free groups Dex. (0.1μM, 3.5h) caused an increase in the expression of ir-LC1 on the outer surface of the pituitary cells when compared to control groups. The response was unaffected by LC1 sense and the scrambled nucleotide (50nM, 3.5h); in contrast the increase in LC1 expression induced by the steroid was not evident in those groups treated with the LC1 antisense (50nM, 3.5h).

For *in vivo* studies adult male CFY rats (≈200g) which had been handled daily for 10 days were injected subcutaneously on two successive days with either a purified polyclonal anti-LC1 antibody (LC1-Ab) or a corresponding volume (1ml/kg) of similarly purified non-immune sheep serum (NSS). On the following morning, rats from each group (n=12-16) were given either Dex. (10μg/kg, i.p.) or its saline vehicle (1ml/kg, i.p.) followed 1h 15 min later by IL-1ß (3μg/kg., i.p) or its saline vehicle (1ml/kg, i.p.). Blood was collected 75min. later and assayed for ir-GH; data were analysed by Scheffé's test. IL-1ß produced significant ($P<0.05$) rises in the serum ir-GH concentration in NSS and anti-LC1-Ab treated animals (NSS treated 300±25 vs. 140±12 ng/ml: anti-LC1 Ab treated 490±50 vs 180±18 ng/ml). The rises in ir-GH release precipitated in NSS treated rats by IL-1ß were abolished by Dex. (185±25 vs. 300±25 ng/ml, $P<0.05$). By contrast, in animals treated with anti-LC1 Ab the inhibitory effects of the Dex. were not apparent (410±45 vs. 490±50 ng/ml, N.S.).

The results suggest a role for LC1 as a mediator of the regulatory actions of glucocorticoids on GH release in the rat.

We are grateful to the Wellcome Trust (grant 041943/Z/94/Z) for financial support.

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346P LACK OF EFFECT OF POTASSIUM CHANNEL MODULATORS ON INTRA-CELLULAR CALCIUM RELEASE AND UPTAKE IN RABBIT ISOLATED MESENTERIC ARTERIES

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Previous studies have suggested that agents which modulate the conductance of ATP-sensitive potassium (K_{ATP}) channels also effect the release from and uptake of calcium (Ca^{2+}) by the sarcoplasmic reticulum (SR: Bray *et al*, 1991; Chopra *et al*, 1992). Ca^{2+} can be released from the SR of smooth muscle cells by either inositol 1,4,5 trisphosphate (IP_3) or caffeine (Iino, 1990). The present study investigated the direct effects of levcromakalim and glibenclamide, a K_{ATP} channel opener and blocker respectively, on IP_3- and caffeine- stimulated Ca^{2+} release from, and Ca^{2+} uptake into the SR of permeabilized mesenteric artery smooth muscle cells.

Female New Zealand White rabbits were injected with sodium pentobarbitone (60 $mgkg^{-1}$) and killed by rapid exsanguination. The third branch of the mesenteric artery was removed and a strip cut, 2mm long and 200 μm in diameter, and attached to a sensitive force transducer. The strip was permeabilized with *Staphylococcus aureus* α-toxin as previously described (Parsons *et al*, 1995). Data are expressed in mN as mean ± s.e.mean.

Both IP_3 and caffeine stimulated reproducible, transient contractions in permeabilized artery strips, providing the strip was incubated in a mock intracellular solution containing 0.08 μM Ca^{2+} for 15 mins between each exposure. The mean response to IP_3 (100 μM), in the presence of GTP (100 μM), was 0.11 ± 0.3 mN (n = 9). Neither levcromakalim (1 μM) nor glibenclamide (1 μM) altered the amplitude of contraction to IP_3/GTP (0.12 ± 0.08 mN and 0.12 ± 0.10 mM, respectively; n = 4, $p> 0.05$). The mean response to caffeine (10 mM) was 0.29 ± 0.05 mN (n = 15), which was also unaltered by either levcromakalim (1 μM) or glibenclamide (1 μM: 0.29 ± 0.1 mN and 0.30 ± 0.03 mN, respectively; n = 4; $p> 0.05$).

Ca^{2+} uptake into IP_3 and caffeine sensitive stores (measured by the magnitude of contraction) was unaltered by levcromakalim or glibenclamide. Mean responses to IP_3 (100 μM) when either levcromakalim (1 μM) or glibenclamide (1 μM) were included in the 0.08 μM Ca^{2+} solution, were 0.11 ± 0.1 mN and 0.10 ± 0.1 mN, respectively (n=4; $p>0.05$). Similarly, Ca^{2+} uptake into caffeine-sensitive stores was unaltered by either levcromakalim or glibenclamide with mean responses of 0.27 ± 0.13 mN and 0.28 ± 0.14 mN, respectively (n=4; $p>0.05$). Increasing the concentration of levcromakalim and glibenclamide to 10 μM failed to inhibit responses to IP_3 or caffeine, or Ca^{2+} uptake into the SR.

These data indicate that levcromakalim and glibenclamide, which modulate the conductance state of K_{ATP} channels, do not also indirectly modify the intracellular Ca^{2+} handling in mesenteric artery smooth muscle cells.

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347P ENDOTHELIN INCREASES INTRACELLULAR Ca^{2+} IN SK-N-MC CELLS BY AT LEAST TWO DIFFERENT MECHANISMS

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The human neuroblastoma cell-line SK-N-MC contains endothelin-receptors (ET-R) that couple to inositol phosphate (IP) formation and increases in intracellular Ca^{2+} (Wilkes & Boarder. 1991; Huggins et al.. 1994). This study aimed to further characterize the mechanism underlying these effects.For this purpose we assessed in SK-N-MC cells the effects of several ET-R agonists on intracellular Ca^{2+} (determined by Fura-2 fluorescence. Michel et al.. 1992) and on IP-formation (determined as accumulation of total [^{3}H]-IP's in [^{3}H]myo-inositol prelabelled cells during a 30 min incubation at 37°C in Krebs-Henseleit solution that contained 10 mM LiCl) in the absence and presence of the ET_A-R antagonist BQ-123 (Moreland. 1994).

ET-R agonists increased IP-formation (max. increase at 10 nM ET-1: 149±43% above basal. n=6) with an order of potency ET-1 (pEC_{50} 9.8±0.3) > sarafotoxin 6b (Sf6b pEC_{50}: 7.1±0.25) > ET-3; Sf6c had no effect. The same order of potency (pEC_{50} ET-1 8.3±0.4; Sf6b 6.3±0.3) was obtained for ET-R agonists induced increase in intracellular Ca^{2+} (max. increase at 1 µM ET-1: 125±23 nM. n=6). BQ-123 (1nM-10µM) antagonized ET-1 induced increases in IP-formation (apparent pK_B 8.3±0.3) and in Ca^{2+} (apparent pK_B 8.6±0.5) indicating that predominantly ET_A-R are involved. Chelation of extracellular Ca^{2+} by 5 mM EGTA decreased ET-1 evoked max. increase in Ca^{2+} by 59±7% (n=3). Pretreatment of the cells with 500ng/ml pertussis toxin (PTX) overnight completely inactivated PTX-substrates (as assessed by ADP-ribosylation); this did not attenuate but rather enhanced ET-1 induced IP-formation (max. increase in not-PTX-treated cells: 116±4%.in PTX-treated cells: 175±28%. n=3).In contrast. PTX-treatment significantly reduced ET-1 induced max. increase in intracellular Ca^{2+} by 45±6% (n=3).

ET-1 induced Ca^{2+} increases returned towards baseline levels within a few min although ET-1 was continuously present in incubation medium. Subsequent addition of ET-1 did not increase Ca^{2+} suggesting rapid desensitization. A similar rapid desensitization was observed for carbachol that is known to increase intracellular Ca^{2+} in SK-N-MC cells (Michel et al., 1992). On the other hand. carbachol-effects were only slightly attenuated after the ET-challenge as was the ET-effect only slightly attenuated after the carbachol challenge indicating that homologous desensitization was more pronounced than heterologous desensitization.

In conclusion: in SK-N-MC cells stimulation of ET_A-R causes increases in intracelullar Ca^{2+} via at least two different mechanisms: a PTX-sensitive influx and a PTX-insensitive mobilisation of Ca^{2+} from intracellular stores.ET_A-R undergo rapid desensitization following short-term activation by ET-1.

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348P EXCITOTOXIC LESIONS OF THE NUCLEUS ACCUMBENS DO NOT AFFECT THE HYPERPHAGIA INDUCED BY 8-OH-DPAT

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Infusion of the non-selective dopamine antagonist α-flupenthixol into the rat nucleus accumbens blocks the increase in feeding observed after application of 8-OH-DPAT to either the DRN or MRN (Fletcher, 1991). To further assess the role of the ventral striatum in mediating 8-OH-DPAT-induced hyperphagia, the present experiment investigated the effect of bilateral excitotoxic lesions of the nucleus accumbens (n.acc.) on the feeding induced by 8-OH-DPAT.

24, male Lister hooded rats (initially 300-440g) were anaesthetised with O_2/N_2O and halothane (3%). 1µl of 10 mg/ml quinolinic acid (or phosphate-buffered saline for controls) was administered bilaterally at the following co-ordinates (relative to bregma, incisor bar -3.5mm): AP +1.2, L ±1.5, DV -7.0 from the dura.

Measurement of food intake following 8-OH-DPAT (vehicle, 0.03, 0.1, 0.3 mg/kg s.c.) began one week after surgery. After dosing, animals were returned to the home cage and allowed access to weighed hoppers of standard laboratory chow for two hours. All animals received each dose of 8-OH-DPAT in a counterbalanced order. One week was allowed between successive drug administrations. Brains were removed and fixed in formal saline for histological processing. Lesions were evaluated blind to the behavioural data. Only those animals with bilateral damage limited to the n.acc. were included in the study. One control animal was removed from analysis due to the presence of gliosis in the n.acc..

The results demonstrate (see Table 1) that systemic administration of 8-OH-DPAT leads to an increase in food intake in both control animals and animals with excitotoxic lesions of the n.acc.. At all doses of 8-OH-DPAT, lesioned animals ate less chow than controls ($F(1,14) = 10.99$, $P<0.005$). However, this effect is likely to be attributable to the fact that the lesions led to a significant reduction in body weight over the course of the experiment ($F(1,14) = 26.98$, $P<0.001$).

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Table 1. The effect of excitotoxic lesions of the nucleus accumbens on 8-OH-DPAT-induced hyperphagia.

	8-OH-DPAT				Change in weight
mg/kg	0	0.03	0.1	0.3	-
Control (n=11)	1.95 ± 0.5	2.64 ± 0.6	3.34 ± 0.5	3.85 ± 0.4 *	142 ± 7.2
Lesion (n=5)	0.79 ± 0.4	1.66 ± 0.8	1.48 ± 0.4	3.23 ± 0.7 **	84 ± 14.5 #

Data are the mean food intake (g) ± sem and the mean increase in body weight (g) ± sem. *P <0.05, ** or # P <0.01. * or ** cf. vehicle group, # cf. control group.

349P [^{3}H]PAROXETINE BINDING INDICATES THAT SIBUTRAMINE IS NOT NEUROTOXIC AND, LIKE FLUOXETINE, IT PROTECTS AGAINST THE DEFICITS IN 5-HT REUPTAKE SITES PRODUCED BY *d*-FENFLURAMINE

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Sibutramine is a 5-HT and noradrenaline reuptake inhibitor in vivo (Buckett et al., 1988) which produces weight-loss in obese patients (Weintraub et al., 1991). The action of sibutramine to enhance 5-HT function is shared by other weight reducing agents, including fluoxetine and d-fenfluramine. The 5-HT releasing properties of d-fenfluramine result in persistent deficits in regional brain levels of 5-HT, 5-hydroxyindoleacetic acid and 5-HT reuptake sites in both rodents and primates (McCann et al., 1994). The aim of this study was to determine the effects of d-fenfluramine and sibutramine on brain 5-HT reuptake sites, labelled with [^{3}H]paroxetine. In addition, the ability of fluoxetine and sibutramine to reverse the deficits in [^{3}H]paroxetine binding produced by d-fenfluramine was examined.

Male CD rats (80-100g) were administered sibutramine (9mg/kg po) or d-fenfluramine (1, 3 and 10mg/kg po) for 4 days followed by a 14 day drug-free period. Sibutramine (9mg/kg po), or fluoxetine (10mg/kg ip) were also given alone or 1hr prior to d-fenfluramine (10mg/kg, po) as above. Controls received vehicle. Brain regions were then removed. [^{3}H]Paroxetine binding was measured in one half of the brain (Cheetham et al., 1993) and monoamine concentrations were determined in the other half (Butler et al., this meeting).

Sibutramine (9mg/kg) had no significant effect on the number of 5-HT reuptake sites in the brain regions studied. In contrast, d-fenfluramine (3mg/kg) significantly decreased the number of sites in frontal cortex (35%), hippocampus (47%) and hypothalamus (27%). This effect was dose-dependent with profound decreases in all regions (58-84%) at 10mg/kg. Fluoxetine completely blocked the effect of d-fenfluramine without having any effect on its own (Table 1). Sibutramine also blocked the effect of d-fenfluramine, although the reversal was only partial in frontal cortex, hippocampus and hypothalamus (Table 1).

Sibutramine does not decrease brain 5-HT reuptake sites, unlike d-fenfluramine. The releasing agent, d-fenfluramine, is taken into 5-HT nerve terminals by high affinity reuptake, where it displaces 5-HT from storage vesicles; reuptake inhibitors prevent this process. Thus, sibutramine and fluoxetine protect against the deficits in 5-HT reuptake sites produced by d-fenfluramine. These data provide further evidence that sibutramine is a 5-HT reuptake inhibitor in vivo and it does not have neurotoxic potential.

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TABLE 1. Effect of fluoxetine, sibutramine and d-fenfluramine alone, or in combination, on [^{3}H]paroxetine binding

	Veh	Flu	Fen	Flu/Fen	Veh	Sib	Fen	Sib/Fen
Frontal cortex	650 ± 14	690 ± 15	113 ± 3***	701 ± 6†††	815 ± 24	807 ± 27	167 ± 5***	652 ± 9♦†††
Hippocampus	439 ± 16	437 ± 15	92 ± 3***	461 ± 6†††	475 ± 14	482 ± 15	95 ± 3***	331 ± 10**♦♦♦†††
Hypothalamus	1284 ± 40	1295 ± 40	454 ± 14***	1176 ± 37†††	769 ± 29	865 ± 35	333 ± 13***	592 ± 22♦♦†
Dorsal raphe	735 ± 36	809 ± 35	410 ± 18**	803 ± 35††	747 ± 44	662 ± 37	311 ± 17***	720 ± 40††
Striatum	651 ± 15	626 ± 15	260 ± 6***	625 ± 15†††	768 ± 20	692 ± 19	320 ± 8***	689 ± 18†††

B_{max} values (fmol/mg protein) ± s.e. mean; n = 6 - 10. ** $p < 0.01$, *** $p < 0.001$ versus vehicle (Veh); ♦ $p < 0.05$, ♦♦ $p < 0.01$, ♦♦♦ $p<0.001$ Sibutramine (Sib) vs Sib/d-fenfluramine (Fen). † $p < 0.05$, †† $p < 0.01$, ††† $p<0.001$ interaction between Sib or fluoxetine (Flu) and Fen.

350P *d*-FENFLURAMINE-INDUCED DEPLETION OF RAT BRAIN 5-HT IS PREVENTED BY FLUOXETINE OR SIBUTRAMINE PRETREATMENT

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The weight-loss agent sibutramine (SIB) is a 5-hydroxytryptamine (5-HT) and noradrenaline reuptake inhibitor (Luscombe *et al.*, 1990). Enhanced 5-HT function is not only a property of SIB, but also other weight-reducing drugs, e.g. fluoxetine (FLU) and *d*-fenfluramine (FEN). However, in microdialysis experiments where SIB and FLU cause small increases in extracellular 5-HT, FEN causes large increases via direct release, which can be reversed by pretreatment with SIB or FLU (Gundlah *et al.*, 1996). The 5-HT releasing effect of FEN results in persistent deficits in brain levels of 5-HT, 5-hydroxyindoleacetic acid and 5-HT reuptake sites in both rodents and primates (McCann *et al.*, 1994, Cheetham *et al.*, 1996). We examined the effects of repeated FEN, FLU and SIB treatment on brain 5-HT levels and determined the ability of FLU and SIB to reverse the depleting effects of FEN.

Male Sprague-Dawley rats (80-100g) received vehicle (5 ml/kg), FEN 1, 3, 10 mg/kg p.o., SIB 9 mg/kg p.o. or FLU 10 mg/kg i.p. for 4 days, *b.i.d.*, alone or in combination (SIB or FLU 1 h prior to FEN). Fourteen days later, brains were removed and hemisected. The frontal cortex, hippocampus, striatum and hypothalamus were removed, frozen, then one half assayed for 5-HT (Heal *et al.*, 1993), while [^{3}H]paroxetine binding was measured in the other half (Cheetham *et al.*, 1996) Data analysis was by 1- or 2-way ANOVA followed by Williams' test or Multiple t-test.

FEN (1, 3 [data not shown] & 10 mg/kg [Table 1]) decreased 5-HT levels in all regions except the dorsal raphe, in a dose-related fashion. By contrast, FLU and SIB had no effects on brain 5-HT levels (Table 1). Additionally, both FLU and SIB reversed the FEN-induced decreases in 5-HT in the majority of areas (Table 1).

These data confirm FEN persistently depletes brain 5-HT (McCann *et al.*, 1994). Consistent with their effects as monoamine reuptake inhibitors, FLU and SIB do not deplete brain 5-HT. Furthermore, and in agreement with microdialysis studies (Gundlah *et al.*, 1996), FLU and SIB block the 5-HT depleting effects of FEN because they prevent the entry of FEN into 5-HT nerve terminals.

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Table 1. 5-HT levels 2 weeks after 4 days treatment with *d*-fenfluramine (FEN), fluoxetine (FLU) or sibutramine (SIB) alone, or in combination.

	Saline/water	FLU 10	FEN 10	FLU/FEN 10	Water/water	SIB 9	FEN 10	SIB/FEN 10
Frontal cortex	509 ± 29	513 ± 29	176 ± 10 **	507 ± 29 ††	675 ± 39	644 ± 38	234 ± 14 **	534 ± 31 * ††
Hippocampus	420 ± 32	446 ± 33	151 ± 11 **	448 ± 34 ††	525 ± 30	552 ± 32	214 ± 12 **	480 ± 28 ††
Striatum	446 ± 40	596 ± 54	250 ± 22 **	534 ± 48 †	401 ± 25	346 ± 22	220 ± 13 **	354 ± 22 ††
Hypothalamus	947 ± 55	912 ± 53	673 ± 39 **	873 ± 50	802 ± 43	756 ± 42	579 ± 31 **	704 ± 39 †
Dorsal raphe	1088 ± 65	1158 ± 69	983 ± 59	1049 ± 63	992 ± 108	1012 ± 113	906 ± 99	916 ± 100

5-HT levels (ng/g wet tissue weight) mean ± s.e. mean (n = 8-18). For comparisons with vehicle, *$p<0.05$ **$p<0.01$, Williams' test. For interactions between drugs, †$p<0.05$, ††$p<0.01$, Multiple t-test.

351P COMPARISON OF THE EFFECTS OF SIBUTRAMINE, FLUOXETINE AND *d*-FENFLURAMINE ON EXTRACELLULAR 5-HT IN RAT ANTERIOR HYPOTHALAMUS: AN *IN VIVO* MICRODIALYSIS STUDY

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Sibutramine (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-3-N,N-dimethylamine hydrochloride monohydrate), an *in vitro* and *in vivo* inhibitor of noradrenaline and 5-hydroxytryptamine (5-HT) reuptake (Buckett *et al.*, 1988; Luscombe *et al.*, 1989), reduces food intake in rats (Jackson *et al.*, 1996) and produces a dose-related improvement in the weight loss of obese patients on a restricted calorie diet (Weintraub *et al.*, 1991). We have used *in vivo* microdialysis to measure extracellular 5-HT levels in the rat anterior hypothalamus to compare the effects of sibutramine with the food intake modifiers, fluoxetine (SSRI) and d-fenfluramine (5-HT releasing agent).

Male Wistar rats (265-310 g) were anaesthetised with Equithesin (1 ml, i.p.). A microdialysis probe (CMA/12, 2 mm length, 0.5 mm diameter) was implanted into the anterior hypothalamus (co-ordinates from bregma: AP -1.8 mm, L +0.8 mm, V -9.3 mm, flat skull position). The probe was perfused (1.2 µl/min) with an aqueous salt solution. Sixteen to 20 h after surgery, 20 min dialysates were collected. Basal samples (8.8 ± 0.9 fmol/20 µl, n=55) were collected prior to drug (1-10 mg base/kg i.p.) or saline (2ml/kg i.p.) administration and 20 min samples taken for a further 3 h. Dialysate 5-HT content was determined by HPLC with electrochemical detection. Post-injection 5-HT levels were expressed as percentage mean basal level determined from the four pre-injection control samples. Each treatment was compared to saline vehicle using Williams' test and to other treatments using Newman-Keul's test. Maximum values were compared by one-way analysis of co-variance and times to maximum by Wilcoxon's rank-sum test.

Sibutramine (1-10 mg/kg) evoked a slow, dose-related increase in dialysate 5-HT. The maximum increase (214 ± 36%, P<0.05, n=5) occurred 100 min after the 10 mg/kg dose. Fluoxetine (10 mg/kg) produced a maximum increase of 406 ± 56% (P<0.001, n=4), also 100 min after injection. By contrast, d-fenfluramine (1-10 mg/kg) caused a rapid and far greater elevation of 5-HT. This was maximal (2145 ± 337%; P<0.001, n=5) 40 min after 10 mg/kg and 5-HT levels remained signficantly higher than control for up to 3 h post-injection. Similarly, d-fenfluramine (3 mg/kg) evoked a 528 ± 67% (P<0.001, n=5) increase 40 min after injection.

Although all three drugs elevated dialysate 5-HT, their effects can be differentiated both qualitatively and quantitatively. For example, at equivalent food intake reducing doses (Wong *et al.*, 1988; Jackson *et al.*, 1996), d-fenfluramine (3 mg/kg) produced an increase in 5-HT that was greater (P<0.01) and faster in onset (P<0.05) than either sibutramine (10 mg/kg) or fluoxetine (10 mg/kg). In conclusion, these results show that the *in vivo* effects of sibutramine on extracellular 5-HT are similar to those of the SSRI, fluoxetine, but clearly differ from those of the 5-HT releasing agent, d-fenfluramine.

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352P CHRONIC ADMINISTRATION OF SIBUTRAMINE REDUCES PLASMA GLUCOSE LEVELS IN OBESE HYPERGLYCAEMIC (*ob/ob*) MICE

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Sibutramine is a noradrenaline and 5-hydroxytryptamine reuptake inhibitor (Buckett *et al.*, 1988), being developed to treat obesity and has been shown to reduce weight and improve glycaemic control in overweight non-insulin dependent diabetics (Griffiths *et al.*, 1995). This study explores the effects of sibutramine on plasma glucose and insulin levels in obese *ob/ob* mice (Aston strain), which are hyperglycaemic and hyperinsulinaemic.

Individually-housed male and female *ob/ob* mice (n=24-36) were maintained on reversed phase lighting (lights off 10:00-18:00h) with free access to food and water. Plasma glucose and insulin were measured by the glucose oxidase method (Analox, GM6) and RIA (Biotrak Amersham RPA 547) after a 7 day run-in; after 14 and 28 days of sibutramine treatment (10 mg/kg po daily at 10:00h) or vehicle (water) and 14 days after drug withdrawal. Body weight and food intake were measured every day.

Plasma glucose and insulin levels were not significantly different between the two groups at baseline or after 14 days treatment with sibutramine. However, plasma glucose levels of mice treated with sibutramine for 28 days were significantly reduced compared to the vehicle-treated group (Table 1) and returned to control levels 14 days following withdrawal of sibutramine. Plasma insulin levels showed similar trends but the results were not significant. Sibutramine treatment and its withdrawal had no significant effect (P>0.05, ANOVA and Dunnett's test) on body weight (g, 74±1, 77±3 and 79±5 for vehicle and 73±1, 77±1 and 77±1 for sibutramine) or daily food intake (g/kg, 118±5, 112±7, 98±18 for vehicle and 130±6, 110±5 and 98±8 for sibutramine) at baseline, day 29 and day 43, respectively (values are mean±s.e.mean).

These results demonstrate that chronic administration of sibutramine significantly reduces plasma glucose levels in *ob/ob* mice. At the same time, sibutramine tends to reduce insulin levels. The glucose-lowering effect of sibutramine appears to be independent of its ability to reduce body weight and food intake and could be an important beneficial property of sibutramine in the treatment of obese and, particularly obese diabetic, patients.

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Table 1. Effects of chronic sibutramine treatment and withdrawal on plasma glucose and insulin levels in *ob/ob* mice

	Day of study	Duration of treatment	Plasma glucose (mmol/l)		Plasma insulin (ng/ml)	
			Vehicle	Sibutramine	Vehicle	Sibutramine
Chronic treatment	15	14 days	21.7±0.9	19.0±0.6	49.7±3.5	45.1±2.6
Chronic treatment	29	28 days	26.0±1.4	15.8±0.7**	46.2±4.5	35.1±2.7
Drug withdrawal	43	14 days	22.9±1.7	23.6±1.2	40.6±8.6	77.1±11.3

Measurements were made 24h following chronic drug administration. Results are back-transformed means±s.e.mean adjusted for differences between treatment groups at baseline. **P<0.01 vs. vehicle (one-way ANCOVA followed by the William's test).

353P SIBUTRAMINE AND ITS ACTIVE METABOLITES DO NOT RELEASE [^{3}H]-NORADRENALINE FROM RAT HEART OR BRAIN SLICES *IN VITRO*: COMPARISON WITH OTHER WEIGHT REDUCING AND SYMPATHOMIMETIC DRUGS

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Sibutramine (SIB) is a noradrenaline (NA) and 5-hydroxytryptamine reuptake inhibitor which is an effective weight-loss agent (Weintraub *et al.*, 1991). SIB produces its effects *in vivo* predominantly via its secondary and primary amine metabolites, BTS 54 354 and BTS 54 505 (Luscombe *et al.*, 1989). Here, we determine whether SIB and other weight-reducing agents release NA from brain or act as sympathomimetics.

Tissue slices from rat heart or frontal cortex were loaded with [^{3}H]NA (480 nM) in the presence of pargyline (0.13 mM, and superfused (1 ml/min) with Krebs buffer with or without drug (brain slices: SIB, BTS 54 354, BTS 54 505, *d*-amphetamine, *d*-norfenfluramine or *d*-fenfluramine, 10^{-7}-10^{-5} M; heart slices: 50 mM KCl, *d*-fenfluramine, *d*-norfenfluramine, *d*-amphetamine, ephedrine, desipramine, SIB, BTS 54 354 or BTS 54 505, all 10^{-5}M). After 4 (2 min) basal samples, drug was added for 4 fractions, followed by 5 fractions without drug. Sample 5-13 fractional release was calculated and data were analysed by Dunnett's test, or two-way ANOVA followed by Dunnett's test.

SIB, BTS 54 354 and BTS 54 505 did not evoke release of [^{3}H]NA from either heart or brain slices (Figures 1a and b). In brain slices, *d*-fenfluramine and *d*-norfenfluramine enhanced [^{3}H]NA overflow at 10^{-5} M, whilst *d*-amphetamine dose-dependently released this monoamine (Figure 1a). In rat heart slices, KCl, *d*-fenfluramine, *d*-norfenfluramine, *d*-amphetamine, ephedrine and desipramine all enhanced unstimulated [^{3}H]NA overflow at 10^{-5} M (Figure 1b).

These results provide clear evidence that *in vitro*, SIB, BTS 54 354 and BTS 54 505 are neither NA releasing agents from brain tissue, nor sympathomimetic, i.e. NA releasing, drugs in rat heart. This supports the hypothesis that unlike other weight loss agents, central NA release does not contribute to the pharmacological action of SIB or its metabolites. Furthermore, in contrast to SIB, other weight-loss agents, e.g. *d*-amphetamine and *d*-fenfluramine, show sympathomimetic actions on rat heart tissue *in vitro*.

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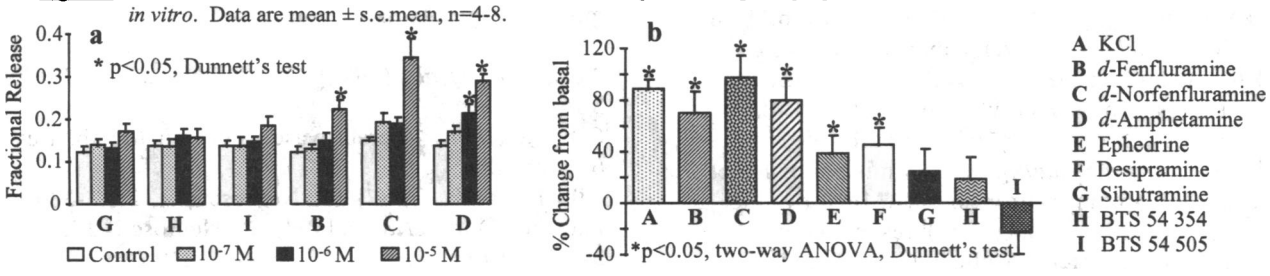
Figure1 Effect of Sibutramine, BTS 54 354, BTS 54 505 and comparator drugs on [^{3}H]NA overflow from rat (**a**) brain, or (**b**) heart slices *in vitro*. Data are mean ± s.e.mean, n=4-8.



354P PRE- AND POST-SYNAPTIC METABOTROPIC GLUTAMATE RECEPTOR PHARMACOLOGY IN GUINEA-PIG OLFACTORY CORTICAL NEURONES *IN VITRO*

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Activation of metabotropic glutamate receptors (mGluRs) in cortical neurones results in a sustained increase in cell excitability, consisting of a prolonged membrane depolarization and the induction of a slow post-stimulus afterdepolarization (sADP) (Constanti & Libri, 1992). However, it is unclear whether these effects are linked to activation of phosphoinositide (PI) hydrolysis (Group I mGluRs) or inhibition of adenyl cyclase (Group II or III mGluRs) (Watkins & Collingridge, 1994). In addition, activation of cortical presynaptic mGluRs leads to a depression in excitatory synaptic transmission (Collins, 1993). In the present study, the pharmacological features of the pre- and post-synaptic mGluR subtype(s) present in the guinea-pig olfactory cortex were examined in brain slices *in vitro* using a conventional single-electrode voltage clamp recording technique.

Transverse slices of guinea-pig olfactory cortex were prepared as previously described (Libri *et al.*, 1996). Stable intracellular recordings were made from neurones in the pyramidal cell layer (II-III) using microelectrodes filled with 4 M potassium acetate (50-70 MΩ). Sub-threshold excitatory post-synaptic potentials (EPSPs) were evoked in response to orthodromic stimuli delivered to local afferent/association fibre terminals, through a bipolar nichrome electrode (50 µm diameter) insulated except at the tip. Measurements were performed before, during and after bath-application of drugs so that each neurone served as its own control.

Bath-application of the mGluR agonist 1S-3R-ACPD (10 µM; 2 min; *n*=44) resulted in a reversible membrane depolarization (10.1 ± 0.9 mV), increase in input resistance (13 ± 3%, measured at -70 mV), intense neuronal discharge (~ 10 Hz frequency) and induction of a post-stimulus inward tail current (I_{ADP}) (0.20 ± 0.01 nA) measured under 'hybrid' voltage clamp. These effects were indistinguishable from those produced by L-glutamate (1 mM, in the presence 20 µM CNQX and 100 µM DL-APV; *n*=3), quisqualate (10 µM in 20 µM CNQX; *n*=5), IBO (25 µM in 100 µM DL-APV; *n*=3) or the selective group I mGluR agonist (S)-3,5-DHPG (10 µM; *n*=5). By contrast, the selective group II mGluR agonist L-CCGI (1 µM; *n*=3) or the selective group III mGluR agonist L-AP4 (up to 1 mM; *n*=5) were inactive. The slow depolarization and I_{ADP} current induced by 10 µM 1S,3R-ACPD were reversibly antagonized by a prior (15 min) preincubation of the slice with the mGluR I/II antagonist (+)-MCPG (0.5-1 mM; *n*=5 at each concentration) or the selective mGluR I antagonist (mGluR II agonist) (S)-4C3HPG (1 mM; *n*=4), but not the selective mGluR III antagonist MAP4 (1 mM; *n*=4). Excitatory postsynaptic potentials (EPSPs) evoked in control (18.3 ± 9.2 mV peak amplitude) at resting membrane potential (-83.4 ± 0.9; *n*=22) were markedly depressed by 10 µM 1S,3R-ACPD (79.6 ± 4.7 %; *n*=9), 10 µM L-AP4 (61.2 ± 3.8 %; *n*=7), 1 mM (S)-4C3HPG (57.6 ± 6.1 %; *n*=3) but not 100 µM (S)-3,5-DHPG (*n*=5). In the presence of 1 mM (+)-MCPG (15 min; *n*=4), the inhibitory effects of 1S,3R-ACPD, but not L-AP4, were invariably abolished, whereas those produced by L-AP4, but not 1S,3R-ACPD, were blocked by 1 mM MAP4 (*n*=3). (+)-MCPG or MAP4 alone had no effect on EPSP amplitude.

We conclude that a PI-coupled group I mGluR is likely to be involved in mediating excitatory post-synaptic responses, whereas two distinct cAMP-linked mGluRs (*e.g.* group II and III mGluRs) might serve as presynaptic inhibitory autoreceptors in the guinea-pig olfactory cortex.

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355P BLOCK OF AMPA RECEPTOR-INDUCED DESENSITISATION BY CYCLOTHIAZIDE DOES NOT POTENTIATE NEURONAL DAMAGE FOLLOWING CEREBRAL ISCHAEMIA

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Glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) subclass mediate synaptic transmission in the mammalian CNS and are also implicated in neuronal damage following cerebral ischaemia (Gill, 1994). Thus, AMPA antagonists reduce neuronal damage following global ischaemia in the gerbil (Gill, 1994). AMPA receptors rapidly desensitise on exposure to glutamate and this desensitisation can be blocked by cyclothiazide (Patneau *et al.*, 1992). To test whether desensitisation of AMPA receptors limits the neurotoxicity of glutamate in ischaemia, we investigated the effects of cyclothiazide.
Using the technique of microiontophoresis on 6 spinal neurones in pentobarbitone-anaesthetised rats (Anis *et al.*, 1983), cyclothiazide (10 mM in 200 mM NaCl; 10-20 nA) enhanced responses to AMPA (10 mM in 200 mM NaCl) by 168 ± 4% but not those to N-methyl-D-aspartate (NMDA; 200mM). Administration of cyclothiazide, 1 mg/kg i.v. and 25 mg/kg p.o., similarly enhanced responses of spinal neurones to AMPA by 119 ± 28 (n=6) and 54 ± 6 % (n=4) respectively.
Bilateral carotid artery occlusion for 2 and 3 min, in the halothane-anaesthetised Mongolian gerbil (10 per group; Gill *et al.*, 1987), resulted in submaximal damage in the CA1 area of the hippocampus. Cyclothiazide, 5 mg/kg i.p. given 30 min before the occlusion, followed by 3 doses of 2.5 mg/kg i.p. at 3 hourly intervals, did not potentiate the damage. So, in gerbils occluded for 2 and 3 min respectively, the number of viable pyramidal neurones per mm of CA1 were 210 ± 12 and 27 ± 5 in the untreated group and 221 ± 10 and 47 ± 6 in the cyclothiazide group with 220 ± 4 in the sham group.
From the electrophysiology data, it appears that cyclothiazide is able to reduce desensitisation of central AMPA receptors and yet, from the histopathology data, does not enhance neuronal damage in the gerbil following bilateral carotid artery occlusion. We therefore conclude that desensitisation of AMPA receptors does not limit the glutamate-induced neuropathology in this model of global ischaemia.

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356P GENISTEIN, A TYROSINE KINASE INHIBITOR, BLOCKS THE SECOND WINDOW OF PROTECTION 48 HOURS AFTER ISCHAEMIC PRECONDITIONING

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Repetitive brief myocardial ischaemia (ischaemic preconditioning, IP) confers an enhancement of tolerance to ischaemia 24-72 h later (Yellon & Baxter, 1995). This phenomenon is known as the second window of protection (SWOP) or delayed preconditioning. Although protein kinase C (PKC) is involved in this delayed protection (Baxter et al., 1995), it is possible that other kinase cascades are activated by IP. We therefore hypothesised that tyrosine kinase activation could be involved in the delayed myocardial protection by IP. To verify our hypothesis, we examined the effect of genistein, a tyrosine kinase inhibitor (Akiyama et al., 1987), on SWOP in a rabbit model of myocardial infarction.

Male New Zealand White rabbits (2.1 - 3.1 kg) underwent a midline sternotomy under Hypnorm/diazepam anaesthesia. The myocardium was preconditioned with four 5 min coronary occlusions each separated by a 10-min reperfusion while control animals had a sham operation (Baxter et al., 1994). Genistein (5mg/kg) or its vehicle was given i.v. 5 min before IP. The animals were allowed to recover for 48 h before re-occluding the artery for 30 min. After 2 h reperfusion, risk area (R) was determined by fluorescent microspheres and infarct zone (I) by tetrazolium staining. Infarct size was calculated as the ratio of infarct zone to risk area (I/R) (Baxter et al., 1994).

I/R after coronary occlusion and reperfusion was significantly reduced by IP 48 h earlier. Genistein abolished this delayed myocardial protection. Genistein per se did not have any significant effects on infarction 48 h later. R were not significantly different between groups. There were no differences in haemodynamic parameters between groups throughout the experimental period. [Table 1.] Our results suggest that activation of tyrosine kinase leads to increased tolerance to ischaemia 48 h later. These data suggest that the signalling cascade underlying SWOP could involve both tyrosine kinase activation as well as PKC in the rabbit heart.

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Table 1. Risk area and infarct size data

	Sham + Veh	IP + Veh	Sham + Gen	PC + Gen
Number	6	6	6	6
R(cm3)	1.0±0.07	1.0±0.06	1.1±0.11	1.1±0.09
I/R (%)	39.6±3.3	18.0±3.7*	37.0±4.0	39.0±3.4†

Data presented as mean ± s.e.mean. * $P < 0.01$ vs. Sham, † $P < 0.01$ vs. IP (ANOVA). Sham; sham-operated, Veh; vehicle, Gen; genistein.

357P EVALUATION OF THE NITRIC OXIDE SYNTHASE INHIBITORS 7-NITROINDAZOLE, 3-BROMO-7-NITROINDAZOLE AND L-NAME IN GERBIL GLOBAL CEREBRAL ISCHAEMIA

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Recently, the role of nitric oxide (NO) in cerebral ischaemia has been investigated. High concentrations of NO are toxic and increased NO· and O_2· interact to form the toxic peroxynitrite anion ($ONOO^-$). 7-nitroindazole (7-NI) is an inhibitor of NO synthase which inhibits rat cerebellar NO synthase with an IC_{50} of 0.9μM. Preliminary studies have reported that 7-NI inhibits neuronal NO synthase without any effect on blood pressure and is neuroprotective in a rat model of focal cerebral ischaemia (Yoshida et al., 1994).

In the present studies we have evaluated the effects of 7-NI (20 or 40 mg/kg i.p.) immediately after occlusion followed by three further doses (10 or 20 mg/kg, respectively) at 3, 6 and 24 h post-occlusion in the gerbil model of transient global cerebral ischaemia. Using the same protocol we examined the effects of 3-bromo-7-nitroindazole (40 mg/kg i.p.) and N^G-nitro-L-arginine methyl ester (L-NAME) (10 mg/kg i.p.) administered immediately after occlusion followed by three further doses (20 or 5 mg/kg, respectively) at 3, 6 and 24 h post-occlusion.

The results were compared with those of the N-methyl-D-aspartate (NMDA) antagonist dizocilpine (MK-801), eliprodil (a polyamine site antagonist) and the α-amino-3-hydroxy-5-methyl-4-isoazole propionate (AMPA) antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benz(F)-quinoxaline (NBQX).

Male Mongolian gerbils (60-80g) were used in these experiments. Ischaemia was induced by 5 min of bilateral carotid artery occlusion under halothane anaesthesia. Sham operated animals were included as controls. 5 days after surgery the animals were perfused transcardially with 0.9% saline followed by 10% buffered formalin. For histological evaluation 5 μm coronal brain sections were cut, stained with haematoxylin and eosin and the viable pyramidal cells in the CA1 hippocampal region were counted (n = 8 animals per group).

The number of viable cells (mean ± SEM) per 1 mm of the CA1 was 223 ± 7 (sham operated), 11 ± 2 (ischaemic control), 28 ± 5 (7-NI 40mg/kg), 48 ± 11 (L-NAME 10mg/kg) and 41 ± 12 (3-bromo-7-nitroindazole 40mg/kg). Therefore, all three NO synthase inhibitors provided significant neuroprotection ($P < 0.05$) against the ischaemia-induced neuronal death. The neuroprotection was similar to that provided by MK-801 (49 ± 11) and eliprodil (37 ± 6), but not as good as that observed with NBQX (90 ± 12).

These results indicate that nitric oxide plays a role in ischaemic cell death and that selective neuronal nitric oxide synthase inhibitors can protect against ischaemic brain damage.

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358P A FREE RADICAL SCAVENGER, U-74389G, ATTENUATES SEIZURES AND NEURONAL DAMAGE INDUCED BY INTRAHIPPOCAMPAL INJECTION OF DENDROTOXIN-K INTO RAT

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In the mammalian brain, excessive activation of glutamate receptors elevates intraneuronal Ca^{++} concentration which in turn leads to metabolic stress, accumulation of oxygen free radicals, membrane lipid peroxidation and neuronal death (Choi, 1988). Recently, we have shown that in rats intra-hippocampal (i.h.) injection of dendrotoxin-K (DTx-K), a snake neurotoxin which preferentially blocks a non-inactivating voltage dependent K^+ current in ganglionic neurones (Owen *et al.*, 1990), produces seizures and hippocampal damage via a mechanism which involves N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors (Bagetta *et al*, 1996). Here we report that DTx-K-induced neuropathological effects are minimized by U-74389G, a free radical scavenger which belongs to the group of lazaroids (Katz *et al.*, 1995).

Under chloral hydrate (400 mg/kg, i.p.) anaesthesia, male Wistar rats (250-280 g) were implanted with a guide cannula into one dorsal hippocampus and brain cortical electrodes as previously described (Bagetta *et al.*, 1996); neuronal brain damage and cell loss were assessed as previously reported (see Bagetta *et al.*, 1996). Unilateral (i.h.) injection of DTx-K (35 pmol in 1μl) produced in all rats (n=6) motor and electrocortical (ECoG) seizures which were apparent after a latency of 5.4±1.4 min; during the observation period (2 h) 58.0±2.8 trains of spikes were counted. Examination of brain coronal sections (10 μm; n=6 per brain) revealed significant ($p<0.05$ unpaired t-test) neuronal loss in the CA1 (4.8±0.9; mean cell number in the treated side) and CA4 (2.9±0.4; mean cell number for the treated side) pyramidal cell layer relative to the corresponding brain regions (30.5±0.9 and 14.0±0.1 in the CA1 and CA4 areas, respectively) of bovine serum albumin (BSA, control; 300 ng)-treated rats (n=6). Pretreatment with U-74389G (5 mg/kg i.p.; given 30 min beforehand) delayed the onset (latency = 17.5±3.9 min) and reduced significantly ($P<0.05$ vs control) the number of trains of ECoG spikes (15.0±3.3) typically elicited by DTx-K; this treatment also attenuated the -neuronal loss inflicted to the CA1 (mean cell number = 29.8±0.7) and CA4 (mean cell number = 16.9±0.5) hippocampal areas by the toxin. A lower dose (0.5 mg/kg i.p.) of U-74389G failed to affect significantly the latency (3.3±0.6 min) and number of ECoG seizures (53.3±0.3) and the loss of neurones. In conclusion, the present data support a role for excessive accumulation of oxygen free radicals in the mechanisms of seizures and hippocampal damage induced by DTx-K in rats.

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359P ANTICONVULSANT AND PROCONVULSANT EFFECTS OF COMPOUNDS ACTING ON DIFFERENT ADEOSINE RECEPTOR SUBTYPES IN DBA/2 MICE

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Adenosine has neuromodulatory actions within central nervous system. Administration of adenosine analogues has anticonvulsant effects, whilst adenosine antagonists such as xanthines are proconvulsant or convulsant (Adami et al., 1995). In an attemp to better understand the role of adenosine in epilepsy we have studied the actions of adenosine agonists and antagonists in mice. In particular, the effects of some selective adenosine receptor agonists (Ongini and Fredholm, 1996) CCPA, CGS 21680, 2HE-NECA, NECA, N^6-2-(4-aminophenyl)ethyladenosine (APNEA) and N^6(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) and of some adenosine receptor antagonists DPCPX, CGS 15943, 5-amino-7-(2-phenyl-ethyl)-2-(2-furyl)-pyrazolo-[4,3-e]1,2,4-triazolo[1,5c]pyrimidine (SCH 58261), DMPX and (E,18%-Z,82%)7-methyl-8-(3,4-dimethoxystyryl)-1,3-dipropylxanthine (KF 17837) were compared in the genetic audiogenic sensible DBA/2 mice. The compounds studied were injected intraperitoneally 30 min before testing in DBA/2 mice (male and female, 6-12g) of 22-26 days old (at least 40 mice were used for each compound). The anticonvulsant effects were evaluated on seizures evoked by means of auditory stimulation (109 dB, 12-16 Hz) as previously described (De Sarro *et al.*, 1994). The ED_{50} values were calculated according to Litchfield and Wilcoxon test (1949). All the agonists studied were able to protect against the audiogenic seizures with the exception of the A_3 agonist, IB-MECA. The ED_{50} values against the clonic phase of the audiogenic seizures were: NECA 0.54 μg/kg, CGS 21680 24 μg/kg, 2HE-NECA 28 μg/kg, CCPA 56 μg/kg and APNEA 670 μg/kg. The ED_{50} values against the tonic phase of the audiogenic seizures were: NECA 0.35 μg/kg, CGS 21680 18 μg/kg, 2HE-NECA 16 μg/kg, CCPA 52 μg/kg and APNEA 540 μg/kg. Following a subconvulsant audiogenic stimulus of 83 dB all the adenosine receptor antagonists studied showed proconvulsant properties. The ED_{50} values for convulsant effects, described by De Sarro et al., (1994), were 0.29 mg/kg for CGS 15943, 0.075 mg/kg for SCH 58261, 0.57 mg/kg for KF 17837, 0.022 mg/kg for DMPX and 5.84 mg/kg for DPCPX. The present data suggest that adenosine agonists acting on both adenosine A_1 and A_2 receptors were more potent as anticonvulsant than the compounds acting selectively on adenosine A_1 receptors. The adenosine A_3 receptors seem to be unable to affect audiogenic seizures in DBA/2 mice. Indeed the antagonists on adenosine A_2 receptors appeared more potent as proconvulsant agents than DPCPX an antagonist acting on A_1 adenosine receptors. However, the fact that DPCPX does not penetrate the brain in sufficient quantity has to be considered.

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360P GR196429, A NON-INDOLIC AGONIST AT HIGH AFFINITY MELATONIN RECEPTORS WHICH MEDIATE THE ACTIONS OF MELATONIN ON THE RAT CIRCADIAN CLOCK

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The indole-based hormone melatonin is secreted from the pineal gland at night and has a wide variety of biological actions (see Arendt, 1995). Of particular interest is its putative role as an internal zeitgeber (time giver) for the circadian clock in the suprachiasmatic nucleus (SCN) of the brain. In mammals, melatonin exerts some of its effects through specific G-protein coupled receptors (ML_1), two subtypes of which have been identified in human by cDNA cloning, termed Mel_{1A} and Mel_{1B} (Reppert *et al.*, 1996). To date, only Mel_{1A} receptor mRNA has been detected in the SCN. Here, we describe the circadian actions of a new melatonin receptor agonist, GR196429.

GR196429, (N-[2-[2,3,7,8-tetrahydro-1H-furo(2,3-g)indol-1-yl]-ethyl]acetamide) is a novel, non-indolic melatonin receptor agonist. GR196429 competes with high affinity for [^{3}H]-melatonin binding sites in membranes prepared from chinese hamster ovary cells expressing recombinant human Mel_{1A} receptors (pKi 9.8 ± 0.1, n=3) or Mel_{1B} receptors (pKi 9.7 ± 0.1, n=3). It has similar affinity for 2-[^{125}I]-iodomelatonin binding sites in membranes prepared from human cerebellum (pKi 9.3 ± 0.1, n=8) and guinea-pig hypothalamus (pKi 9.5 ± 0.2, n=3). In contrast, GR196429 is inactive in a wide range of other hormone and neurotransmitter receptor radioligand binding assays (pKi < 6).

In slices of rat brain hypothalamus, there is a circadian peak in firing rate of SCN neurones at zeitgeber time 7 (ZT7; Starkey *et al.*, 1995). Exposure of the slice to GR196429 (1 fM - 10 nM, 1 concentration per slice) by bath perfusion for 1 h at ZT10 produced a concentration-related phase advance of the peak in electrical activity measured on the following day. GR196429 evoked a maximum phase advance of 2.5 & 3.0 h at 10 pM (n=2). Curve fitting (ALLFIT; n=15 slices) generated an EC_{50} of 0.6 pM. For comparison, at ZT10, melatonin behaved in a similar manner, evoking a maximum phase advance of 3 h and EC_{50} of 1.2 pM (Starkey *et al.*, 1995).

Rats (male Lister hooded, 300-350 g) housed in photocell cages (12:12 h light-dark cycle) demonstrated robust circadian rest-activity rhythms. When subjected to an 8 h phase advance in their light-dark cycle (Redman & Armstrong, 1988), 6/6 rats treated with vehicle (1% ethanol in saline s.c.), given at the pre-phase shift dark onset for 12 days, re-entrained to the new cycle by delaying their onset of activity. In contrast, rats treated with either GR196429 (0.2-5 mg/kg s.c.) or melatonin (1 mg/kg s.c.) re-entrained by advancing their activity rhythms into the new light-dark cycle. Thus, 5/6 rats treated with GR196429 (5 mg/kg) and 6/6 rats treated with melatonin, phase advanced into the new light-dark cycle (both $P < 0.01$ vs vehicle, Fishers exact test).

In conclusion, GR196429, a potent agonist at ML_1 receptors, mimics the phase advancing actions of melatonin on the rat circadian clock.

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361P PHARMACOLOGICAL CHARACTERISATION OF [^{3}H]-MELATONIN BINDING TO HUMAN RECOMBINANT MELATONIN Mel_{1A} AND Mel_{1B} RECEPTORS

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The pineal hormone melatonin is the principle hormone involved in the regulation of the mammalian circadian system (see Arendt, 1995). To date, three cDNAs encoding melatonin receptors have been cloned, two of which have been identified in human, termed Mel_{1A} and Mel_{1B} (Reppert *et al.*, 1996). Here we compare the pharmacological characteristics of recombinant human Mel_{1A} and Mel_{1B} receptors expressed in Chinese hamster ovary (CHO) cells.

Human Mel_{1A} and Mel_{1B} receptors were stably expressed in CHO cells using the calcium phosphate transfection method and gentamycin selection. Membranes (25ug) were incubated with [^{3}H]-melatonin (0.3 nM) at 37°C for 120 min. Bound radioactivity was separated by rapid filtration using a Brandel cell harvester and counted by liquid scintillation spectrometry.

Saturation binding studies indicated that [^{3}H]-melatonin bound in a specific and saturable manner to both Mel_{1A} and Mel_{1B} receptors, with log equilibrium dissociation constants (pK_D) of 9.89 ± 0.13 and 9.56 ± 0.03, respectively (mean ± s.e. mean; n=3). Maximal binding capacities were 1.20 ± 0.10 and 0.82 ± 0.06 pmol/mg protein respectively. Hill coefficients were 1.02 ± 0.13 and 1.06 ± 0.06 respectively, suggesting interactions with single populations of binding sites.

The abilities of melatonin analogues to compete for [^{3}H]-melatonin at Mel_{1A} and Mel_{1B} receptors are shown in Table 1. All Hill slopes were not significantly different from unity, with the exception of 2-I-melatonin at Mel_{1A} receptors (1.38 ± 0.09, $P < 0.05$). Most compounds, including melatonin and the non-indolic melatonin receptor agonists GR196429 (Beresford *et al.*, This Meeting) and S20098 (Yous *et al.*, 1992) bound with similar affinities to both receptor subtypes. 2-iodomelatonin had 5-fold higher affinity for Mel_{1A} receptors, whilst 6-chloromelatonin had 5-fold higher affinity for Mel_{1B} receptors. The most selective compound tested was the melatonin receptor antagonist luzindole (Dubocovich, 1989), which was 17 fold more selective for Mel_{1B} compared to Mel_{1A} receptors.

Table 1: Affinities of melatonin analogues for human Mel_{1A} and Mel_{1B} receptors.

	CHO Mel_{1A} pKi	CHO Mel_{1B} pKi	Ki_{1A}/Ki_{1B}
2-I-melatonin	10.91 ± 0.04	10.28 ± 0.05	0.2
S20098	10.36 ± 0.04	10.47 ± 0.07	1.3
Melatonin	10.08 ± 0.05	9.72 ± 0.11	0.4
GR196429	9.85 ± 0.06	9.79 ± 0.07	0.9
6-Cl-melatonin	9.17 ± 0.06	9.84 ± 0.08	4.7
6-OH-melatonin	9.26 ± 0.03	8.77 ± 0.06	0.3
Luzindole	6.60 ± 0.05	7.84 ± 0.08	17.1
N-acetylserotonin	7.10 ± 0.05	6.83 ± 0.04	0.6

Values are mean ± s.e.mean. $n \geq 3$.

This is the first characterisation of recombinant human Mel_{1A} and Mel_{1B} receptors using [^{3}H]-melatonin. These studies will now enable the development of subtype selective ligands.

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362P CHARACTERISATION OF THE TACHYKININ NK RECEPTOR IN DOG CORTEX

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We have demonstrated that the tachykinin NK_1 receptor antagonists are broad spectrum enti-emetics in a number of animal models, including dog (Gardner, C.J. *et al.* 1995). As the dog may prove to be an important model in the development of novel anti emetics, we were interested to characterise the tachykinin NK_1 receptor in dog brain

Membranes were prepared and binding experiments carrired out as described previously (Beattie *et al* 1995). K_D and B_{max} were determined from saturation binding. Affinity estimates were determined for substance P, a range of non-peptide tachykinin NK_1 receptor antagonists selective for either human (CP-96345, CP-99,994, GR203040 see Beattie *et al* 1995, Beresford *et al* 1991) or rat (RP67580, see Hawcock *et al* 1995) receptors, and a non-selective peptide tachykinin NK_1 receptor antagonists (GR82334, see Hawcock *et al* 1995) Table 1.

Saturation analysis indicated that [^{3}H]-Substance P bound in a saturable manner to a single population of binding sites with a pK_D of 9.58±0.12 and a B_{max} of 45±4.4 fmol/mg protein (n = 3). Competition studies yielded inhibition curves with slopes not significantly different to unity. There was an excellent correlation (coefficient = 0.94) between affinity estimates at [^{3}H]-Substance P binding sites in dog cortex and at membranes prepared from CHO cells transfected with the human tachykinin NK_1 receptor. There was a lower correlation (coefficient = 0.13) between affinity estimates at [^{3}H]-Substance P binding sites in dog cortex and at membranes prepared from rat cortex.

Table 1 Affinity estimates at dog, rat and human (pK_i ± SEM, n = 3-5) tachykinin NK_1 receptors. n.d. = not determined.

	Affinity estimate (pK_i)		
	Dog Cortex	Rat Cortex	CHO hNK_1
CP-99,994	9.6 ±0.39	7.3±0.35	9.8±0.13
CP-96,345	9.5±0.24	6.8±0.08	10.2±0.16
RP67580	7.4±0.02	8.2±0.18	8.1±0.09
GR203040	10.6±0.15	8.3±0.25	10.3±0.19
GR82334	6.3±0.09	n.d.	6.3±0.09
Substance P	9.3±0.13	9.1±0.16	9.6±0.05

This is the first study describing tachykinin NK_1 receptor binding in dog cortex. Results obtained from this indicate that the tachykinin NK_1 receptor in dog brain is similar to that in human, but different to that in rat. Emesis in the dog could therefore prove to be a usefull model for tachykinin NK_1 receptor antagonists as novel anti emetics.

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363P PREDICTION OF THE ANTI-EMETIC ACTIVITY OF NK_1 RECEPTOR ANTAGONISTS IN FERRETS BY THEIR ABILITY TO INHIBIT GR73632-INDUCED FOOT TAPPING IN GERBILS

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The relationship between receptor binding affinity, CNS penetration and anti-emetic activity of a range of structurally diverse NK_1 receptor antagonists was assessed using *in vitro* and *in vivo* assays.

Binding assays were performed using intact CHO cells expressing the human NK_1 receptor as described by Cascieri et al (1992). ^{125}I-Tyr^8-substance P (0.1 nM, 2000 Ci/mmol) was incubated in the presence or absence of test compounds (dissolved in 5 µl DMSO) with $5x10^4$ cells; nonspecific binding was defined using excess substance P. Affinity for the ferret NK_1 receptor was similarly determined using synaptosomal membranes (50 µg) purified from ferret brain cortex as described by Cascieri et al (1985). CNS penetration was determined by the inhibition of NK_1 agonist-induced foot tapping in gerbils (Rupniak & Williams, 1994). Under isoflurane anaesthesia, test compounds were injected i.v. in male or female Mongolian gerbils (40-70 g) immediately before i.c.v. infusion of the NK_1 agonist GR73632 (3 pmol in 5 µl). On recovery from anaesthesia, the duration of foot tapping was recorded for 5 min (n = 3-9). Acute emesis studies were performed as described by Tattersall et al (1993). Under halothane anaesthesia, test compounds were administered i.v. to male ferrets (1.1-1.8 kg) followed 3 min later by cisplatin (10 mg/kg i.v.). Retching was recorded continuously for 4 h thereafter (n = 3-6).

(+)-GR203040, CP-99,994 and L-742,694 were potent inhibitors of GR73632-induced foot tapping in gerbils (ID_{50} 0.04, 0.06 and 0.85 mg/kg, respectively). (+)-GR203040, CP-99,994 and L-742,694 were potent inhibitors of the acute retching response to cisplatin in ferrets (ID_{50} 0.05, 0.18 and 0.18 mg/kg, respectively). Higher doses of RPR100893 were required to prevent retching (ID_{50} 4.1 mg/kg); this compound caused only weak inhibition of foot tapping (< 14% at 10 mg/kg). Relatively high doses (3-10 mg/kg) of CGP 49823, FK888 and LY303870 were required to inhibit foot tapping, and these agents were not anti-emetic at doses of 1 (FK888) or 10 mg/kg (CGP 49823 and LY303870) in ferrets. SR140333 (3-10 mg/kg) failed to inhibit foot tapping or emesis, indicating poor CNS penetration of this compound.

The affinities of these compounds for the human and ferret NK_1 receptor were highly correlated (r = 0.93, p < 0.001). However, NK_1 receptor binding affinity *in vitro* was not predictive of anti-emetic activity *in vivo* (r < 0.28, p > 0.4), whilst the ability of NK_1 receptor antagonists to inhibit foot tapping in gerbils was correlated with their anti-emetic activity in ferrets (r = 0.79, p = 0.03).

These findings confirm that the anti-emetic activity of NK_1 receptor antagonists is critically dependent on their ability to cross the blood-brain barrier, and that the gerbil foot tapping assay provides a simple *in vivo* assay for CNS penetration which is predictive of anti-emetic activity against cisplatin in ferrets.

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364P COMPARISON OF BEHAVIOURAL EFFECTS OF CORTICOTROPIN-RELEASING FACTOR AND THE NOVEL NEUROPEPTIDE, UROCORTIN

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Urocortin (Ucn), a novel member of the corticotropin-releasing factor (CRF) family of peptides, has been found in rat and man (see Donaldson et al., 1996). Compared with human/rat CRF (h/rCRF), human and rat Ucn (hUcn and rUcn, respectively) have higher affinities for $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors and approximately 10 fold greater potency to stimulate cAMP in cell lines expressing these receptors. In a previous study (Jones et al., 1996b), intracerebroventricular (ICV) rUcn caused motor activation in a familiar environment and hypophagia in hungry rats in a similar manner to h/rCRF and other CRF-related peptides (Jones et al., 1996a). In the present study, we compared the effects of h/rCRF and rUcn following ICV administration in: *i*) the elevated X-maze, *ii*) observation of gross behavioural change.

Studies used male Sprague Dawley rats (250-275g) with a guide cannula directed towards the lateral cerebral ventricle. In the X-maze test (5 min test period, data analysed by Videotrack™, CPL Systems), rUcn and h/rCRF (both 0.1, 0.3 or 1.0µg ICV, 30 min pre-treatment) had an anxiogenic-like profile (data shown as mean ± SEM). For example, open arm entries were reduced from 4.4±0.6 (Veh) to 1.8 ± 0.4 (rUcn, 1.0µg, P<0.05) and from 7.7 ± 0.7 (Veh) to 3.4 ± 0.7 (h/rCRF, 1.0µg, P<0.05). h/rCRF reduced %time on the open arms e.g. from 32.8 ± 1.8 % (Veh) to 17.5 ± 3.3 % (h/rCRF, 1.0µg, P<0.05); the effects of rUcn on this measure just failed to reach significance (F[3,53]=2.54, P=0.066) e.g., from 21.2 ± 1.6 % (Veh) to 12 ± 2.5 % (rUcn, 1.0µg).

To determine the effects of the peptides upon gross behaviour, rats were observed in clear cages for 30s periods every 5 min (from 10 to 60 min post-dose; Veh, h/rCRF or rUcn, both 3µg ICV). Time spent grooming and number of rears during each period were recorded (total mean ± SEM). Other behaviours were recorded as either present or absent during each 30s period (mean no. of periods [± SEM] in which behaviour present). Total body weight change and number of faecal pellets were also recorded. Both peptides increased grooming (from 14.4 ± 5 s [Veh] to 41 ± 10 s [rUcn, P<0.05] and 41 ± 6 s [h/rCRF, P<0.05]). However, only h/rCRF increased defaecation (1.2 ± 0.5 [Veh] and 5.13 ± 1.2 [h/rCRF, P<0.05]). Neither peptide altered rearing. Other changes included increased activity (3.0 ± 0.6 [Veh], 6.3 ± 0.4 [h/rCRF, P<0.05] and 5.2 ± 0.7 [rUcn, P<0.05]), decreased frequency of motionless (7.7 ± 0.5 [Veh], 1.9±0.4 [h/rCRF, P<0.05] and 4.0 ± 0.8 [rUcn, P<0.05]). However, h/rCRF, but not rUcn, increased oral movements (0 [Veh] and 2.9 ± 0.7 [h/rCRF, P<0.05]) and body weight change (-3.0 ± 0.4 g [Veh] and -6.0 ± 0.9 g [h/rCRF, P<0.05]). Further, h/rCRF had a greater effect than rUcn on forepaw tremor (0 [Veh], 4.3 ± 0.5 [h/rCRF, P<0.05 *cf* Veh and rUcn] and 1.4 ± 0.5 [rUcn, P<0.05]).

In summary, rUcn and h/rCRF have a number of behavioural effects in common, including anxiogenesis, motor activation and hypophagia (Jones et al., 1996b). However, there are some differences which require further investigation including the induction of oral movements and body weight change.

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365P HYPOTHERMIC AND HYPERTHERMIC ACTIONS OF CRF: INFLUENCE OF ENVIRONMENTAL CONDITIONS

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The neuropeptide corticotrophin-releasing factor (CRF) is an important mediator of stress responses in the brain, including changes in body temperature. Studies in experimental animals have demonstrated that injection of CRF into the brain induces an increase in body temperature (Rothwell, 1990), whereas other studies have shown antipyretic effects (Bernardini *et al.*, 1984). The aim of the present investigation was to attempt to resolve these apparently conflicting findings and identify specific factors which may explain the different effects of CRF on body temperature.

Unrestrained, male, Sprague-Dawley rats (275-325g) housed at an ambient temperature of 22"1◦C, were injected (2µl) intracerebroventricularly (i.c.v.) with CRF (0.3, 3 or 30µg) or vehicle. The effects on core body temperature were measured:
(i) using either remote radiotelemetry via pre-implanted, abdominal transmitters, or by colonic probe,
(ii) in rats housed either individually or in groups.
Data were analysed using either MANOVA for comparison over the experimental time-course, or ANOVA for individual time-points.

Basal temperatures of all animlas ranged from 36.9-37.2◦C. Injection (i.c.v.) of CRF in individually-housed, telemetry-monitored animals produced dose-dependent hypothermia after 20min. Injection of 3µg CRF (n=7) produced optimal significant ($P<0.01$) hypothermia, declining to a minimum temperature (36.2"0.2◦C) after 1h. CRF (3µg) also produced significant ($P<0.01$) hypothermia (36.3"0.1◦C) when core body temperature was measured using a colonic probe in individually-housed animals (n=4). In addition, significant ($P<0.01$) hypothermia (35.8"0.1◦C) was observed in group-housed animals injected (i.c.v.) with CRF (3µg), when body temperature was measured using telemetry (n=5). However, i.c.v. injection of CRF (3µg) in group-housed animals whose core body temperature was measured using colonic probe (n=4) produced an initial significant ($P<0.05$) hypothermia (36.6"0.1◦C) at 2h, followed by a significant ($P<0.01$) increase in body temperature (38.6"0.2◦C) 3h after injection.

The observations made in this study indicate that central injection of CRF can elicit either hypothermia or hyperthermia. These differences may be attributable to the stress incurred by housing conditions and the method of measuring core body temperature. The results indicate that whereas alteration of housing or the method of temperature-measurement alone, do not influence the hypothermic response, a combination of these conditions dramatically reverse the resulting temperature change. These data illustrate the importance of experimental conditions when measuring the thermoregulatory responses to CRF.

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366P THE GABA UPTAKE INHIBITOR, NO-711, INDUCES DEPOLARIZATIONS IN MOUSE CORTICAL WEDGES

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NO-711, (1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine carboxylic acid) is a potent and selective inhibitor of the GABA transporter, GAT1 (Suzdak *et al.*, 1992). This action, resulting in increased synaptic GABA levels, would be expected to lead to hyperpolarization mediated through increased Cl^- flux through $GABA_A$ receptors. However, GABA has also been shown to depolarize neurons (Michelson & Wong, 1991) and this study has investigated the action of NO-711 on mouse cortical wedges.

Cortical wedges used in this study were prepared from audiogenic seizure-prone male or female DBA/2 mice, 21-50 days old. However, in some control experiments BALB/c mice, of similar age, were also used. Coronal slices (500µm) were cut and cortical wedges prepared and placed in a two-compartment bath with a grease seal isolating the cortical matter from the callosum. The cortical side was perfused with gassed (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) at $2ml^{-min}$ at room temperature, 20-22°C (Hu & Davies, 1995). Following equilibration of the wedges for 60 minutes, NO-711 (25 µM) was perfused for 15 minutes. Composition of aCSF (mM): NaCl 124, KCl 5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, D-glucose 10 and pH was 7.4. Bicarbonate-free aCSF contained HEPES (26mM) gassed with 100% O_2, at pH 7.3.

NO-711 induced repetitive, depolarizations with a duration of 50-60s which continued for 4-5 hours with little decrement in frequency or amplitude. These had a mean onset of 10 ± 1.7 min, an interval of 14.4 ± 2.2 min and an amplitude of 173 ± 26 µV (mean ± s.e. mean, n=15). There was no difference in the responses between wedges prepared from DBA/2 or BALBc mice (n=8). Bicuculline (10 µM) abolished NO-711-induced depolarizations (n=5) and the removal of calcium from the aCSF also reversibly inhibited these depolarizations (n=6), while tetrodotoxin (0.5µM, TTX, n=4) irreversibly inhibited the responses. The spontaneous depolarizations were abolished during perfusion with aCSF containing HEPES and recovered within 20-30 minutes of reinstating normal aCSF (n=8). GABA (25 µM-32mM), perfused for 1 minute, depolarized the wedges in a concentration-dependent manner (EC_{50} 1.17 ± 0.19 mM; n=11). Application of GABA (5 µM-2 mM), following pretreatment with NO-711(25 µM), resulted in significant potentiation (EC_{50} 111 ± 24 µM; $p<0.001$; n=9).

These results, using mouse cortical wedges, demonstrate a depolarizing effect of GABA, elicited either by blocking re-uptake or by perfusing exogenous GABA. $GABA_A$ receptors are implicated as bicuculline blocked the responses to both drugs. The blockade of NO-711-induced depolariza-tions by TTX and Ca^{2+} implies the involvement of propagated events and possibly the release of an excitatory transmitter. The inhibitory effect of perfusion with HEPES suggests that the initiation of these depolarizations could be the efflux of HCO_3^- through $GABA_A$ receptor- operated channels.

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367P GABA-INDUCED DEPOLARISATIONS IN THE RAT CORTICAL WEDGE PREPARATION ARE MEDIATED BY $GABA_A$ RECEPTORS AND MAY INVOLVE HCO_3 IONS

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Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian CNS. Activation of postsynaptic $GABA_A$ receptors mediates an inward Cl^- conductance, hyperpolarising cells and inhibiting neuronal activity (Krnjevic & Schwartz, 1967). More recently, GABA has also been reported to exert excitatory actions on neurones *in vitro* (Avoli & Perreault, 1987; Grover *et al.*, 1993; Michelson & Wong, 1991). We have investigated the actions of GABA on the rat cortical wedge preparation *in vitro*.

Cortical wedges, cut from 500μm coronal sections of hemibrains removed from halothane-anaesthetised male Sprague-Dawley rats (80-120g; Charles River), were placed in a two-compartment chamber such that the cortical side was isolated from the callosal side by a grease-seal. Both sides of the chamber were perfused with aCSF composed of (mM): NaCl 124, KCl 3.3, KH_2PO_4 1.22, $NaHCO_3$ 25.5, $CaCl_2$ 2.5, $MgSO_4$ 1, D-glucose 10. The experimental protocol was as previously described (Phillips *et al.*, 1996). The role of HCO_3^- ions was determined by replacing $NaHCO_3$ with 10mM HEPES, and gassing with 100% O_2. Following two-way analysis of variance, comparisons between control and drug groups were made using Williams' test.

The application of GABA (10^{-4}M - $3x10^{-2}$M) to the cortical chamber resulted in depolarisations (47 - 811μV respectively) which were concentration-dependent, and remained consistent over a 5 hour recording period. From the concentration-response curves generated, an EC_{50} of 1.5mM (95% confidence limits: 0.3, 8.3; n=3) was determined. GABA-evoked (10^{-4}M - $3x10^{-2}$M) depolarisations were signficantly ($p<0.05$) attenuated by up to 60% when wedges were perfused with bicarbonate-free HEPES buffer; mean response to 1mM GABA (n=4): 396 ± 64μV in HCO_3^- buffer and 179 ± 42μV in HEPES buffer. In normal aCSF, the response to 1mM GABA was concentration-dependently attenuated by (-)-bicuculline and picrotoxin. (IC_{50} [95% confidence limits]: 13μM [5.4, 31.4; $p<0.01$, n=4] and 28.3μM [21.5, 37.3; $p<0.001$, n=4] respectively). NO-711, a GABA re-uptake inhibitor (Suzdak *et al.*, 1992) and pentobarbitone potentiated responses to 1mM GABA in a concentration-dependent manner, and by 111 ± 41%, $p<0.001$ and 40 ± 16%, $p<0.001$ (n=4) respectively at 10^{-4}M.

These results in the rat cortical wedge, demonstrating that GABA evokes depolarising potentials which can be blocked by the $GABA_A$ receptor antagonist (-)-bicuculline and by the Cl^- channel blocker picrotoxin, and which can be potentiated by the GABA re-uptake inhibitor NO-711 and by pentobarbitone, indicate that these responses are mediated via the $GABA_A$ receptor. Furthermore, the experiments in the presence of HEPES buffer indicate that approximately 50% of the response is attributable to HCO_3^- ions which can also pass through the $GABA_A$ receptor ionophore. This finding is in general agreement with the data of Grover *et al.* (1993), who reported a 72% reduction in the amplitude of depolarising GABA responses in bicarbonate-free buffer possibly as a result of an activity-dependent shift of the $GABA_A$ reversal potential (E_{GABA}) toward E_{HCO3}^- (Staley *et al.*, 1995). The remaining component of the depolarisation seems likely to be due to Cl^- efflux from cells, possibly due to a collapse of the Cl^- gradient resulting from Cl^- ion loading of the cells during preparation of wedges.

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368P DETERMINATION OF CORTICAL LEVELS OF MULTIPLE NEUROTRANSMITTER AMINO ACIDS: AN *IN VIVO* MICRODIALYSIS STUDY IN CONSCIOUS RATS

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Amino acid neurotransmitters play an important role in the CNS in normal function and potentially in various disease states, eg cerebral ischaemia (Rothman & Olney, 1986) and Alzheimer's disease (Maragos *et al.*, 1987). The objective of this study was to resolve multiple neurotransmitter and also metabolic amino acids by HPLC with isocratic elution. Using microdialysis, the K^+-stimulation and Ca^{2+}-dependence of the neurotransmitter amino acids in the cortex of conscious rats was examined.

Male Sprague Dawley rats (300-350g) were anaesthetised with halothane in N_2O/O_2. Microdialysis probes (Hospal membrane; 4 mm) were implanted into the cortex (mm; AP +0.2, L -5.2, V -7.0, jawbar -3.3) and rats were left to recover for at least 20 h. Four basal samples (5 min) were collected with artificial CSF (aCSF; 2μl/min) followed by 5 min of 100mM K^+ or 100mM K^+/Ca^{2+}-free aCSF. HPLC analysis was modified from the methods of Smith & Sharpe (1992) and Rowley *et al.* (1995) to improve resolution. Separation was achieved with a Phenosphere 1-ODS, 25cm, 3μm reversed-phase C18 column (Phenomenex) at 30°C. Mobile phase was 0.1M sodium phosphate, 5mM EDTA and 10% methanol, pH 5.25, pumped at 0.6 ml/min. Amino acids were detected electrochemically with a glassy carbon electrode set at +0.85 V versus an Ag/AgCl reference electrode. The derivitising reagent was *o*-phthalaldehyde plus 1M sodium sulphite and 0.1M sodium tetraborate (pH 9.9). Statistical analysis of data was performed using one sample t-test. All data are quoted as mean ± s.e. mean.

The following amino acid standards were resolvable aspartate, glutamate, glycine, GABA, taurine, serine, B-alanine, arginine and glutamine. The detector response was linear ($r^2>0.985$) from 1.2nM to 6μM and all, except arginine, were present in cortical microdialysates. Basal levels of these amino acids (not corrected for *in vitro* recovery), were 73±32nM for aspartate, 266±134nM for glutamate, 226±169nM for glycine, 30±19nM for GABA, 2±0.88μM for taurine, 198±122nM for serine, 43±17nM for B-alanine, and 40±13nM for glutamine. When compared with basal levels, 100mM K^+ elevated aspartate (186±47%; $P<0.01$; n=6), glutamate (165±31%; $P<0.01$; n=6), glycine (358±48%; $P<0.001$; n=6), GABA (916±51%; $P<0.001$; n=4), taurine (624±143%; $P<0.001$; n=6), serine (439±80%; $P<0.001$; n=6) and B-alanine (313±55%; $P<0.05$; n=3); glutamine was unaltered. Removal of Ca^{2+} abolished the K^+-induced increases of aspartate, glutamate, glycine, GABA, serine and B-alanine and reduced that of taurine to 167±35% ($P<0.05$).

A new method for the resolution of seven neurotransmitter and two metabolic amino acids using HPLC with isocratic elution and a run time of less than 25 min has been developed. We have applied this technique to microdialysates from rat cortex and shown depolarisation-induced elevations of all neurotransmitter amino acids and the Ca^{2+}-dependence of this stimulation.

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369P EFFECT OF THE ACETYLCHOLINESTERASE INHIBITOR E2020 IN TESTS OF SPATIAL AND VISUAL RECOGNITION MEMORY IN RHESUS MONKEYS

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Hepatotoxicity limits clinical use of the cholinesterase inhibitor tacrine in Alzheimer's disease. Clinical trials with E2020 indicate improvements in cognition without liver toxicity, suggesting a significant advantage over tacrine. The effect of E2020 on cognition in rhesus monkeys using tasks used previously to evaluate tacrine is now described.

For the spatial memory task, male rhesus monkeys (4-5 kg; n=5) were required to remember the location of a white square (3x3 cm) displayed at random in one of 9 possible positions on a touch-sensitive screen for retention intervals of 2, 10 or 20 s (30 randomised trials at each delay). The ability of E2020 (0.01-1.75 mg/kg i.m.) to reverse the cognitive impairment induced by scopolamine (0.03 mg/kg i.m.) was asssessed by coadministration of both drugs 30 min before testing. In the visual recognition task, monkeys (n=4) were presented a with up to 60 visually distinct 3-dimensional objects, shown individually every 15 s. During the retention test, members of this list were presented in pairs with novel objects, and animals were required to select the "nonmatching" objects in order to obtain a food reward. The list length was calibrated for each animal to give a baseline performance level of approximately 60% correct. E2020 (0.003-0.06 mg/kg i.m.) was administered 20 min before behavioural testing; each animal received every dose of E2020 examined in order to construct a dose-response curve. E2020 (1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl] methylpiperidine hydrochloride) was synthesised by the Department of Medicinal Chemistry, MSD, Harlow.

In the spatial memory task, choice accuracy was > 93% correct across all retention intervals on days when animals received no drug treatment, and it was therefore not possible to detect drug-induced improvements in performance against this baseline. Administration of scopolamine (0.03 mg/kg) caused a delay-dependent reduction in choice accuracy to 84 $\pm$ 7% correct for trials using a 2 s retention interval, 37 $\pm$ 7% for trials with a delay of 10 s, and 22 $\pm$ 5 s for trials employing a 20 s retention interval ($P < 0.05$ compared with control performance at delays of 10 and 20 s, paired contrast analysis). Coadministration of E2020 in the dose range 0.5-1.75 mg/kg caused a dose-dependent reversal of the scopolamine-induced deficit (10 s: $F_{6,28}$ 7.9, $P < 0.001$; 20 s: $F_{6,28}$ 10.0, $P < 0.001$, ANOVA). In animals receiving 1.75 mg/kg of E2020, performance approached control levels (100% correct for retention intervals of 2 s, 88 $\pm$ 4% for 10 s delays, and 74 $\pm$ 4% for retention intervals of 20 s). In this dose range, E2020 was well tolerated, but at the higher dose of 2 mg/kg, cholinergic side-effects were observed and 2 out of 5 monkeys failed to complete the task.

In the visual recognition task, the mean baseline choice accuracy in untreated animals was 59 $\pm$ 1% correct. The active dose range for E2020 in this task was lower than in the spatial memory test since scopolamine was not required to impair performance. Administration of E2020 increased choice accuracy to 71 $\pm$ 2% correct at 0.03 mg/kg ($F_{1,3}$ 29.5, P = 0.01, paired contrast analysis), and to 64 $\pm$ 2% at 0.05 mg/kg ($F_{1,3}$ 23.7, P = 0.02). Performance did not differ from control levels at the higher dose of 0.06 mg/kg (60 $\pm$ 4%). No observable adverse effects were apparent in this dose range.

The ability of E2020 to improve performance in these cognitive tasks resembles the profile of other acetylcholinesterase inhibitors, including tacrine, that also improve cognitive function in Alzheimer's disease (Rupniak et al, 1990). However, as with other agents of this pharmacological class, cholinergic side-effects appear to be dose-limiting for E2020 (SCRIP, 1996). Nonetheless, because of its improved clinical safety profile, E2020 may represent a significantly improved palliative therapy for dementia.

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370P PREFERENTIAL BLOCKADE OF CCK-8S-INDUCED RISES IN EXTRACELLULAR ASPARTATE BY THE CCK_B ANTAGONIST, L-365,260, IN THE FRONTAL CORTEX OF THE THE ANAESTHETISED RAT

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Local application *in vivo* of sulphated cholecystokinin octapeptide (CCK-8S) into various brain regions promotes an increase in the extracellular content of some amino acids and in particular, that of aspartate, when measured by microdialysis (Freedman *et al.*, 1994; You *et al.*, 1994; Godukhin *et al.*, 1995). In both the caudate-putamen and frontal cortex of anaesthetised rats, these rises in aspartate have been reported to be offset or even prevented by CCK_B-selective antagonists but not by CCK_A-selective antagonists (Godukhin *et al.*, 1995).

In the present study, we have used intracerebral microdialysis to assess the influence of systemic CCK antagonist delivery on changes in sampled amino acids caused by CCK-8S infused at the sampling site in the rat frontal cortex. Adult male Wistar rats (250-300 g) were anaesthetised with chloral hydrate (0.4 g kg^{-1}, i.p.) and placed in a stereotaxic frame. A microdialysis probe (310 µm o.d., 4 mm Hospal AN69 membrane) was inserted into the frontal cortex of one hemisphere (A +0.7, L 1.5, V 7.0 relative to bregma) and perfused at 2 µl min^{-1} with buffer at 20°C of the following composition (mM): NaCl 126, KCl 2.4, $CaCl_2$ 1.1, $MgCl_2$ 1.3, KH_2PO_4 0.5, Na_2HPO_4 0.49, $NaHCO_3$ 27.4, (+)-glucose 7.0. Amino acids were assayed in 40 µl dialysates by *o*-phthaldialdehyde derivatisation, HPLC separation and fluorimetric detection (Kilpatrick & Mozley, 1986).

The mean contents of the 4 'basal' samples preceding the systemic treatments were for aspartate, 4.36 ± 0.35 pmol/40 µl and for glutamate, 23.6 ± 3.8 pmol/40 µl (n=15). In response to 10 µM CCK-8S (Bachem) given via the probe, aspartate values were increased 60 min later to a maximum of 265 ± 16 % of basal values ($p<0.01$; n=5; 2-way ANOVA followed by Dunnett's test). At the same time, glutamate levels rose to a near maximum of 168 ± 7 % of basal values ($p<0.01$; n=5). Levels of serine, glutamine, glycine, arginine, taurine and tyrosine were unaltered. Given 40 min prior to the cortical infusion of 10 µM CCK-8S, the CCK_B antagonist, L-365,260 (20 mg/kg s.c.), limited the rise in cortical aspartate by over half to 170 ± 10 % of basal values ($p<0.05$; n=5). However, this same dose of L-365,260 still allowed CCK-8S to increase glutamate by 44 ± 15 % above basal values ($p<0.05$; n=5). Whereas the enhanced glutamate levels were totally unaffected by systemic delivery of the CCK_A antagonist, L-364,718 (-40 min, 20 mg/kg s.c.), this treatment was able to limit the elevation in aspartate to 220 ± 4 % of basal levels ($p<0.05$; n=5).

These data reinforce those of Godukhin *et al.* (1995) concerning the selectivity of L-365,260 as an inhibitor of CCK-evoked increases in extracellular aspartate. For reasons that are yet unclear, they are at variance with the same report in which L-364,718 was found to prevent CCK-induced rises in cortical glutamate but was without influence on cortical aspartate.

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371P INCREASED HIPPOCAMPAL CALCIUM CURRENTS DURING WITHDRAWAL FROM CHRONIC ETHANOL TREATMENT

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Withdrawal from chronic ethanol treatment produces a syndrome of tremor, anxiety, and convulsions. The calcium channel antagonists, nitrendipine, nimodipine and isradipine, have been found to prevent these behavioural signs of withdrawal (Littleton et al., 1990; Whittington and Little, 1991a). Nitrendipine and isradipine prevented the electrophysiological signs of hyperexcitability in field potentials recorded from isolated hippocampal slices prepared after chronic ethanol administration (Whittington & Little 1991b; Ripley and Little, 1995). This evidence, plus the fact that the density of dihydropyridine binding sites (thought to correspond to L-type calcium channels) were increased by chronic ethanol treatment (Dolin et al., 1987), has suggested that ethanol withdrawal hyperexcitability involves increases in calcium currents. The present study directly examines the effects of prolonged ethanol administration on the functioning of calcium channels in isolated hippocampal slices.

Male C57 strain mice, 25-30 g, were used for hippocampal slice preparation. Ethanol was administered for 12-18 weeks as the sole drinking fluid; ethanol intake was 20-22 g/kg/24h. Hippocampal slices were prepared immediately on withdrawal from the ethanol treatment; recordings were made between 5 and 6h after slice preparation (i.e. from ethanol withdrawal). Blind whole cell patch clamping was used to record currents from CA1 pyramidal cells. The holding potential was -60 mV and two pulse protocols, one in 15 steps of 10 mV, from -80 mV, and the second a ramp from +40 mV to -60 mV were applied, 3 min after each solution change. The bathing medium was buffered with HEPES. Calcium chloride 2 mM was used for the first 5 min, then barium chloride, 2 mM, replaced the calcium chloride for the rest of the testing period. Intracellular potassium was replaced by caesium ions. Statistical analysis was by Student's t test, n = 8 for all data (one cell and one drug per slice, one slice per mouse).

Cell depolarisation evoked a fast transient inward current, sensitive to tetrodotoxin (300 nM), and a slow transient inward current, which was blocked by cadmium chloride, 20 µM. Replacing extracellular calcium with barium removed inactivation of the slow inward current. Table 1 gives the maximal steady state currents. The barium currents in slices prepared after ethanol treatment were significantly larger than those in control preparations ($P < 0.05$). The corresponding P value for the calcium currents was 0.07.

These results provide direct evidence that ethanol withdrawal hyperexcitability is associated with increased amplitude of neuronal calcium currents in hippocampal pyramidal cells.

Dolin et al. (1987) *Neuropharmacology*, 26, 275 - 279.
Littleton et al. (1990) *Psychopharmacology*, 100, 387 - 392.
Ripley, T. L. *et al.*, (1996) *Alc. and Alcoholism*, 31, 347-358.
Whittington, M. A. and Little, H. J. (1991a) *Br. J. Pharmacol.*, 103, 1677-1684.
Whittington, M. A. and Little, H. J. (1991b) *Br. J. Pharmacol.*, 103, 1313-1320.

Table 1. Maximum amplitude of voltage activated inward currents, nA, mean + s.e.m.* $P < 0.05$ compared with control values.

	calcium	barium
Controls	-1.93 ± 0.22	-1.42 ± 0.18
Ethanol treatment	-2.85 ± 0.4	-2.10 ± 0.24 *

372P PROLONGED INCREASES IN SENSITIVITY TO ADENOSINE LIGANDS FOLLOWING WITHDRAWAL FROM ETHANOL TREATMENT

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Decreases in adenosine uptake may be involved in the behavioural effects of ethanol. Tolerance occurs to this effect and changes in adenosine systems occur during chronic ethanol intake. We have previously demonstrated changes in the sensitivity to adenosine ligands during the acute phase of ethanol withdrawal (Manley & Little, 1995). Investigations into prolonged changes following ethanol treatment may be of relevance to the phenomena of craving and relapse in alcoholics. The present study investigated the effects of the adenosine receptor A1 selective agonist, CPA and the A1 antagonist, DPCPX (Collis & Hourani, 1993), tested after a prolonged interval from cessation of chronic ethanol treatment.

Male TO mice (25-30g) were administered ethanol by liquid diet. Ethanol-treated mice first received control diet for 3 days, followed by 2 days 3.5% v/v ethanol in the diet, then 9 days 5% ethanol, followed by 9 days 8% ethanol. Controls were pair-fed an isocaloritic control diet. Handling induced hyperexcitability was rated 3-6h after cessation of ethanol treatment to confirm physical dependence. Animals were withdrawn from ethanol for 6 days before further drug administration. CPA (0.1mg/kg), DPCPX (2.5mg/kg), both drugs or vehicle (Tween 80, 0.5%) were then injected i.p., 10 min (CPA) or 15 min (DPCPX) before testing. The CPA dose was in the middle of the range for depression of locomotor activity. Ambulatory locomotor activity was measured for 10 min, in single mice, by the breaking of infra-red beams (n=7 per treatment group).

CPA significantly decreased locomotor activity in ethanol treated animals ($P< 0.05$) compared with vehicle treated animals but there was no significant difference in controls. DPCPX caused a significant increase in locomotor activity of ethanol treated animals when compared with control animals ($P< 0.05$). Concomittant administration of CPA and DPCPX produced no changes in the locomotor activity of ethanol treated or controls when compared with vehicle treated animals. There were no differences between the activity of ethanol treated mice and controls after vehicle injection.

The results show that chronic ethanol intake can cause increases in the sensitivity to adenosine receptor ligands which far outlast the withdrawal hyperexcitability. They provide further evidence that a complex sequence of changes occurs during ethanol withdrawal. Increases in A1 receptor binding have been reported after chronic ethanol (Daly et al,. 1994) but such long-term changes have not previously been reported. Investigation of such prolonged effects of ethanol may aid research into the relapse after abstinence, which is the major problem in alcohol dependence.

Collis, M.G. & Hourani, S.M.O. (1993) *Trends Pharm. Sci.*14, 360-366
Daly et al. (1994) *Brain Res.*, 650, 153-156
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Table 1. Locomotor activity measurements, mean ± s.e.m., comparisons (*a priori*) by Student's t-test.
Con = controls; Eth = chronic ethanol; * $P< 0.05$ cf. Eth + vehicle, † $P< 0.05$ cf. Con + DPCPX

Con+vehicle	Con+CPA	Con+DPCPX	Con+CPA+DPCPX	Eth+vehicle	E/th+CPA	Eth+DPCPX	Eth+CPA+DPCPX
1863 ±180	1205±343	1381±284	1622±307	1789±240	895±244 *	2361±278 †	1420±289

373P CLASSICALLY CONDITIONED REWARD RESULTS IN POTENTIATED STARTLE IN RATS

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In the traditional potentiated startle paradigm, the amplitude of the startle response in rats is increased when the startle stimulus is preceded by a light which previously had been paired with the delivery of a shock. Thus, the potentiated startle paradigm has been described as a paradigm for studying the influence of classically-conditioned fear on the startle response (McAllister & McAllister, 1971; Davis et al., 1993). Whether this enhancement is in fact fear-specific has not been thoroughly addressed. Therefore, the present study determined whether the acoustic startle response would be potentiated in animals in which a rewarding stimulus, rather than a punishing stimulus, had been paired with the presentation of a light.

Four food-restricted (85-90% free-feeding weights) female Sprague Dawley rats were exposed to potentiated startle training sessions on two consecutive days. On 10 occasions each training session, a light (15-watt incandescant) was presented for 3500 msec; at the time of light offset, a food pellet dispenser was electronically activated and a 45 mg food pellet was provided to the rat. The 10 occurrences of the light + food pairings occurred on a random interval 240-second schedule. Twenty-four hours after the second training session, the subjects were tested for their startle response; 70 acoustic startle stimuli were presented at a 30-second ISI. The first 10 trials (habituation component) were presented in the dark and the stimulus intensity was 115 dB. The remaining 60 trials were arrayed in a 3 x 2 factorial design in which acoustic stimuli of 95, 105 and 115 dB were presented either in the absence of the light (Noise Alone trials) or 3500 msec after the onset of a 15-watt incandescant light (Light + Noise trials). The data obtained during the latter 60 trials were analyzed using a 3 x 2 factorial ANOVA, followed by post hoc analyses using the Student Neuman Keuls (SNK) test.

Figure 1 illustrates the results. As expected, the magnitude of the startle response was related to the intensity of the acoustic stimulus ($F[2,12]=36.96$, $p<0.05$). More important, there was a significant Stimulus Intensity x Trial Type interaction ($F[2,12]=13.68$, $p<0.05$) and post hoc SNK tests revealed that, for the 105 dB and 115 dB intensities, startle amplitude in the presence of the light was significantly greater than startle amplitude in the absence of the light.

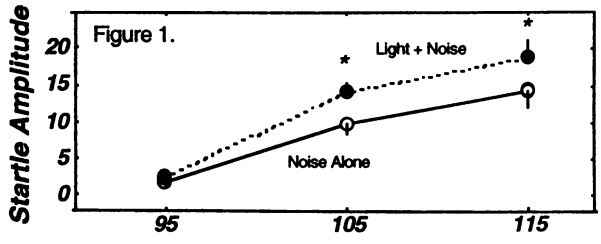

Figure 1. *Startle amplitude produced by 95, 105 or 115 dB Noise bursts presented alone (open circles) or in the presence of a light which previously had been paired with food presentation (filled circles). * p<0.05, post hoc SNK test following factorial ANOVA.*

Thus, it appears that the acoustic startle response can be enhanced by preceding the startle stimulus with a stimulus which has been associated with reward. Future studies comparing the neuropharmacology and neuronal circuitry of the traditional fear-potentiated startle and this newly-identified reward-potentiated startle will allow for a better understanding of the similarities and distinctions among "fear-based", "reward-based" and "conditioning-based" mechanisms with respect to the potentiated startle paradigm.

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McAllister, W.R. & McAllister, D.E. (1971) in *Aversive Conditioning and Learning*, ed. Brush, F.R. pp 105-179. Academic Press.

374P HYPER-RESPONSIVENESS TO ISOPRENALINE ON THE ATRIA, BUT NOT VENTRICLES, OF PREHYPERTENSIVE RATS

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Cardiac output is increased in the early stages of human and rat hypertension by a poorly defined mechanism. We have determined the concentration-response curves to isoprenaline on the isolated left ventricle, right atrium and left atrium of 5 week and 6 month old Wistar Kyoto rats (WKY) and Spontaneously Hypertensive rats (SHRs) using contractility methods. At 5 weeks the SHRs are prehypertensive with a systolic blood pressure of 106 mmHg ± 3 (16); WKY 107 mmHg ± 2 (14). The sensitivity of the left ventricles to isoprenaline was similar on the 5 week and 6 month old WKY and SHRs (Table 1). At 5 weeks resting heart rates of the WKY and SHRs isolated right atriums were similar [(384 beats^{-1} ± 14 (13) and 382 beats^{-1} ± 14 (14), respectively] but the sensitivity of the atria to isoprenaline was greater on the SHRs than WKY (Table 1). The basal heart rates decreased with age in the WKY to 335 ± 12 (10) at 6 months. There was a further decrease in basal heart rates in the presence of hypertension with the SHR isolated right atrium having a rate of 293 beats^{-1} ± 15 (10) at 6 months. At 6 months the sensitivities to isoprenaline had become similar on the atria of WKY and SHRs (Table 1). In summary, there is a hyper-responsiveness of the atria, but not the ventricles, to isoprenaline in the prehypertension stage of the SHR which may be responsible for the increased cardiac output observed at this stage. The hyper-responsiveness to isoprenaline is not observed in the presence of sustained hypertension.

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Table 1: Isoprenaline pD_2 Values

5 weeks	WKY	SHR
Left ventricle	8.50 ± 0.19 (9)	8.30 ± 0.13 (13)
Right atrium	9.07 ± 0.18 (16)	9.75 ± 0.10 (14)*
Left atrium	9.59 ± 0.20 (9)	10.08 ± 0.16 (10)*
6 months		
Left ventricle	8.39 ± 0.19 (14)	8.62 ± 0.15 (15)
Right atrium	8.89 ± 0.12 (8)	9.10 ± 0.19 (10)*
Left atrium	9.44 ± 0.38 (9)	9.47 ± 0.17 (16)

* P<0.01, Student's unpaired t-test