1P THE POTENT EFFECTS OF MODULATING INTERLEUKIN-1 ON NEURODEGENERATION IN THE RAT

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Interleukin-1 (IL-1) exerts diverse actions in the brain, and recent studies implicate this cytokine in neurodegeneration. Administration of IL-1β exacerbates ischaemic damage (Minami *et al*, 1992), whereas inhibition of endogenous IL-1, by administration of a recombinant human IL-1 receptor antagonist (IL-1ra), dramatically reduces ischaemic damage (Relton and Rothwell, 1993). Given that IL-1 is a potent pyrogen, and body temperature can markedly affect ischaemic damage, the significance of these observations is unknown. We investigated the affects of IL-1β and IL-1ra on body temperature during focal ischaemic brain damage. Following the observation that rhIL-1ra is neuroprotective, we determined the timecourse of action of IL-1ra, and whether it prevents, or merely delays the process of infarction.

Guide cannulae were stereotaxically implanted into the lateral cerebral ventricle of male, Sprague Dawley rats (180-220g) under pentabarbitone anaesthesia (60mg/kg, ip) to permit subsequent injections into the lateral ventricle of the brain (icv). In some of these animals radiotransmitters were implanted (ip) to allow measurement of core temperature (Tc) by remote radio telemetry. Seven days later, under halothane anaesthesia, ischaemia was induced by permanent occlusion of the left middle cerebral artery (MCAo) proximal to the lenticulostriate branch, by electrocoagulation. Unless stated otherwise, damage was assessed 24h later by incubating 500μm slices of fresh tissue in a 2,4 triphenyl tetrazolium medium. The infarct areas of slices 500μm apart was measured, then integrated to obtain the infarct volume. Results, presented as mean % inhibition (see Table 1), were analysed using Student's unpaired t-test for infarct volume, and MANOVA for temperature data. Control infarct volumes were similar in all experiments ($105mm^3$-$130mm^3$).

2.5ng (500IU) of IL-1β, administered icv 30min and 2h after MCAo, dramatically potentiated ischaemic damage, (92%, $P<0.001$) and caused a large (1.5°C) and significant ($P<0.001$) elevation of Tc, MCAo alone had no affect on Tc. 10μg of IL-1ra (Synergen, USA), administered icv 30min before and 10min after MCAo, dramatically reduced ischaemic damage, in both cortex and striatum, but had no significant affect on Tc throughout the 24h after ischaemia. Similar cortical protection resulted from administration (10μg, icv) at 0h only, when damage was assessed either 24h, or 7days after MCAo (by Haematoxylin & Eosin staining of 15μm frozen sections), though protection in the striatum was slightly reduced. Delayed treatment with IL-1ra (10μg, icv, 30min after MCAo) significantly protected cortical tissue, but no significant protection of striatal tissue was observed.

Table 1.

Experiment	% Inhibition of infarct volume Total	Cortex	Striatum
IL-1ra,-30,+10min	54 ($P<0.001$)	55 ($P<0.001$)	52 ($P<0.001$)
IL-1ra,0h (24h)	56 ($P<0.001$)	60 ($P<0.001$)	41 ($P<0.001$)
IL-1ra,0h (7day)	53 ($P<0.001$)	55 ($P<0.001$)	37 ($P<0.01$)
IL-1ra, +30min	26 ($P<0.05$)	29 ($P<0.05$)	15 ($P>0.05$)

These results confirm that IL-1β exacerbates ischaemic damage, but this may be partly due to an indirect effect on Tc. IL-1ra protects striatal and cortical tissue measured after 7days as well as 24h, and its action is not dependent on changes in Tc, suggesting that endogenous IL-1 plays a significant role in the development of ischaemic brain damage.

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2P EFFECTS OF A PROTEIN KINASE C INHIBITOR, BISINDOLYLMALEIMIDE I, ON RESPIRATORY BURST RESPONSES TO PLATELET ACTIVATING FACTOR AND PHORBOL ESTER IN HUMAN EOSINOPHILS

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Human eosinophils produce toxic oxygen metabolites, such as superoxide anion (O_2^-) and hydrogen peroxide, in response to a variety of stimuli, including platelet activating factor (PAF), which also stimulates adherence to endothelium, chemotaxis, degranulation and arachidonic acid metabolism in these cells (Kroegel *et al.*, 1994). The signal transduction mechanisms involved in eosinophil activation are poorly characterized. This study aimed to determine whether protein kinase C is involved in the respiratory burst response of human eosinophils to PAF.

Eosinophils were purified from peripheral blood of non-asthmatic male volunteers (age range 22 - 63 years) by differential centrifugation over Ficoll-Paque followed by immunomagnetic separation using anti-CD16-coated paramagnetic beads (Hansel *et al.*, 1989). Eosinophil preparations were > 90% pure (93 ± 0.6%, arithmetic mean ± s.e.m.; n = 17) and > 97% viable (98 ± 0.3%). Cells were incubated in HEPES-BSA buffer (pH 7.4) containing 100 µM ferricytochrome c at 37°C for 10 min in the presence or absence of the specific protein kinase C inhibitor bisindolylmaleimide I (BIM), after which buffer, PAF or the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) was added and the cells were incubated for a further 15 min at 37°C. Superoxide production was measured as the superoxide dismutase (60 U/ml)-inhibitable reduction of cytochrome c, assessed by extinction at 550 nm.

PAF and PMA induced concentration-dependent production of O_2^-, with respective EC_{50} values of 390 nM (geometric mean, 95% c.i. 260 - 610 nM; n = 6) and 2.8 fM (0.39 - 20 fM; n = 6) and respective maximal responses of 17 ± 2.2 and 35 ± 1.5 nmol 10^6 $cells^{-1}$ $15\ min^{-1}$. Responses to 1 µM PAF and 1 fM PMA were both significantly greater than baseline ($p < 0.05$) and were not significantly different from each other. Inhibition of basal, PAF-stimulated and PMA-stimulated O_2^- generation by BIM is shown in Table 1.

	Basal	PAF 1 µM	PMA 1 fM
Control	6.3 ± 1.3	12 ± 1.6	14 ± 3.2
BIM 0.1 µM	5.8 ± 1.2	12 ± 1.8	9.9 ± 2.2
" 1 µM	5.4 ± 1.3	8.6 ± 1.7*	3.6 ± 1.4**
" 10 µM	1.2 ± 0.5**	0.5 ± 0.3**	0.1 ± 0.9**

Table 1. Basal, PAF-stimulated and PMA-stimulated O_2^- generation by human eosinophils pre-treated with buffer (Control) or BIM. Data are expressed as nmol cytochrome c reduced per 10^6 cells in 15 min; mean ± s.e.m., n = 6. * $p < 0.05$, ** $p < 0.01$, compared to control.

The inhibition of the PMA response by 1 µM BIM (74 ± 7.9%) was significantly greater than the inhibition of the response to PAF (29 ± 7.5%; $p < 0.005$). These data may indicate that either (a) PAF-induced respiratory burst in human eosinophils is only partially dependent upon protein kinase C or (b) protein kinase C activation leads to a phosphorylation of the PAF receptor - with consequent loss of PAF sensitivity - that is blocked by BIM.

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3P DIRECT CYTOTOXICITY OF INTERFERON α-2b ON THE LLC-PK$_1$ RENAL CELL LINE

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Interferonα-2b (IFNα-2b), a member of the cytokine family, has been used as an anti-tumour agent. Its clinical use is limited by side-effects, including nephrotoxicity (Kurschel et al, 1991). The aims of this study were to investigate possible direct mechanisms of IFNα-2b nephrotoxicity. Comparisons of alterations in cellular integrity induced by IFNα-2b in cells from different areas of the nephron were carried out to investigate a possible site-selective action of the drug.

The established renal epithelial cell lines MDCK (distal tubular/collecting duct origin) and LLC-PK$_1$ (proximal tubular origin) were used as the model system. Changes in cellular viability and morphology were assessed using the MTT viability assay and phase contrast microscopy, respectively, following treatment from 24 to 72 hours with doses of IFNα-2b ranging from $8x10^1$ to $8x10^4$ iu/ml. These doses approximate those used in the clinical situation. Assessment of the effect of IFNα-2b on Na/hexose co-transport in LLC-PK$_1$ cells was carried out using the radiolabelled glucose analogue ^{14}C-methyl (α-D-U-> glucopyranoside (AMG). ^{3}H-Thymidine incorporation into cells following exposure to IFNα-2b was used as an index of mitotic cell division.Cell cycle changes were measured using the fluorescent dye propidium iodide in a B.D. Facstar Plus Flow Cytometer (Carter *et al*, 1990).

A dose- and time- dependent loss of cellular viability was seen in the LLC-PK$_1$ cells, but not MDCK cells for all doses of IFNα-2b ($p<.01$). Differences in sensitivities of LLC-PK$_1$ and MDCK cells to IFNα-2b were also detected in morphological changes. A decrease in cellular proliferation, cellular elongation and extensive vacuolisation was seen in LLC-PK$_1$ cells treated for 48 hours with IFNα-2b. No morphological effects of IFNα-2b were seen in MDCK cells. IFNα-2b treatment for 24 hours reduced ^{14}C-AMG incorporation into LLC-PK$_1$ cells from control values of 10.76±1.3 pmoles/mg protein to 5.07±0.64 pmoles/mg protein with $8x10^4$ iu/ml IFNα-2b. The same treatment decreased ^{3}H-Thymidine incorporation into LLC-PK$_1$ cells from 100% (control) to 31.91±1.87% with $8x10^4$ iu/ml IFNα-2b. No effect of IFNα-2b on ^{3}H-Thymidine incorporation into MDCK cells was observed. The drug induced a cell cycle blockade at the G_2/M phase in LLC-PK$_1$ cells treated for 24, 48 and 72 hours. At 72 hours, for example, the percentage of cells in the G_2/M phase increased from 3.97±0.76 (control) to 24.97±1.08 ($8x10^4$ iu/ml IFNα-2b). The drug was seen to have no effect on the cell cycle of MDCK cells.

In summary, IFNα-2b was found to be cytotoxic to LLC-PK$_1$ cells but not to MDCK cells. The effects of IFNα-2b included a blockade in G_2/M phase of the cell cycle. The greater susceptibility of LLC-PK$_1$ cells to IFNα-2b induced damage indicates a possible site-selective toxicity of IFNα-2b to the proximal tubular region of the kidney. However, a species-specific effect cannot be ruled out.

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4P DEXAMETHASONE AND CYCLOSPORIN A INHIBIT CYTOKINE RELEASE FROM HUMAN T-LYMPHOCYTES

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Marked airway eosinophilia, governed by interleukin-5 (IL-5), is a feature of intrinsic and extrinsic asthma (Anderson & Coyle, 1994). Corticosteroids are currently the most effective therapy for asthma, but their mechanism of action is still not certain. Wilson et al. (1994) demonstrated that activated lymphocytes in the airways of allergic asthmatics were downregulated by beclomethasone, and Schmidt et al. (1994) showed that dexamethasone and the immunosuppressant, cyclosporin A, (which improves lung function in asthma) inhibited IL-4 and IL-5 release from murine T_H2-type cells.

In this study we investigated the effects of dexamethasone, cyclosporin A, cetirizine, cromoglycate and salbutamol, on release of IL-2, IL-4, IL-5 and TNFα from human peripheral blood T-lymphocytes obtained from healthy donors. Cytokines were measured in supernatants from phytohaemagglutinin (PHA, 20ug/ml)-stimulated T-lymphocytes (>90% pure) incubated for 24h at 37°C.

Dexamethasone (Fig. 1a) inhibited release of IL-2, IL-4, IL-5 and TNFα (IC50 values of 1.9, 0.02, 0.02 and 0.2μM respectively) whereas cyclosporin A (Fig. 1b) inhibited release of IL-2, IL-4 and IL-5 (IC50 values of 0.03, 0.01 and 0.03μM) but not TNFα. Cetirizine, cromoglycate and salbutamol (up to 10-5M) did not significantly ($p>0.05$) alter cytokine release.

In summary, agents which are most effective in asthma treatment inhibit cytokine release from human T-lymphocytes with a wide spectrum of activity.

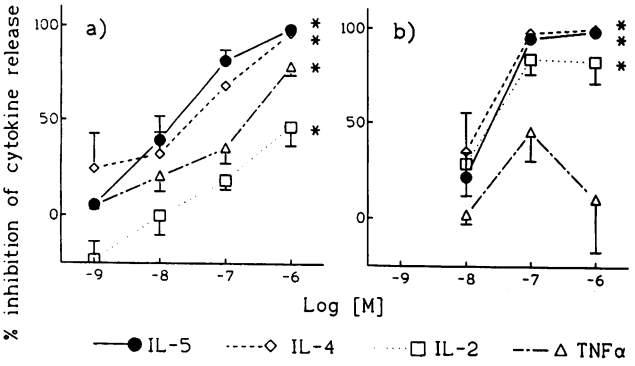

Fig. 1 Effects of (a) dexamethasone and (b) cyclosporin A on IL-2, IL-4, IL-5 and TNFα release from PHA-stimulated peripheral blood T-lymphocytes from healthy donors (Mean ± s.e.mean, n=3-14), *slope significant ($p<0.05$).

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5P SUPPRESSION OF ACUTE LUNG INJURY IN THE MOUSE BY THE PHOSPHODIESTERASE IV INHIBITOR, ROLIPRAM

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We have previously shown that combined LPS and zymosan treatment induces neutrophil-dependent acute lung injury in the mouse which is characterised by neutrophil sequestration and increased pulmonary vascular permeability. Lung injury is dependent on the leukocyte cell adhesion molecule CD11b (Miotla *et al*, 1993) and also vascular endothelial ICAM-1 (Miotla *et al*, 1994). Inhibitors of phosphodiesterase type IV (PDE-IV) have been shown to inhibit granulocyte function (Torphy *et al*, 1991). We have therefore investigated the effect of the PDE-IV inhibitor, rolipram, on lung injury in our model.

All experiments were carried out in anaesthetised BALB/c mice (18-20g; Hypnorm/Diazepam, 16mg/kg, i.p.). Animals were pretreated with LPS (*E. Coli* 0111:B4) at a dose of 3mg/kg, i.v. and left for 2 hours. Acute lung injury was induced by i.v. injection of zymosan A (10mg/kg) along with ^{125}I-human serum albumin (^{125}I-HSA). ^{125}I-HSA was used to measure changes in vascular permeability. After 30 minutes, ^{131}I-HSA as injected i.v. 5 minutes before sacrifice, as a marker of pulmonary intravascular volume. The activities of ^{125}I-HSA and ^{131}I-HSA in whole lung tissue were measured in a gamma counter and compared with that in plasma. Albumin accumulation was expressed as μl of plasma retained extravascularly in whole lung. Pretreatment with rolipram (5mg/kg, i.p.) or vehicle control was 30 minutes prior to LPS or, in a separate study, after LPS and 30 minutes before the zymosan. All lung tissue was processed for examination by light microscopy and neutrophil sequestration was quantified in separate animals by assaying myeloperoxidase (MPO) activity. Serum TNFα levels were measured by ELISA (Endogen).

Albumin accumulation in control saline treated mice was $2.3\pm0.9\mu$l (n=3). Treatment with combined LPS and zymosan treatment led to significant extravascular albumin accumulation in lung tissue ($10.6\pm3.6\mu$l, n=6; $p<0.01$). This was accompanied by a significant sequestration of neutrophils in pulmonary microvessels as seen by light microscopy and assessed by MPO activity (5-10 fold increase). Serum TNFα levels at 2.5 hours were elevated in LPS and zymosan treated mice (112 ± 41pg/ml, n=3) compared with saline-treated controls (<10pg/ml). Pretreatment with vehicle had no significant affect on lung injury induced by LPS and zymosan ($10.3\pm1.8\mu$l, n=5), whereas rolipram significantly reduced leakage to $3.5\pm0.9\mu$l (n=6; $p<0.01$). In addition, neutrophil sequestration was reduced by 71% ($p<0.05$) and the serum TNFα levels were decreased by 75% ($p=0.051$). When rolipram was given after LPS and 30 minutes before zymosan, albumin leakage was inhibited completely ($0.3\pm0.2\mu$l, n=7; $p<0.01$) when compared with vehicle treated mice ($10.2\pm2.6\mu$l, n=5).

These results demonstrate that rolipram blocks LPS-induced neutrophil sequestration in lung tissue and the associated vascular permeability. Some of these effects may be related to inhibition of TNFα production and down-regulation of ICAM-1. Rolipram also attenuates zymosan-induced activation of sequestered neutrophils. These data suggest that PDE-IV inhibitors may be useful in the treatment of acute lung injury.

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6P SUPPRESSION OF ANTIGEN-INDUCED AIRWAY RESPONSES AND BRONCHIAL HYPERRESPONSIVENESS BY CDP840, A NOVEL STEREO-SELECTIVE INHIBITOR OF PHOSPHODIESTERASE TYPE IV

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Phosphodiesterase type IV (PDE IV) controls the hydrolysis of cAMP in a number of tissues including airway smooth muscle, sensory neurones and inflammatory leukocytes and it has been proposed that PDE IV inhibitors will be of therapeutic value in asthma (Torphy and Undem, 1991). CDP840 (R-(+)-4-[2-(3-Cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl]pyridine) is a novel PDE IV inhibitor with IC_{50}s in the range of 1-10nM against partially purified natural PDE IV and human recombinant PDE IV. This inhibitor is stereo-selective since its S(-) enantiomer is approximately 40 times less active against the enzyme. Both enantiomers are inactive (IC_{50}s >100μM) against PDE types I, II, III and V. In leukocytes and neuronal cells, CDP840 (1-1000nM) causes a concentration-dependent, enantiomer-selective elevation of intracellular cAMP. We have now investigated the effects of CDP840 in airway responses in vivo.

In male Dunkin-Hartley guinea pigs (300-600g) sensitised to ovalbumin (Sanjar et al, 1990), antigen challenge caused a dose-related acute bronchoconstriction. Nebulised ovalbumin (5mg.ml^{-1}) delivered to the airways of anaesthetised animals caused an increase in pulmonary inflation pressure of 405 ± 41% (mean ± s.e mean, n=10) which was reduced to 164 ± 56% (n = 6) by administration of CDP840 (1.0mg.kg^{-1}, i.p.) 1h prior to challenge. CDP840 (3mg.kg^{-1}) did not reverse histamine-induced bronchoconstriction.

In a separate study, conscious animals were exposed to aerosolised ovalbumin (1mg.ml^{-1}) for 30 min. Bronchial alveolar lavage fluids collected 48h after challenge contained elevated numbers of eosinophils ($13.2 \pm 1.0 \times 10^6$ml^{-1}) compared to non-sensitised controls ($4.1 \pm 2.7 \times 10^6$ml^{-1}). Administration of CDP840 (0.01-1.0mg.kg^{-1} i.p.) 2h prior to challenge, 6h later and 24h before lavage caused a dose-dependent reduction in eosinophil numbers with maximal inhibition reached at 0.1mg.kg^{-1} ($7.1 \pm 1.6 \times 10^6$ml^{-1}).

Ozone induces bronchial hyperresponsiveness (BHR) to histamine with increased sensitivity and enhanced maximal responses (Holbrook & Hughes, 1992). The dose of inhaled histamine required to increase airway resistance by 200% in normal animals was 175± 33μg.ml^{-1} for 10s (n = 13) and this was reduced to 25± 6μg.ml^{-1} for 10s (n = 25) in animals exposed to ozone (3ppm) for 30 min. CDP840 (0.001-0.1mg.kg^{-1} i.p.) administered 1h before ozone caused a dose-dependent reversal of BHR and doses greater than 0.01mg.kg^{-1} completely abolished BHR.

CDP840 was more potent than its S(-) enantiomer in suppressing antigen or ozone-induced responses in guinea pig airways. The different potency of CDP840 in these airway responses indicates that it is working in different tissues to elevate cAMP which in turn regulates BHR, eosinophil recruitment and antigen-induced bronchoconstriction. CDP840 is currently being evaluated in human asthma.

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7P CELL ACCUMULATION, TNF-ALPHA AND NITRITE PRODUCTION IN A RAT MODEL OF LPS-INDUCED PLEURISY

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Intrapleural injection of bacterial lipopolysaccharide (LPS) to experimental animals causes an inflammatory reaction mainly characterized by leucocyte infiltration (Bozza et al., 1993). Tumor necrosis factor alpha (TNF) has been described to be an early mediator of LPS-induced inflammation and to be produced by LPS-stimulated monocytes/macrophages and PMN leucocytes, both in vitro and in vivo. Nitric oxide synthase (NOS) is also induced in many different organs and cell types by LPS. Here we have examined and characterized the time course of cell accumulation, TNF and nitrite production in a rat model of LPS induced pleurisy. LPS from E. coli (0.25×10^6 units per cavity; serotype 0127:B8, Sigma) was injected intrapleurally to male Wistar rats (Charles River, 220-300g) under enflurane light anaesthesia. Rats were sacrificed after 2,6,24 and 48h following LPS injection and the pleural cavity opened and washed with 2 ml of sterile saline. In the pleural washing, total and differential cell count was evaluated by light microscopy. Cells obtained from the pleural cavity were incubated for 24h to determine nitrite production by Griess reaction. In the cell free pleural washing, TNF and protein content were evaluated. Following LPS intrapleural administration, total leucocyte count was significantly increased from control value only at 48h point (control $4.87 \times 10^6 \pm 1.21$, mean±s.e.mean, n=4; LPS $12.27 \times 10^6 \pm 0.96$, n=3; $p<0.01$, Student's t test). Conversely, 6h after LPS injection there was a significant reduction in the total leucocyte count (control $5.37 \times 10^6 \pm 0.99$, n=6; LPS $2.94 \times 10^6 \pm 0.32$, n=7, $p<0.05$). The differential cell count showed that mononuclear cell count was significantly increased after 48h (control $4.52 \times 10^6 \pm 1.11$, n=4; LPS $7.8 \times 10^6 \pm 0.82$, n=3, $p< 0.05$), while it was reduced at 6h (control $3.79 \times 10^6 \pm 0.61$ n=6, ; LPS $0.84 \times 10^6 \pm 0.41$, n=7, $p<0.01$) and 24h (control $4.21 \times 10^6 \pm 0.31$, n=3; LPS $2.83 \times 10^6 \pm 0.21$, n=3, $p<0.05$). After 2h there was no significant difference. Polymorphonuclear cell count was increased at 2,24 and 48h when compared to control values (2h, $4.47 \times 10^6 \pm 1.33$, n=5, vs $1.79 \times 10^6 \pm 0.46$, n=6, $p<0.05$; 24h, $3.55 \times 10^6 \pm 0.38$, n=3, vs $0.90 \times 10^6 \pm 0.31$, n=3 $p<0.05$; 48h, $4.47 \times 10^6 \pm$, n=3, vs $0.36 \times 10^6 \pm 0.1$, n=4, $p<0.01$). Cells obtained from pleural washing 6h after LPS injection produced the highest amount of nitrites, after being cultured for 24h. The highest amount of TNF in the cell free pleural washing was obtained 2h after LPS injection (control, 56.38±32.1 U ml^{-1} n=6; LPS 3492±1882 U ml^{-1}, n=5, $p< 0.01$, Mann-Whitney test) and declined at 6h. At 24h and 48h after LPS injection TNF was undetectable.

Our results indicate that in endotoxin-induced pleurisy there is a time dependent TNF and nitrite production. TNF peaks at 2h while nitrites peak at 6h. These findings are in agreement with other studies demonstrating the induction of NOS by TNF. We suggest that TNF and nitrite production may be due to stimulation of resident pulmonary cells. Following this stimulation, activated cells could infiltrate lungs and initiate inflammatory events.

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8P THE EFFECT OF A NOVEL INHIBITOR OF TUMOUR NECROSIS FACTOR α (TNFα) PROCESSING, BB-1101, IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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BB-1101 (2S-allyl-N^1-hydroxy-3R-isobutyl-N^4-(1S-methylcarbamoyl-2-phenylethyl)-succinamide) is a member of a novel class of broad spectrum inhibitors of matrix metalloproteinases which have also been shown to be capable of inhibiting the processing of tumour necrosis factor alpha (TNFα), (Gearing *et al*, 1994). The aim of this work was to investigate the *in vivo* effects of BB-1101 against lipopolysaccharide (LPS) induced increases in serum TNFα in the anaesthetised rat, and in experimental autoimmune encephalomyelitis (EAE).

The *in vivo* activity of BB-1101 against LPS (*E. coli* 0111:B4 Difcolabs, U.S.A.) induced increases in TNFα, was assessed following i.v. and i.p. administration. Male Sprague Dawley rats (400-500g) were anaesthetised with sodium pentobarbitone/thiopental mixture i.p. ($22.5 mg.kg^{-1}$ pentobarbitone, $63 mg.kg^{-1}$ thiopental). The trachea, both jugular veins and left carotid artery, were cannulated. BB-1101 was infused intravenously ($0.25 mg.kg^{-1}.hr^{-1}$), 10min after a blood sample had been taken. Fifteen minutes later a single intravenous bolus dose of LPS (E.Coli 0111:B4, Difco Labs, USA), $0.5 mg.kg^{-1}$, was given via the right jugular vein. A further blood sample was taken one hour after administration of LPS. When the effect of i.p. BB-1101 was assessed, the same experimental procedures were used except that 55 minutes after administration of BB-1101, ($2 mg.kg^{-1}$ i.p.), a blood sample was taken and 5 minutes later LPS was given. A further blood sample was taken 1 hour after LPS. Serum was then frozen at -20°C, and TNFα levels determined using a murine TNFα ELISA kit (Genzyme USA). BB-1101 at $0.25 mg\ kg^{-1}.hr^{-1}$ i.v. and $2 mg.kg^{-1}$ i.p. significantly ($p<0.01$; Student's t-test) inhibited the increase in serum concentration of TNFα from $11.5 \pm 1.8 \times 10^3$ (mean±s.e.m. $units.ml^{-1}$; n=5) for controls to $1.8 \pm 0.4 \times 10^3$ (n=6) for i.v. BB-1101 treated (85% inhibition), and from $15.1 \pm 2.7 \times 10^3$ (n=4) for controls to $2.6 \pm 1.0 \times 10^3$ (n=6) for i.p. BB-1101 treated (83% inhibition).

BB-1101 was investigated in EAE in male Lewis rats (200-300g), which were inoculated at the base of the tail under halothane anaesthesia with guinea pig myelin basic protein $40 \mu g.kg^{-1}$, formulated in supplemented complete Freunds adjuvant (mycobacterium tuberculosis $= 1 mg.ml^{-1}$). On days 6-17 post-inoculation, animals were dosed b.i.d. with BB-1101, $2 mg.kg^{-1}$ i.p., or vehicle, phosphate buffered saline with 0.01%(v/v) Tween 80. Animals were scored daily for disease severity using a modification of the clinical scoring system of Sedgwick *et al*, 1987. Analysis of 5 separate studies (n=6-9 for each study) showed that the peak clinical score (mean±s.e.m. = 2.70±0.40) of control animals occurred on day 13. Treatment with BB-1101 $2 mg.kg^{-1}$ i.p. significantly ($p<0.05$; Student's t-test) reduced this clinical score to 1.54±0.30 accompanied by a reduction in bodyweight loss: mean day 13 weight for BB-1101 treated animals was 239±5g compared to controls 228±7g. This indicates that BB-1101 can inhibit the disease in this model.

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9P BB10010, A DEMULTIMERISED VARIANT OF LD 78, PRODUCES A RAPID INCREASE IN CIRCULATING WHITE BLOOD CELLS AND CELLS WITH BONE MARROW REPOPULATING ABILITY

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BB-10010 is a recombinant demultimerised variant of LD78, the human homologue of murine macrophage inflammatory protein (mMIP-1α). It has a reduced tendency to aggregate in physiological solution and therefore has advantages for manufacture, formulation and clinical studies. mMIP-1α is able to inhibit the cycling status of early haematopoietic progenitor cells (spleen colony forming units) in the mouse and therefore can protect cells against cell cycle specific cytotoxic agents used in cancer chemotherapy (Lord et al., 1992). Similarly, we have shown that BB-10010 can inhibit the killing of multi-lineage progenitor cells (methylcellulose colony forming units, CFU) by hydroxyurea. On day 1 following hydroxyurea (1g.kg^{-1} i.p. at 0 and 7h on day 0), CFU per femur fell from 13000±707 to 4992±1186 in vehicle treated mice, whereas in mice (B6D2F1, 8-12 weeks old) treated with BB-10010 (1μg.kg^{-1} s.c at 3 and 6h on day 0) progenitor number remained unaffected at 16466±2292 (Values represent mean±s.e.m. of triplicate plates from blood pooled from 5 animals).

In addition to this bone marrow protection in C57BL/6J mice (8-12 weeks old), BB-10010 (100μg.kg^{-1} s.c., 30min prior to sampling) produced a rapid and transient increase in blood neutrophil count from 0.42±0.07 to 3.39±0.06 x10^9.l^{-1} ($p<0.01$, Students t-test, n=5). Blood CFU were also increased by BB-10010 from 12.5±1.8 to 40.5±3.7 CFU.ml^{-1} blood (n=5). Further studies have revealed that the release of neutrophils and CFU can be enhanced by pre-treatment with granulocyte colony stimulating factor (G-CSF). G-CSF alone (100μg.kg^{-1} s.c. *b.i.d.* for 4 days) produced an increase in neutrophil count from 0.42±0.07 to 2.29±0.59 x10^9.l^{-1} ($p<0.01$ Students t-test, n=5). When BB-10010 was administered 30 min prior to sampling, neutrophil count was increased to 14.55±1.77 x10^9.l^{-1} ($p<0.01$ Students t-test compared to PBS controls, n=5). CFU.ml^{-1} blood count in G-CSF treated mice was increased from 35.5±13 to 2643±376 ($p<0.01$ Students t-test) compared to PBS controls. When BB-10010 was administered 30 min prior to sampling, CFU.ml^{-1} blood counts were increased to 6182±487 ($p<0.01$ Students t-test compared to PBS controls). (All values represent mean±s.e.m, n=5 pooled blood samples each from 5 animals).

Blood from female mice (B6D2F1, 6-8 weeks old) treated with either G-CSF or with G-CSF and BB-10010 treated mice can reconstitute the bone marrow of male B6D2F1 mice (8-12 weeks old) lethally irradiated (8.2Gy total body irradiation from an x-ray source) in a manner similar to autologous bone marrow. Mice injected with 0.15ml control blood showed no sign of haematopoietic recovery and were dead by day 13 following irradiation. Mice injected with blood from G-CSF mobilised donors showed recovery with neutrophil counts > 0.05 x 10^9.l^{-1} seen by day 18. Mice receiving blood from G-CSF and BB-10010 mobilised donors also showed recovery of haematopoiesis with neutrophil counts > 0.05x10^9.l^{-1} on day 15, n=2 for each day of sampling. The data suggests that BB-10010 protects stem cells against cytotoxic damage. In addition the stem cell mobilisation properties of BB-10010 may be useful for peripheral blood stem cell transplantation.

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10P THROMBOLYTIC ACTIVITY OF THROMBIN-ACTIVATABLE PLASMINOGEN IN THE ANAESTHETISED RABBIT

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Thrombin-activatable plasminogen (TAPgen) is an engineered form of human plasminogen designed to be activated by the blood clotting system to produce thrombus-selective thrombolytic activity. TAPgen has been shown to lyse clots *in vitro* (Dawson et. al., 1994). The aim of the present study was to show that the TAPgen BB-10153 has thrombus selectivity *in vivo* and that this selectivity confers an advantage over current thrombolytic therapy in that it produces thrombus lysis without systemic activation and associated bleeding complications.

The *in vivo* thrombolytic activity of the TAPgen BB-10153 was examined in a model of arterial thrombosis in the anaesthetised (sodium pentobarbitone 35mg.kg^{-1} i.v.) male New Zealand White rabbit (2.9 - 3.5kg) and its activity has been compared to that of tissue plasminogen activator (tPA). A coil of copper wire placed in the left femoral artery was used to induce thrombus formation. BB-10153 and tPA were administered 30 minutes after thrombus formation, as measured by cessation of blood flow in the femoral artery. In addition, the effects of BB-10153 and tPA on bleeding time have been studied in the anaesthetised rabbit. Bleeding times were assessed by a standard cut (Surgicutt®) of the marginal ear vein 30 mins and 120 mins after administration of the thrombolytic proteins.

Administration of tPA at 1mg.kg^{-1} i.v. resulted in transient reperfusion in 2/4 rabbits whereas at 3mg.kg^{-1} i.v. it produced prolonged periods of flow in 3/4 rabbits. However, reocclusion occurred in 2 of these 3. BB-10153 at 10mg.kg^{-1} i.v. resulted in reperfusion in 4/4 rabbits with no reocclusion up to 4 hours after administration. The thrombolytic activity of BB-10153 was not accompanied by systemic plasmin production as there was no change in the concentration of circulating fibrinogen and α_2-antiplasmin. In contrast, tPA at 3mg.kg^{-1}, although less effective at thrombolysis, resulted in decreases in circulating fibrinogen of 68.6 ± 7.7% and α_2-antiplasmin of 72.3 ± 2.4% at 30 minutes after dosing. (Values are mean ± s.e.m.)

Pretreatment bleeding times were 3.7 ± 0.6 and 3.0 ± 0.3 mins (n=4) for tPA and BB-10153 treated animals respectively. tPA at 3mg.kg^{-1} (n=4) increased bleeding time to 24.8 ± 4.4 mins ($p<0.01$, Students t-test) at 30 mins and 6.6 ± 0.92 mins ($p>0.05$, Students t-test) at 120 mins after dosing. In contrast, BB-10153 at 10mg.kg^{-1} (n=4) did not significantly ($p>0.05$, Students t-test) affect bleeding time (4.2 ± 0.5 mins at 30 mins after dosing and 5.4 ± 1.1 at 120 mins after dosing) demonstrating the thrombus selectivity of BB-10153. (Values are mean ± s.e.m.). The results indicate that TAPgen has potential as a novel thrombus-selective thrombolytic therapy with a reduced risk of inducing haemorrhage.

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11P MONO(ADP-RIBOSYL)TRANSFERASE ACTIVITY IN HUMAN NEUTROPHILS: A POSSIBLE ROLE IN CHEMOTAXIS

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Eukaryotic mono(ADP-ribosyl)transferases are the functional homologues of the enzymes expressed in *V cholerae, B pertussis* and numerous other bacterial toxins, whose activity includes mono(ADP-ribosyl)ation of G proteins. Few of the substrates of the eukaryotic enzymes in intact cells have been identified, although they appear to include $G_{\alpha\alpha}$ (Donnelly *et al* 1992) and integrin α7 (Zolkiewska and Moss 1993). We have recently demonstrated Arg-specific mono(ADP-ribosyl)transferase activity in human polymorphonuclear neutrophil leucocytes (PMNs). The catalytic activity is expressed on the cell surface, and is probably GPI-linked, as it is released by the addition of PI-specific phospholipase C. The Km for NAD^+ in the ADP-ribosylation of agmatine is 100 ± 30.4 μM (n=4 ± SEM), and the Vmax is 1.4 ± 0.2 pmoles ADP-ribosylagmatine/h/10^6 cells. The possibility of non-enzymatic ADP-ribosylation of the α-NH_2 group of agmatine was excluded as diethylamino(benzylidineamino)guanidine, which is devoid of an α-NH_2 group, was not ADP-ribosylated in the absence of PMNs. Therefore the ADP-ribosylation of agmatine is on the guanidino nitrogen.

The physiological role of this enzyme in PMNs remains unresolved. However, we now show that inhibition of the enzyme with nicotinamide, novobiocin, vitamin K_1 or vitamin K_3 blocks both microfilament actin polymerization and chemotaxis following exposure of PMNs to FMLP, PAF or C5a. There are close linear correlations between the Ki values for enzyme inhibition and inhibition of actin polymerization (r^2 = 0.98) or inhibition of chemotaxis (r^2 = 0.94) (Table 1.). The inhibitors of mono(ADP-ribosyl)transferase activity had no effect on the magnitude of Ca^{2+} transients in PMNs following exposure of the cells to chemotaxin.

Table 1. IC_{50} values of inhibitors of mono(ADP-ribosyl)transferase, and their effects on actin polymerization.

Inhibitor	Published IC_{50} (μM)	Actin polymerization IC_{50} (μM)
Vitamin K_1	1.9	1.3±0.9
Vitamin K_3	10	5.5±0.5
Nicotinamide	3500	11500±4500
Novobiocin	280	263±117

The signal transduction pathway mediating receptor dependent actin polymerization in PMNs is unknown. There was no consistent inhibition of receptor dependent actin polymerization by a panel of PKC or tyr kinase inhibitors, although calphostin C and tyrphostin A1 attenuated the response. Inhibitors of PKA, PKG, myosin light chain kinase or phosphatases had no effect.

We propose that mono(ADP-ribosyl)transferase activity of PMNs has a role in the control of microfilament actin polymerization, which is an essential step in the continuous re-alignment of the cytoskeleton during chemotaxis.

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12P SLOWLY DEVELOPING BLOCK OF THE NEURONAL STIMULANT ACTIONS OF CICAPROST BY BMY 45778

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The non-prostanoid prostacyclin mimetic BMY 45778 inhibits ADP-induced platelet aggregation in human platelet-rich plasma with an IC_{50} value of 27 nM; this is probably an underestimate of its agonist potency at IP-receptors due to protein binding (see Meanwell *et al.*, 1994). BMY 45778 also fully relaxes human pulmonary artery rings (IC_{50} = 2.5 nM, n = 3), being only 2 - 5 times less potent than the stable prostacyclin analogue cicaprost (this communication). In contrast, we have recently shown that BMY 45778 behaves as a low potency partial agonist (maximum = 15%) on the rat isolated colon (Wise *et al.*, 1995). IP agonists such as cicaprost and iloprost inhibit colon spontaneous activity by releasing NANC transmitters, one of which is nitric oxide; the effect is abolished by tetrodotoxin (Qian & Jones, 1995).

We now report that prolonged exposure of neuronal IP preparations to BMY 45778 produces a slow onset/slow offset block of cicaprost action. Results for the rat colon are shown in Figure. 1. For comparison, the lack of effect of a related analogue, BMY 42393, is also shown (DMSO conc. = 0.01% for both); the slight increase in sensitivity to cicaprost is very similar to that found for cicaprost alone. Inhibition of colon motility by 3 μM nicotine (also due to activation of NANC neurones) was unaffected by BMY 45778.

A similar inhibitory profile for BMY 45778 (1 μM) was found on guinea-pig isolated ileum, where cicaprost (1 - 20 nM) produces contraction by release of ACh and a substance P-like transmitter (Jones & Lawrence, 1993). Again the neuronal stimulant action of nicotine was unaffected. Finally, BMY 45778 (1 μM) blocked potentiation of electrical field stimulation responses of guinea-pig vas deferens by cicaprost (1 - 50 nM). This action of cicaprost is probably due to potentiation of neuronal ATP release.

Thus the inhibitory profile of BMY 45778 on the neuronal preparations differs markedly from its agonist actions on human platelets and pulmonary vessels. Its slow onset may reflect the low abundance of a particular "active" conformer, due to restricted rotational mobility of the bis-oxazole unit.

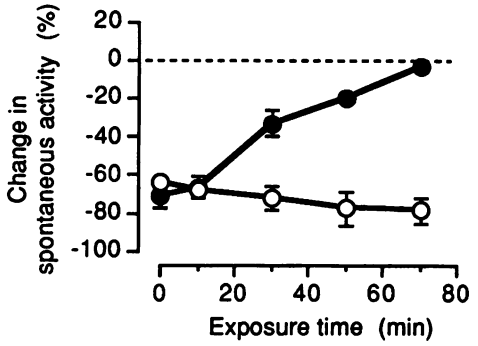

Figure 1. Inhibition of rat colon motility by 10 nM cicaprost: effects of continuous exposure to 1 μM BMY 45778 (●) and 1 μM BMY 42393 (○). Means ± s.e.mean, n = 5.

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13P TOTAL BODY PROSTACYCLIN SYNTHESIS IS ENHANCED IN ADJUVANT ARTHRITIC RATS

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Studies concerning the involvement of prostaglandins in inflammatory diseases mainly focussed on the E prostaglandins; only Brodie *et al.* (1980) considered prostacyclin to be an important pro-inflammatory mediator. To investigate the role of prostacyclin in a disease with systemical inflammatory activity, we measured in 12 rats with adjuvant arthritis (male Sprague Dawley, 20 days after induction) the urinary excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$, 2,3-dinor-thromboxane-B_2 and 7α-hydroxy-5,11-dioxo-tetranor-prosta-1,16-dioic acid (PGE-M), reflecting total body synthesis of prostacyclin, thromboxane and the E prostaglandins, respectively. At the same time (day 20 after induction of arthritis) arthritis score and body weight were assessed. 12 healthy rats of the same strain and age served as control.

The urinary prostanoid metabolites were measured by gas chromatography/tandem mass spectrometry using stable prostanoid isotopes as internal standards and corrected by urinary creatinine excretion (Fauler *et al.*, 1994). Arthritis score (maximal value/animal = 16) was examined as described previously (Stichtenoth *et al.*, 1994).

Urinary 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and PGE-M excretions were significant ($p < 0.001$, 2-tailed unpaired t-test) higher in adjuvant arthritic rats as compared to non-arthritic control rats (2,3-dinor-6-oxo-$PGF_{1\alpha}$ mean 7.8 ± s.d. 3.8 nmol/mmol creatinine versus 0.7 ± 0.2 nmol/mmol creatinine; PGE-M 51.3 ± 18.1 nmol/mmol creatinine versus 9.4 ± 2.3 nmol/mmol creatinine). The urinary 2,3-dinor-thromboxane-B_2 excretion was slightly, but not significantly higher in arthritic rats than in non-arthritic control rats (mean 268 ± s.d. 126 pmol/mmol creatinine versus 185 ± 114 pmol/mmol creatinine). There was no significant difference in urinary creatinine excretion between arthritic rats (mean 70 ± s.d. 12 µmol/d) and healthy control rats (mean 68 ± s.d. 12 µmol/d). The mean arthritis score in the arthritic rats was 10 ± s.d. 2; in the non-arthritic control rats no signs of inflammation could be examined, therefore the arthritis score of each rat was 0. Body weight was lower ($p < 0.001$, 2-tailed unpaired t-test) in arthritic rats than in healthy control rats (mean 218 ± s.d. 18 g versus 242 ± 13 g).

This study clearly shows, that in adjuvant arthritic rats total body synthesis of prostacyclin is increased 11-fold as compared to healthy controls, whereas the total body synthesis of the E prostaglandins increased only 5-fold and thromboxane synthesis remained unchanged. Our data suggest, that prostacyclin plays a role in generalized inflammatory diseases, comparable to that of the E-prostaglandins.

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14P THE EFFECT OF CAPSAICIN ON SUBSTANCE P EXPRESSION IN RATS WITH CHRONIC ARTHRITIS

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The sensory nervous system is implicated in the aetiology of inflammatory joint disease. We have investigated the effect of capsaicin-induced sensory denervation in adult rats on changes in the levels of substance P and nerve growth factor (NGF) in chronic arthritis.

Under fentanyl citrate: fluanisone (32 µg/kg: 1 mg/kg i.m.) recovery anaesthesia two groups of adult rats were injected with capsaicin (50 mg/kg s.c.). Five days later polyarthritis was induced (under anaesthesia as before) in one group of capsaicin-treated and one group of untreated rats by intradermal injection of 100 µl of 10 mg/ml suspension of adjuvant (heat-killed *Mycobacterium tuberculosis* in sterile paraffin oil) into the base of the tail. On day 21 arthritis (26 days after capsaicin treatment), substance P levels were measured by radioimmunoassay in L_4/L_5 dorsal root ganglia (DRG), sciatic nerve and hindpaw footskin samples. Additionally, mRNA expression of γ-preprotachykinin (PPT), which encodes for substance P, and trkA, the high affinity receptor of NGF were measured by northern blot hybridisation. Levels of NGF were measured in sciatic nerve and footskin. Data are mean ± SEM, analysed by unpaired t-test (footpad diameters) or by one-way analysis of variance with post-hoc Duncan's multiple range tests.

Capsaicin treatment reduced substance P in DRG from 143±14 (control, n=7) to 92±3 pg/L_4/L_5 DRG (n=7), in sciatic nerve from 85±5 (control) to 49±3 pg/cm nerve and in footskin from 28±2 (control) to 17±1 pg/cm^2; all $p<0.01$ vs control. NGF levels in footskin were unchanged by capsaicin treatment whereas sciatic nerve levels were significantly reduced from 25.2±2.4 (control, n=8) to 18.5 ±0.9 pg/cm nerve (n=7); $p< 0.05$ vs control. In contrast PPT mRNA and trkA mRNA were increased 2.7±0.4 and 2.8±0.5 fold respectively after capsaicin treatment (n=5; both $p<0.01$ vs control). Twenty one days after induction of arthritis, hindpaw footpad diameter had increased from 5.7±0.1 mm to 9.2±0.3 mm in untreated arthritic rats (n=9). Arthritis increased PPT mRNA expression 2-fold (nsd vs control) and substance P to 174±23 and 104±9 in DRG and sciatic nerve ($p<0.05$) respectively. Footskin substance P in arthritic rats was not significantly different from control values. NGF levels in sciatic nerve and footskin and trkA mRNA expression were unaltered after 21 days arthritis. Footpad diameter in capsaicin-treated arthritic rats increased to 7.6 ± 0.2 mm (n=8; $p<0.01$ vs untreated arthritic) on day 21 arthritis. Arthritis in capsaicin-treated rats did not significantly alter PPT mRNA expression or substance P levels in DRG and footskin compared to capsaicin-treated control rats. Sciatic nerve substance P was increased to 61±4 pg/cm nerve ($p<0.05$ vs capsaicin-treated control) but was still significantly below untreated control levels ($p<0.01$). NGF levels and trkA mRNA expression were also unaltered compared to capsaicin-treated controls. Our results show that pretreatment of adult rats with capsaicin significantly attenuates the development of arthritis suggesting an important neurosensory influence in the aetiology of inflammatory joint disease. Interestingly, whilst capsaicin treatment reduced peripheral tissue levels of substance P, expression of its precursor PPT mRNA was increased almost three-fold, suggesting that capsaicin may interfere with the biosynthesis of substance P by some translational modification.

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Endogenous glucocorticoids are known to influence immune and inflammatory responses through their immunosuppressive and anti-inflammatory actions. In contrast, recent evidence suggests that another steroid hormone, dehydroepiandrosterone (DHEA) provides an immunostimulatory influence, opposing these effects of glucocorticoids (Daynes, *et al.*, 1990). DHEA circulates as its inactive sulphated form, dehydroepiandrosterone sulphate (DHEAS), requiring conversion to DHEA by a steroid sulphatase for biological activity. Therefore inhibition of steroid sulphatase activity may affect immune responses allowing endogenous glucocorticoid effects to predominate. Using a model of contact sensitisation (CS), the effects of these steroids and a steroid sulphatase inhibitor, 3-estronesulphamate (CT2251) (Howarth, *et al.*,1994) were investigated. Male Balb/c mice (Harlan UK Ltd), 16-20g, were shaved on their right flank on day -1, and 50µl 2.5% oxazalone (Sigma) in 4:1 acetone:olive oil, or vehicle only topically applied on day 0. On day 5 the animals were challenged on their right ear with 25µl 0.25% oxazalone. Ear measurements were taken prior to and 24 post challenge. Results were expressed as change from prior measurement, and analysed as percentage inhibition from the vehicle control. Groups of mice (n=7-14) were treated with either DHEA ± CT2251, DHEAS ± CT2251 or dexamethasone (DEX). Steroids were given subcutaneously at 5mg/kg in olive oil on days 0 and 4. CT2251 was given subcutaneously at 10 & 0.1mg/kg in olive oil on days 0 and 4. Augmentation of the CS response was seen at 24 hours with DHEA (43.2% ± 19.4) (mean ± SEM) and DHEAS (45% ± 18.4) ($p=0.05$ ANOVA). In combination with CT2251 (10 & 0.1 mg/kg), DHEA augmented the response by 41% ± 18.1 and 22% ± 16.8 respectively ($p<0.05$). In contrast CT2251 inhibited the DHEAS augmented response by 48.9% ± 18.5 & 35% ± 15.0 respectively ($p<0.05$). CT2251 alone at 10 and 0.1mg/kg inhibited the response by 61.6% ± 8.03 & 38.6% ± 13.95 ($p<0.05$). DEX significantly inhibited the response by 82.4% ± 4.97. In a further study, the inhibitory effect of 0.1mg/kg CT2251 (51.3% ± 7.89) ($p<0.05$) could be reversed by increasing doses of DHEAS, with 50mg/kg completely reversing the effect and showing an augmentation of 35.8% ± 9.49 ($p<0.05$). In conclusion, the inhibitory effect of CT2251 on its own and on DHEAS, but not DHEA, augmented responses confirms the regulatory role of steroid sulphatase enzyme in this model.

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16P PROSTAGLANDIN OVERPRODUCTION IN DRUG-INDUCED CYTOLYSIS OF MACROPHAGES WITHOUT COX-2 INDUCTION

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During a study of the upregulation by cytokines of the inducible form of cyclo-oxygenase (COX-2) in macrophages, we found a paradoxical increase in PGE_2 released into the medium after adding agents designed to prevent transcription or translation of the COX-2. We show here that enhanced PGE_2 generation is associated with drug-induced cell death, without enhanced expression of COX-2.

Resident peritoneal macrophages from Swiss Webster mice or cells from the mouse macrophage-like J774 line were plated at 5×10^5 cells/ml, cultured in DMEM and the amounts of PGE_2 released into the medium measured by radioimmunoassay. Expression of COX-2 within the cells was determined by Western blotting using specific anti-mouse COX-2 antibody from Cayman.

Peritoneal macrophages cultured for 1h or 42h without stimulants or with 25 ng/ml hrTNFα released 408 ± 11 and 1975 ± 83 pg/ml PGE_2 (control) and 468 ± 19 and 3150 ± 87 pg/ml (TNFα). Pretreatment of control cells with actinomycin D (2×10^{-6}M) or verapamil (2×10^{-4}M), both added 45 min before the culture period caused release of 333 ± 26 and 7200 ± 115 pg/ml at 1h and 42h (actinomycin) and 3050 ± 210 and 10625 ± 1068 pg/ml (verapamil). The slow onset increase in PGE_2 release after actinomycin and the rapid onset increase after verapamil were both associated with cell death, as observed microscopically. There was no evidence for COX-2 protein in these cells. Further studies showed a time-dependent loss of cell viability with these drugs accompanied by an increase in PGE_2 release into the culture medium. Cultured J774 cells also exhibit PG overproduction accompanying cell death. After 16h culture with the bisbenzylisoquinoline alkaloid tetrandrine at doses ranging from 10^{-7}M to 10^{-4}M, there was dose-dependent cell death as measured using the MTT assay for mitochondrial respiratory integrity ($IC_{50} \approx 5 \times 10^{-6}$M). In cells in which mitochondrial integrity was ≥80%, PGE_2 levels were 300-520 pg/ml, whereas for integrity ≤25% PGE_2 release was 1250-5000 pg/ml. At 10^{-5}M tetrandrine, mitochondrial integrity declined to 56% at 2h, 24% at 16h and 8% at 24h, accompanied by increases in PGE_2 from 736 ± 60, 2480 ± 79 and 3280 ± 124 pg/ml, respectively. J774 cells treated with tetrandrine do not contain COX-2.

These data show that verapamil, tetrandrine and actinomycin D cause death of macrophages and that this is accompanied by release of large quantities of PGE_2 via a non-COX-2 pathway. These cells are assumed to contain COX-1, but it is surprising that a non-specific event such as cytolysis can so effectively activate PLA_2 enzyme(s) and that the released arachidonate is efficiently converted to biologically relevant products.

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17P ACTIVATION OF COX-2 BY ENDOGENOUSLY FORMED NITRIC OXIDE IN J774 MACROPHAGES STIMULATED BY LIPOPOLYSACCHARIDE AND INTERFERON-γ: COMPARISON WITH EXOGENOUS NO FROM DONORS SNAP AND SNP

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Macrophages generate both nitric oxide (NO) and eicosanoids such as PGE_2 via consititutive and inducible enzyme pathways. For example, culture with lipolysaccharide (LPS) causes increased expression of both iNOS (inducible nitric oxide synthase) and COX-2 (inducible isoform of cyclo-oxygenase). In RAW 264.7 macrophages NO generated via the iNOS pathway directly activates COX, implying a mechanism for exacerbating the inflammatory effects of these mediators (Salvemini et al., 1993). We have investigated this in J774 mouse macrophages by stimulating them with LPS or interferon-γ (IFN-γ), comparing the effects on COX activity of NO generated endogenously with that provided exogenously by the NO donors SNAP (S-nitrosopenicillamine) or SNP (sodium nitroprusside).

IFN-γ (3-300 U/ml) incubated for 16h with J774 cells caused small increases in NO release (measured as NO_2^-: up to 5.3 μM, basal < 1.0 μM), but no change in COX activity (measured as immunoassayable PGE_2 derived from incubating washed cells with 10^{-6}M arachidonate). LPS (125 ng/ml) increased both NO generation (10.1 μM) and COX activity (7.0 ng/ml, basal 3.1 ng/ml). The mixture of LPS and IFN-γ was strongly synergistic (34.4 μM and 13.5 ng/ml). Measurement of cell-associated iNOS and COX-2 proteins by Western blotting showed that LPS increased amounts of both enzymes, and LPS + IFN-γ enhanced iNOS expression. Inhibition of NOS with 5×10^{-5}M L-NAME or 7-nitroindazole (70-90% reduction in NO_2 released) diminished apparent COX activity in LPS or LPS + IFN-γ-treated cells by 70-90%, consistent with a direct effect of NO on the COX enzyme. Indomethacin abolished COX activity without reducing NO generation, whereas 10^{-5}M dexamethasone suppressed both iNOS and COX activity. The NOS inhibitors and indomethacin did not alter the amount of iNOS and COX-2 protein, whereas dexamethasone prevented their expression. The NO donor SNAP (10^{-4}M) increased COX activity in LPS-treated cells (without altering its expression) and reversed its inhibition in LPS-treated cells by L-NAME. SNP (10^{-4}M) did not augment COX activity; in fact it inhibited COX activity as well as PGE_2 release into the medium.

We conclude that LPS and IFN-γ upregulate the L-arginine:NO pathway in macrophages by more than one mechanism (enabling synergistic upregulation of NO expression) and that this endogenously formed NO directly activates the induced COX-2 protein. However, exogenously added NO does not always mimic this, perhaps because the timing or spatial aspects are not optimal. SNP may also have other detrimental effects on the cells under these experimental conditions.

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18P INHIBITION OF NEUROGENIC OEDEMA BY CP-122,288 IS STEREOSPECIFIC AND IS SENSITIVE TO THE 5-$HT1_D$ ANTAGONIST GR127,935

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Electrical stimulation of the saphenous nerve in the anaesthetised rat leads to neurogenic inflammation in the hind paw skin. We have recently demonstrated that the 5-HT_1 receptor agonist CP-122,288 (Lee and Moskowitz 1993) is a potent inhibitor of oedema formation in rat skin, possibly acting *via* the activation of prejunctional receptors to inhibit neuropeptide release (Kajekar et al., 1995). In the present study we investigate further the mechanism by which CP-122,288 attenuates neurogenic oedema formation. Male Wistar rats (200-250g), anaesthetised with sodium pentobarbitone (50mg kg^{-1}, i.p.), were prepared for electrical stimulation (10V, 1ms, 2Hz for 5min) of the saphenous nerve of one hind paw, whilst the other paw served as a sham control. Oedema formation was assessed in hind paw skin by the extravascular accumulation of i.v. ^{125}I-albumin (Kajekar et al., 1995). Results are presented as the ratio of plasma protein extravasation (per 100 mg tissue) in the skin of the test paw compared with that in the sham paw. CP-122,288 pretreatment (-5 min, i.v.), but not its S-enantiomer (CP-225,809), significantly inhibited oedema formation induced by stimulation of the saphenous nerve (Table 1). Interestingly, the 5-HT_{1D} receptor antagonist, GR127,935, significantly reversed the inhibitory effect of CP-122,288 (Table 1).

Table 1. Modulation of saphenous nerve-induced oedema formation. **P<0.01 cf. vehicle (Bonferroni's modified t test)

Agent i.v. (mol kg^{-1})	Ratio (mean ± s.e.mean)	n
Vehicle	11.4 ± 1.1	6
CP-122,288 ($2x10^{-10}$)	5.8 ± 0.7**	6
CP-122,288 ($2x10^{-10}$) + GR127,935 ($2x10^{-8}$)	9.6 ± 0.9	6
GR127,935 ($2x10^{-8}$)	10.5 ± 1.2	6
Vehicle	10.0 ± 0.8	5
CP-225,809 ($2x10^{-10}$)	9.2 ± 1.2	5

These data demonstrate that a stereoselective preference exists for the inhibitory effect of CP-122,288 in the rat saphenous nerve preparation, a property which is common to many drug-receptor interactions. In addition, the antagonist actions of GR127,935 suggests an action of CP-122,288 at the 5-HT_{1D} receptor. However, the low potency of sumatriptan in this model relative to CP-122,288 (Kajekar et al., 1995) is not consistent with a 5-HT_{1D} site of action. Therefore, the possibility that CP-122,288 activates a novel receptor subtype should be considered.

RK is the recipient of a Pfizer Ph.D. studentship.

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19P THE EFFECT OF CYCLOHEXIMIDE ON IP PROSTANOID RECEPTOR DOWN-REGULATION IN NG108-15 CELLS

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Functional desensitization of IP prostanoid receptor responses can be produced by pretreatment of NG108-15 cells with either IP receptor agonists or forskolin and, in both cases, this is accompanied by a loss of IP receptors (Kelly et al., 1990; Keen et al., 1992). It has previously been reported that inhibition of protein synthesis with cycloheximide does not block the functional desensitization produced by pretreatment with an IP receptor agonist in these cells (Kenimer and Nirenberg, 1981). In this study we have investigated the effect of cycloheximide on IP receptor down-regulation produced by iloprost or forskolin.

Confluent NG108-15 cells (passage 19-25) were pretreated for 17h in the absence or presence of 10 $mg.ml^{-1}$ cycloheximide in Dulbecco's modified medium containing 10μM iloprost, 10μM forskolin or vehicle as control. Cells were subsequently harvested and washed and and [^{3}H]-iloprost binding activity was measured as previously described (Keen et al., 1992). Data were analysed using a paired *t*-test, $P<0.05$ was considered significant.

The specific binding of [^{3}H]-iloprost to homogenates of control and treated cells is shown in Table 1. Cycloheximide alone had no effect on [^{3}H]-iloprost binding, suggesting that IP receptor turnover is not particularly rapid in these cells. The iloprost mediated loss of binding activity was reduced from 77% to 56% by cycloheximide, whereas cycloheximide appeared to abolish the 19% reduction in binding produced by forskolin.

Table 1. The specific binding of ca. 10nM [^{3}H]-iloprost (fmol.mg $protein^{-1}$) to NG108-15 cell membranes. Each value is mean±s.e.mean, n=4. * Significantly different from no desensitizing agent. $^+$ Significantly different from no cycloheximide.

	no iloprost	+ iloprost
no cycloheximide	75.8 ± 14.0	17.2 ± 3.9*
+ cycloheximide	83.0 ± 21.7	36.3 ± 4.6$^+$
	no forskolin	**+ forskolin**
no cycloheximide	85.6 ± 9.6	69.5 ± 14.2*
+ cycloheximide	70.4 ± 16.5	93.7 ± 22.6

Thus forskolin-mediated receptor down-regulation appears dependent on protein synthesis and may reflect decreased receptor synthesis rather than increased breakdown. However, while cycloheximide did significantly reduce the degree of down-regulation produced by iloprost, the loss of IP receptors appeared not to be abolished under these conditions. It therefore seems likely that the major component of agonist-mediated IP receptor down-regulation is due to increased breakdown of existing receptors.

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20P THE HUMAN SOMATOSTATIN sst_2 RECEPTOR MEDIATES INCREASES IN THE RATE OF EXTRACELLULAR ACIDIFICATION IN A MOUSE FIBROBLAST (Ltk$^-$) CELL LINE

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The Cytosensor microphysiometer measures rates of extracellular acidification as a means of monitoring cell metabolism. We have used this approach to investigate the functional characteristics of the human somatostatin sst_2 receptor transfected into an Ltk$^-$ cell line (clone LSSRII/13; Castro *et al.*, 1994).

Cells were plated out into microphysiometer cups at a density of 750,000 per cup, 24 h prior to the experiment. Bicarbonate-free DMEM (pH 7.4) containing bacitracin (0.2mg/ml) was perfused over the cells at approx. 120 μl/min. The rate of extracellular acidification was measured intermittently for 30 s while perfusion was stopped, with a washout period of 60 s between each measurement. Basal acidification rates were 100 - 280μV/s (0.1 - 0.28 pH units/min). UTP (3μM) was added at the start of the experiment for 3 min to confirm cell viability. Somatostatin (SRIF) doses were added for 10 s, prior to acidification rate measurement, every 21 min. Concentration-effect (c-e) curves to SRIF were constructed, followed 42 min later by a second c-e curve to SRIF or test agonist. Data were analysed as percentage of the first SRIF c-e curve maximum. EC_{50} values shown are for the second c-e curves only.

UTP (3μM) produced increases in extracellular acidification rates in LSSRII/13 cells of 52.7 ± 1.4 % (n=89) above baseline. Concentration-dependent increases in rate were also observed with SRIF (0.3 - 3000nM), with increases of 11.6 ± 0.4 % (n=89) above baseline seen at maximum concentrations for the first SRIF c-e curve. The EC_{50} values for first and second SRIF curves were 8.1nM [6.4 - 10.3] and 6.8nM [5.6-8.2], respectively (mean [95% confidence limits], n=30).

A number of SRIF analogues, including the putatively selective sst_2, sst_3 and sst_5 compounds, BIM23027, BIM23056 and L362,855, respectively (Raynor *et al.*, 1993a, b), were examined for their ability to increase extracellular acidification rates (Table 1). BIM23027 and MK678 were the most potent compounds, L362,855 had a lower potency, whilst BIM23056 produced only a small effect.

The functional data (EC_{50} values) from these studies correlate well (r = 0.94) with corresponding affinity values obtained from ligand binding

Table 1. Potencies of SRIF analogues at increasing extracellular acidification rates (EC_{50} values, nM [95% confidence limits], n = 4 - 30).

	EC_{50} (nM)	Hill slope ± SEM	Max response ± SEM (% 1st SRIF max)
SRIF	6.8 [5.6 - 8.2]	1.1 ± 0.1	108 ± 4
SRIF28	25.4 [14.8 - 43.4]	0.9 ± 0.1	149 ± 12
Octreotide	4.9 [2.9 - 8.4]	1.1 ± 0.1	138 ± 11
BIM-23027	0.2 [0.1 - 0.4]	0.9 ± 0.1	124 ± 17
MK-678	0.3 [0.1 - 0.6]	1.1 ± 0.2	129 ± 22
L-362,855	203 [65.6 - 632]	1.2 ± 0.1	187 ± 14
D-Trp8-SS14	3.1 [1.8 - 5.4]	0.9 ± 0.1	118 ± 17
Angiopeptin	18.5 [8.9 - 39.1]	1.3 ± 0.3	110 ± 11
CGP23996	17.2 [5.0 - 8.7]	1.2 ± 0.2	102 ± 5
RC-160	9.5 [3.7 - 24.7]	1.1 ± 0.2	103 ± 6
BIM-23056	>10,000	-	40 ± 12
CPP	>10,000	-	21 ± 12

experiments (Castro *et al.*, 1994). The putative somatostatin receptor antagonist, CPP, had little effect in these cells, and neither BIM23056 nor CPP (1μM) antagonised SRIF-mediated increases in acidification. The maximum response to SRIF, but not UTP, was markedly reduced when cells were preincubated for 18 h with 100ng/ml pertussis toxin (14.3 ± 0.6% vs 5.5 ± 0.5% of the UTP response). The UTP response was 67.3 ± 2.4 % and 68.0 ± 1.2 % above baseline in the absence and presence of pertussis toxin, respectively (n=14).

Using the microphysiometer, we have shown that SRIF, and a number of analogues known to bind to somatostatin receptors, activate the human sst_2 receptor expressed in LSSRII/13 cells, resulting in increases in rates of extracellular acidification. The transduction mechanism involved in producing these increases in these cells is currently under investigation. However, initial studies indicate that the response, in part at least, is mediated by an event involving a pertussis toxin-sensitive G-protein.

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21P SOMATOSTATIN sst_2 RECEPTORS MEDIATE INHIBITION OF DIMAPRIT- AND ISOBUTYL METHYLXANTHINE-INDUCED ACID SECRETION IN RAT ISOLATED GASTRIC MUCOSA

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Gastrin-induced acid secretion can be inhibited by somatostatin (SRIF) by inhibiting gastrin-induced histamine release from enterochromaffin-like (ECL) cells (Prinz et al., 1994). This inhibitory effect of SRIF involves stimulation of sst_2 receptors (Prinz et al., 1994; Feniuk et al., 1994). In the present study, we have investigated the operational characteristics of the SRIF-receptor type mediating inhibition of acid secretion at the level of the parietal cell using a range of selective SRIF analogues including BIM-23027, BIM-23056 and L-362855, which are selective for sst_2, sst_3 and sst_5 receptors, respectively (Raynor et al., 1993).

Acid secretion was measured from rat isolated gastric mucosa (RGM) (Reeves and Stables, 1985). The cumulative administration of isobutyl methylxanthine (IBMX) (0.1μM-10mM) or dimaprit (1μM-200μM) caused a concentration-dependent increase in H^+ output from the RGM. Ranitidine (10μM, 30 min pre-incubation) abolished the effect of dimaprit but had no effect on IBMX-induced acid secretion (concentration-ratio 2.2 (1.5-3.3; n=4).

In the absence of ranitidine, the IBMX concentration ratio was 1.6 (0.9-2.7). The cumulative administration of SRIF and a range of SRIF analogues (1-1000nM) inhibited IBMX (30μM)- and dimaprit (200μM)-induced H^+ secretion in a concentration-dependent manner (Table 1). The increase in acid output produced by IBMX (30μM) and dimaprit (200μM) was 60±4 nmol/min (n=34) and 39±6 nmol/min (n=28), respectively. BIM-23027, octreotide and seglitide were of similar potency and more potent than SRIF at inhibiting IBMX- and dimaprit-induced H^+ secretion. The sst_3 and sst_5 receptor selective ligands, BIM-23056 and L-362855, were both inactive in concentrations up to 300nM.

The high agonist potency of seglitide, octreotide and BIM-23027 at inhibiting dimaprit- and IBMX-induced acid secretion suggests that sst_2 receptors mediate inhibition of acid secretion at the level of the parietal cell, as also at ECL cells, in rat isolated gastric mucosa.

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Table 1: Inhibitory effect of SRIF analogues on IBMX and dimaprit-induced acid secretion in rat gastric mucosa

	Inhibition of IBMX-induced H^+ secretion			*Inhibition of Dimaprit-induced H^+ secretion*		
Agonist	EC_{50} (nM)	max % inhibition	n	EC_{50} (nM)	max % inhibition	n
$SRIF_{14}$	167 (98-283)	50 ± 9	8	54 (37-79)	81 ± 6	5
$SRIF_{28}$	252 (87-729)	26 ± 9	4	295 (85-1023)	85 ± 9	7
Seglitide	13 (4-41)	64 ± 2	4	17 (11-26)	77 ± 7	5
BIM-23027	11 (9-13)	36 ± 3	4	16 (11-25)	78 ± 9	5
Octreotide	22 (9-51)	62 ± 11	4	13 (8-23)	84 ± 6	4
L-362855	≥300	≥32	5	≥1000	≥8	4
BIM-23056	>1000	≥8	5	>1000	≥9	3

22P STABLE EXPRESSION AND CHARACTERISATION OF CLONED HUMAN BOMBESIN RECEPTORS TRANSFECTED INTO CHINESE HAMSTER OVARY CELLS

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Recent radioligand binding and molecular cloning studies have revealed two pharmacologically distinct receptors for bombesin and related peptides. In this study, we have characterised cloned human brain NMB and GRP receptors stably expressed in Chinese Hamster Ovary (CHO-K1) cells and have investigated the structure-activity requirements of bombesin for the two respective receptor types.

DNA encoding either GRP or NMB bombesin receptors types was obtained by PCR amplification from human cortex and caudate cDNA respectively. Stable cell lines were obtained by transfection of CHO-K1 cells with cDNA, and selection with G418 sulphate. Single clones expressing high levels of each receptor were identified and used in radioligand binding studies to characterise both receptors as described by Guard et al. (1993). Saturation studies using [^{125}I][Tyr^4]bombesin in CH0-K1 cells yielded linear scatchard plots indicating binding to single, saturable sites (K_D = 287 pM (range 172-393) and 36 pM (range 15-62); Bmax = 76 ± 16 and 67 ± 14 fmol/10^6 cells, for NMB and GRP receptors respectively; n=4). Kinetic studies showed that association was rapid and reversible. A steady state was reached within 30 min and maintained for up to 150 min for both receptors (k_{obs} = 0.34 and 0.074 $min^{-1}M^{-1}$; k_{-1} = 0.144 and 0.018 min^{-1}; kinetic K_D = 66pM and 29 pM for NMB and GRP receptors respectively; n=3). The rank order of affinities of bombesin receptor ligands for cloned human receptors (Table 1) was similar to that previously obtained in rat tissues. The relative importance of each amino acid residue present in bombesin was assessed by carrying out an alanine scan of the minimum active fragment of bombesin, acetyl-bombesin$_{7-14}$, (Guard et al., 1993). The rank order of affinity of these peptides against human receptors was again similar to data previously obtained using rat tissues. Thus, replacement of Trp and Leu with Ala resulted in >1400-fold decrease in affinity as compared to acetyl-bombesin$_{7-14}$ (Ki = 2nM), indicating that these residues are critical for high affinity binding at human NMB and GRP receptors.

In summary, the pharmacology of cloned human NMB and GRP receptors with respect to the ligands used in this study, is generally similar to that described for rat bombesin receptors.

Guard et al (1993) Eur J Pharmacol. 240, 177-184

Table 1. Binding affinities (Ki nM; mean and range;n=3-6) of bombesin receptor ligands for cloned human bombesin receptors.

	NMB	GRP
Bombesin	2.0 (1.7-2.5)	0.15 (0.08-0.4)
GRP	9 (4-17)	0.04 (0.02-0.06)
Neuromedin B	0.05 (0.03-0.09)	56 (24-180)
Neuromedin C	28 (17-43)	1.3 (0.4-2.4)
[D-Phe^6]$BN_{6-13}EtNH_2$	680 (539-836)	2.1 (1.6-2.5)
[D-Phe^6]$BN_{6-13}EtOH$	621 (474-855)	0.14 (0.07-0.28)
Litorin	0.08 (0.07-0.08)	1.7 (1.1-2.7)

23P CHARACTERISATION OF THE $[Ca^{2+}]_i$ AND ACIDIFICATION RESPONSE TO BOMBESIN IN CHO CELLS EXPRESSING THE HUMAN NMB-RECEPTOR

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Bombesin is a 14 amino acid peptide that is an agonist for two mammalian peptide receptors, the neuromedin B (NMB) and gastrin releasing peptide (GRP) receptor. In the present study we have investigated the mechanism by which agonist peptides produce an increase in $[Ca^{2+}]_i$ and acidification response in CHO cells expressing the human NMB receptor.

A description of the methods for transfection of the CHO cells with NMB receptors is given in an accompanying communication (Suman-Chauhan *et al.*, 1995). For $[Ca^{2+}]_i$ measurements CHO cells were grown on glass cover slips at a density of $10^4/cm^2$. These were loaded with Fura-2 by incubation with 2μM Fura-2AM and then viewed on a fluorescence microscope. Standard ratiometric methods were used to determine $[Ca^{2+}]_i$. For acidification response experiments the cells were grown on microporous polycarbonate membranes (3μM pore) inside disposable capsule cups. The acidification response was measured using a cytosensor (Molecular Devices).

Bombesin related peptides produced a robust and reversible increase in $[Ca^{2+}]_i$ in all CHO cells expressing the receptors. The GRP receptor antagonist [D-Phe^6, des-Met^{14}]BN(6-14)ethylamide had agonist like actions. EC_{50} values (geometric means) for the peptides were as follows: NMB; 68pM (range 2-110, n=4), NMC; 240pM (range 68-546 n=4). [D-Phe^6, des-Met^{14}]BN(6-14)ethylamide ; 72nM (range 54-110 n=3). All three peptides caused an acidification response as measured with the cytosensor with the same rank order of potency as seen in the $[Ca^{2+}]_i$ experiments.

Removal of external Ca^{2+} did not immediately abolish the $[Ca^{2+}]_i$ response to NMB. Thapsigargin (1μM) caused a large increase in $[Ca^{2+}]_i$ (500nM) followed by a return to near control levels (100nM). After this the response to low concentrations of NMB was absent, however, increasing the concentration of NMB to 1μM caused a depression rather than an increase of $[Ca^{2+}]_i$. This depression of $[Ca^{2+}]_i$ by NMB was not blocked by the PKC inhibitor staurosporine (1μM). The PLC inhibitor U73122 (10μM) applied for 10 minutes blocked the $[Ca^{2+}]_i$ response to NMB. The response could not be recovered by increasing the concentration of NMB up to 1μM. Ryanodine (10μM), which disrupts calcium sensitive calcium stores, did not change the response to NMB. The acidification response to NMB was reduced by 65% by 1mM amiloride. Thapsigargin caused an acidification response of its own following which the response to NMB was reduced by >70%.

These results show that when the human NMB receptor is expressed in CHO cells, the G-protein receptor complex couples to phospholipase. This suggests that the source of the increase in $[Ca^{2+}]_i$ is an internal store which is not sensitive to ryanodine and is probably an IP_3 sensitive store. The metabolic consequence of raising $[Ca^{2+}]_i$ is stimulation of metabolism and H^+ excretion by the Na^+/H^+ antiporter.

Suman-Chauhan, N. *et al* (1995) This meeting.

24P BASIC FIBROBLAST GROWTH FACTOR DECREASES THE GUINEA-PIG CARDIAC DELAYED RECTIFIER POTASSIUM CURRENT

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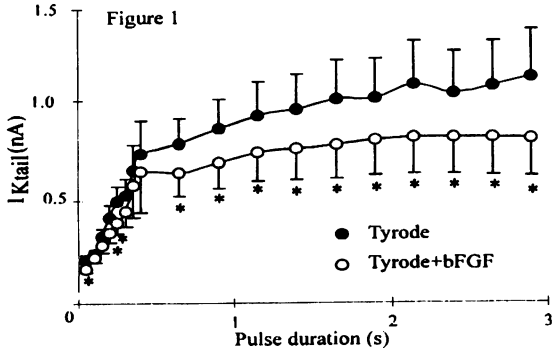
Basic fibroblast growth factor (bFGF) has a variety of cellular effects, causing proliferation, migration and differentiation (Folkman & Klagsbrun, 1987). It is a potent angiogenic factor in cardiac tissue (Miwa *et al.*, 1992). Since bFGF and its receptor are present in heart and bFGF levels may be altered by myocardial ischaemia (Miwa *et al.*, 1992; Paternostro *et al.*, 1993), our aim was to investigate the electrophysiological effects of bFGF in single cardiac cells. Ventricular myocytes were isolated enzymatically from guinea pigs killed by cervical dislocation (no anaesthesia). Delayed rectifier K^+ current (I_K) was measured using the whole-cell voltage clamp technique (pipette solution, in mM, 140 K-aspartate, 5 $MgCl_2$, 5 K_2ATP, 10 EGTA and 5 HEPES). A depolarisation step was applied from-40 mV to +40 mV for 18 durations between 50 ms and 2.9 s.



This envelope of tails test was used to aid discrimination between the 2 subtypes of I_K, known as I_{Kr} and I_{Ks} (Sanguinetti, & Jurkiewicz, 1990). In additional experiments, I_{Kr} was evoked by a 250 ms step from -40 to -10 mV. Cells were superfused with Tyrode solution (33°C), containing nisoldipine (0.3 μM; Bayer, UK), in the presence and absence of 50 ng/ml bFGF (supplied by Farmitalia). Results are expressed as mean±s.e.mean. From Figure 1 it can be seen that bFGF significantly (paired t-test) decreases I_K tail currents (I_{Ktail}) across the range of pulse durations (n=8). However, when current was measured at the end of depolarising pulse, bFGF was ineffective (current at 50 ms, 1.15 s and 2.9 s was 85±20 vs. 80±15 pA, 2.5±0.5 vs. 2.4±0.4 nA and 3.4±0.7 vs. 3.1±0.6 nA in control vs. bFGF treated cells, respectively; p:NS, n=8). These results indicate that bFGF affects the inwardly rectifying component of I_K only, i.e. I_{Kr}. In further experiments, aimed at examining I_{Kr} in isolation, this was found to be the case; bFGF decreased I_{Kr} from 114±39 to 38±17 pA ($p<0.05$, n=6).
When experiments were repeated in the presence of heparin (1 IU/ml, source BDH, UK), which has been shown to potentiate some of the effects of bFGF (Yayon *et al.*, 1991), results were similar. In the presence of heparin, I_{Kr} was decreased by bFGF from 100±18 to -33.8±17 pA ($p<0.05$, n=5).
In conclusion, bFGF at 50 ng/ml decreases the rapid component of I_K, an effect which would be expected to increase action potential duration. Thus, if levels of bFGF are increased during ischaemia, this may serve to protect the heart from arrhythmias.

S.A.R. is a Beit Memorial Fellow.

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25P EFFECTS OF THIOPENTONE ON ATP-SENSITIVE POTASSIUM CURRENTS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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Thiopentone has been shown to suppress the activity of ATP-sensitive potassium channels in CR1-G1 insulin-secreting cells (Kozlowski & Ashford, 1991). The aim of the present study was to investigate actions of thiopentone on ATP-sensitive potassium currents in cardiac myocytes.

Myocytes were isolated from guinea-pig ventricular muscle and superfused with a balanced salt solution containing 2.5 mM Ca and 5.4 mM K (36°C). Patch electrodes (containing, in mM: KCl, 13.2; K glutamate, 109; NaCl, 5; $MgCl_2$, 1; Na_2ATP, 2, EGTA, 1.1; free Ca 10^{-5}; pH 7.2) were used for whole-cell voltage-clamp. Slow ramp depolarizations were applied from -120 to 0 mV (15 s).

Figure 1 shows currents in the absence of drugs (open squares; currents normalised to that at -60 mV) and after exposure to 40 μM cromakalim (thought to open ATP-sensitive potassium channels; open circles). It can be seen that cromakalim induced additional outward current, particularly at potentials positive to -50 mV. 100 μM thiopentone (closed circles), added in the presence of cromakalim, consistently reduced this additional current: at 0 mV the current was 0.4±0.01 (normalised to that at -60 mV) in the absence of drugs, 7.4±1.9 in the presence of cromakalim and 4.1±1.1 in the presence of cromakalim with thiopentone (n=6 cells). When the experiments were repeated with 300 μM thiopentone the currents at 0 mV were: 0.3±0.1 in the absence of drugs, 5.4±1.4 in the presence of cromakalim and 2.0±0.04 in the presence of cromakalim with thiopentone (n=7). In preliminary experiments, activity of single ATP-sensitive channels was recorded in cell-attached patches; in six cells 100 μM thiopentone reduced single channel activity induced by 40 μM cromakalim (in the pipette solution) without altering channel conductance.

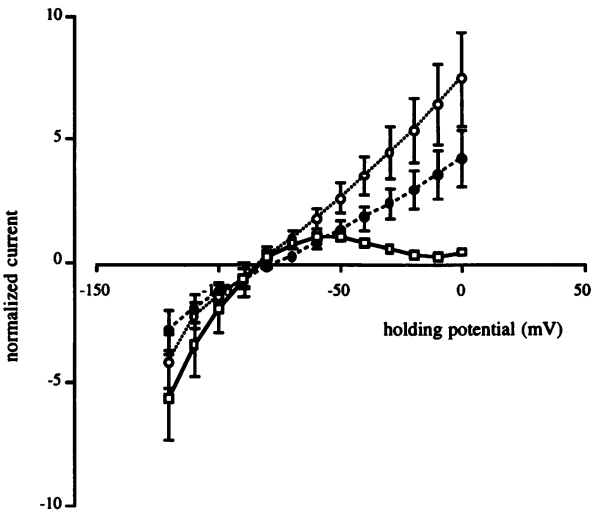

Figure 1. Effect of 100 μM thiopentone (closed circles) on current in the presence of cromakalim (open circles); current before cromakalim shown as open squares.

The observations are consistent with reduction by thiopentone of current through ATP-sensitive potassium channels; such actions are expected to be of importance when thiopentone is administered clinically under conditions of ischaemia.

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26P INFLUENCE OF E4031 ON THE DEACTIVATION OF DELAYED RECTIFIER POTASSIUM CURRENTS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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The effect of E4031 on the time course of the deactivation of the delayed rectifier potassium current (I_K) was studied. Curves were fitted to tail currents in the absence and presence of E4031, a selective blocker of the rapidly activating component of I_K (I_{Kr}; Sanguinetti and Jurkiewicz, 1990).

Single cells were isolated from guinea-pig ventricle. I_K was studied under voltage clamp conditions (balanced salt solution containing 2 μM nifedipine and 2.5 mM Ca at 36°C; micro-electrodes contained 60 mM BAPTA, 0.5 M K_2SO_4 and 10 mM KCl). Cells were clamped at a holding potential of -40 mV and I_K was activated by step depolarizations to +40 mV for either 400 or 1000 ms. I_K was measured as a tail current deactivating upon repolarization to -40 mV before and after exposure to 5 μM E4031. The time constants for decay were fitted to the average of 6 tail currents per cell (n=5 cells) using a modified Levenberg-Marquardt least squares minimisation algorithm (Dempster, 1993) over at least 8 seconds of decay.

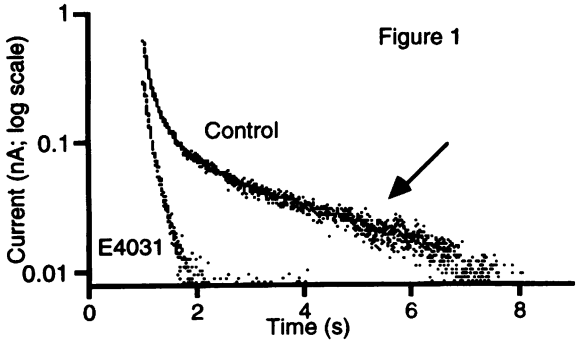

Figure 1 shows the effect of E4031 on the deactivation of the average tail currents from one cell after a 1000 ms depolarization. E4031 appeared to suppress a slow component of deactivation (arrow in control). The table shows that control tail currents after 400 and 1000 ms pulses were best fit by a double exponential (y(t) = a1 *exp(-t/ tau 1) + a2 *exp(-t/tau 2)) with one fast and one slow time constant. Since exposure to 5 μM E4031 is thought to abolish I_{Kr}, the tail current remaining after E4031 is expected to represent the slowly activating component of I_K (I_{Ks}); this was best fit by a single exponential (y(t) = a *exp(-t/tau) with a similar time constant ($p>0.05$, paired t test) to that of the fast component of decay in the absence of drug, but with a slightly reduced amplitude ($p<0.05$).

Pulse width (ms)	Tau 1 (ms)	Amplitude 1 (pA)	Tau 2 (ms)	Amplitude 2 (pA)
400 (control)	99 ± 5	391 ± 46	1653 ± 236	133 ± 17
1000 (control)	100 ± 5	481 ± 46	1767 ± 248	132 ± 15
400 (E4031)	137 ± 23	232 ± 24		
1000 (E4031)	106 ± 16	314 ± 33		

The observations are consistent with the hypothesis that the slowly activating component of I_K (E4031-insensitive current) is fast to deactivate, while the rapidly activating component of I_K (sensitive to E4031) deactivates relatively slowly.

Sanguinetti, M C & Jurkiewicz, N K (1990) *J gen Physiol* 96: 195-215.
Dempster, J (1993) Computer analysis of electrophysiological signals. Academic Press Ltd.

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27P EFFECTS OF GLIBENCLAMIDE ON RESTING HAEMODYNAMICS AND ON RESPONSES TO VASODILATORS IN CONSCIOUS RATS

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Since there is increasing evidence for the involvement of ATP-sensitive K^+ channels (K_{ATP}) in the regulation of vascular tone and in the responses to some vasodilators (Quayle & Standen 1994), we have assessed the effects of the K_{ATP} blocker, glibenclamide, on resting haemodynamics and on responses to acetylcholine (ACh), bradykinin (BK), sodium nitroprusside (SNP) and levcromakalin (LCK) in conscious, Long Evans rats.

Animals (n = 10) were instrumented for recording renal (R), mesenteric (M) and hindquarters (H) haemodynamics, with pulsed Doppler flow probes and intravascular catheters; all surgery was carried out under sodium methohexitone anaesthesia (40-60 mg kg^{-1}, supplemented as required).

ACh (10 μg kg^{-1} min^{-1}), BK (20 μg kg^{-1} min^{-1}), SNP (3.75 μg kg^{-1} min^{-1}) and LK (5 μg kg^{-1} min^{-1}) were given as 3 min i.v. infusions (0.15 ml min^{-1}) before, and starting 15 min after administration of glibenclamide (20 mg kg^{-1}, solubilized in 1% 2-hydroxypropyl-β-cyclodextrin and administered over 5 min). Catheters were flushed with 5% dextrose to avoid hypoglycaemia (blood glucose before glibenclamide = 6.8 ± 0.3 mM; after = 7.8 ± 0.7 mM).

Twenty min after administration of glibenclamide, there was a significant ($P < 0.05$, Wilcoxon's test) increase in mean blood pressure (104 ± 2 to 117 ± 2 mm Hg) and decreases in heart rate (355 ± 7 to 326 ± 7 beats min^{-1}) and vascular conductance in all 3 vascular beds (R = 63 ± 4 to 47 ± 3; M = 77 ±9 to 69 ± 7; H = 44 ± 4 to 32 ± 2 [kHz mm Hg^{-1}]10^3). The vasoconstrictor effect of glibenclamide suggests an influence of K_{ATP} on basal vascular tone, and is consistent with the findings of Moreau *et al* (1994), although in that study there was no increase in blood pressure.

The effects of glibenclamide on responses to the vasodilator challenges are shown in Table 1.

Table 1. Cardiovascular responses (areas under or over curves (0-3 min)) to ACh, BK, SNP and LK in the absence and presence of glibenclamide (G). Values are mean ± s.e. mean; n = 10, * $P < 0.05$, Wilcoxon's test. Units for vascular conductance (VC) are [kHz mm Hg^{-1}]10^3.min.

	ACh	BK	SNP	LCK
HR (beats)	179 ± 21	275 ± 28	140 ± 18	90 ± 8
+G	160 ± 20	270 ± 24	110 ± 17	16 ± 3*
MAP (mmHgmin)	-24 ± 2	-30 ± 5	-25 ± 2	-18 ± 3
+G	-33 ± 4*	-40 ± 5	-19 ± 2	-3 ± 1*
RVC (units)	51 ± 5	54 ± 7	-2 ± 2	21 ± 3
+G	66 ± 5	58 ± 5	0 + 0	4 ± 1*
MVC (units)	-9 ± 4	135 ± 10	37 ± 7	52 ± 6
+G	0 ± 0	151 ± 12	37 ± 6	10 ± 5*
HVC (units)	12 ± 4	54 ± 10	13 ± 4	19 ± 2
+G	21 ± 8	33 ±5*	13 ± 5	4 ± 1*

Glibenclamide clearly attenuated the haemodynamic effects of LK, but had no effect on other vasodilator responses, except the hindquarters response to BK. Since the latter involves a β_2-adrenoceptor-mediated mechanism (Gardiner *et al.*, 1992), these results are consistent with a role for K_{ATP} in that process, as seen with β_2-adrenoceptor-mediated vasodilatation in the mesenteric vascular bed *in vitro* (Randall & McCulloch, 1995).

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28P EFFECTS OF AN ACTIVATOR OF LARGE-CONDUCTANCE CALCIUM ACTIVATED POTASSIUM CHANNELS (NS 1619) ON ION CHANNELS IN ISOLATED CEREBRAL ARTERIAL MYOCYTES

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Large conductance calcium-activated potassium channels (BK_{Ca}) exist in most types of smooth muscle. BK_{Ca} open probability is increased both by cellular depolarisation and by elevation of intracellular calcium, and may act to regulate myogenic tone in cerebral arteries (Brayden and Nelson, 1992). Recently a novel family of compounds have been described that activate these channels in smooth muscle cells (Olesen *et al*, 1994, Edwards *et al*, 1994). In the present study we describe the effects of one of these compounds (NS1619) on ion channels in myocytes from a resistance cerebral artery.

Smooth muscle cells were enzymatically isolated from rat basilar artery. Whole cell currents were measured using perforated-patch or conventional configurations of the patch clamp technique. Single channel recordings were made using inside-out membrane patches. The composition of the solution bathing the cell or cytoplasmic face of the excised patch could be rapidly altered using a multi-channel pressure superfusion system. All electrophysiological experiments were conducted at room temperature. Relaxation experiments were conducted at 37°C using rings of basilar artery contracted by a depolarising (46mM K^+), isotonic solution. Isometric tension was then recorded while NS1619 was cumulatively added to the bath.

In cells held at 0mV NS1619 (20μM) activated a noisy outward current (209±37pA n=8) which was insensitive to glibenclamide and apamin but was rapidly inhibited by iberiotoxin (IBTx) (50-100 nM) and which reversed following washoff. IBTx-sensitive outward currents activated by depolarising potentials from -10mV were increased by application of NS1619 (10-30μM). NS1619 (10-30μM) applied to inside-out patches (0mV) caused a rapid (<5s) increase in the open probability of a Ca^{2+}-dependent channel while having no effect on channel amplitude. Voltage-dependent Ba^{2+} currents recorded using conventional whole-cell techniques were inhibited in a concentration-dependent manner by NS1619 (1-30μM) (IC_{50} 7.3±1.8 μM (n=4)). Following washout of NS1619 the current recovered to approximately 90% of control values. Voltage-dependent potassium currents (K_V) were activated by stepping from a holding potential of -60 mV using the conventional whole cell configuration with 5mM EGTA in the pipette solution and with IBTx (100nM) in the bath. NS1619 (10 & 30μM) caused a reduction in K_V (43% and 62% respectively of control currents at +60mV) which was reversed following washoff. In myograph experiments NS1619 (1-30μM) caused concentration-dependent relaxation of basilar artery rings (IC_{50} 39±0.5 μM (n=4)).

This study provides further evidence that NS1619 can activate BK_{Ca} and inhibit K_V in smooth muscle. Since BK_{Ca} activation was rapid in inside-out patches compared to whole cell, this suggests that NS1619 acts on the internal face of the channel. NS1619 was also a potent inhibitor of voltage-dependent Ca^{2+} channels and evidence from functional studies suggest that this is likely to be the basis of its vasorelaxant effects.

Brayden J. & Nelson M (1992) *Science* 256, 532-535.
Edwards G., Hollenberg A.N., Schneider J. *et al* (1994) *Brit J. Pharmacol.* 113, 1538-1547.
Olesen S.P., Munch E., Moldt P. *et al* (1994) *Eur.J.Pharmacol.* 251, 53-59.

29P THE EFFECTS OF THE POTASSIUM CHANNEL OPENER ZM260384 ON ISCHAEMIC SKELETAL MUSCLE FUNCTION IN THE ANAESTHETISED CAT

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Potassium channel openers accelerate the decline in tension following metabolic inhibition in isolated skeletal muscle (Wesselcouch *et al.*, 1993; Wickenden & Prior, 1994). The purpose of the present study was to investigate whether a potassium channel opener could exert similar effects on ischaemic skeletal muscle *in-vivo*. The low potency of standard agents on skeletal muscle make them unsuitable for *in-vivo* evaluation. The present study employed the potent potassium channel opener, ZM260384 (2-(2,2-bis(difluoromethyl)-6-nitro-3,4-dihydro-2H-1,4-benzox- azine-4-yl) pyridine-N-oxide; Russell *et al.*, 1993)

Male cats were anaesthetised with alphaloxone/alphadolone (12mg Kg^{-1} *iv* followed by infusion 5-15mg Kg^{-1} h^{-1}) and allowed to breathe spontaneously following tracheotomy. Mean systemic arterial blood pressure (MAP) was measured in the left brachial artery. The extensor digitorum longus (EDL) and anterior tibialis (TA) were exposed and connected *in-situ* to an isometric force transducer. Repetitive sub-maximal (20% of the pre-determined maximum) muscle contraction was induced by stimulating the common peroneal nerve with trains of pulses (0.1ms, sub-maximal voltage) at 32.5Hz for 300ms s^{-1}. The lower hindlimb was pump perfused with blood via the femoral artery at a constant rate of 12.5ml min^{-1} (approximately 70% normal resting flow). Hindlimb perfusion pressure (HLPP) was measured via a sidearm of the perfusion circuit. As tension declined with repetitive contractions, stimulation voltage was increased in order to maintain isometric tension at 20% maximum force ± 50g. Stimulation was continued until additional voltage increments no longer increased tension (t_{ex}, min). Recovery of function following repetitive contraction to t_{ex} was assessed by stimulating the common peroneal nerve (0.1ms, supramaximal voltage) to induce twitch contractions at 0.01Hz for 60min. Twitch tension is expressed as a percentage of the pre-drug twitch tension. All results are mean ± s.e.mean.

In the presence of ZM260384 (0.03mg Kg^{-1}), MAP was 58±4mmHg (n=7) and HLPP was 130±6mmHg (n=7). Following ZM260384 (0.03mg Kg^{-1}, *iv*), t_{ex} was 17.5±4min (n=7), maintenance of 20% maximum tension required 1.48±0.14 voltage increments min^{-1} and twitch tension was 25.9±6.2% predrug twitch tension (n=4), 60min post-t_{ex}. In the presence of ZM260384 (3mg Kg^{-1}, *iv*) MAP was 57± 6mmHg (n=6) and HLPP was 120±4mmHg (n=6). These values were not significantly different to those recorded in the presence of the lower dose ($p>0.05$, 2 tail t-test). Despite the similar haemodynamic effects of the two doses, t_{ex} (7.2±0.8min, n=6) was significantly shorter, and the number of voltage increments min^{-1} required to maintain 20% maximum tension (1.93±0.07, n=6) and twitch tension 60min post-t_{ex} (52.1±9.8% predrug tension, n=4) were significantly greater in the presence of ZM260384 (3mg Kg^{-1}, 1 tail t-test).

ZM260384 (3mg Kg^{-1}) increased the rate of tension loss and improved the recovery of function in ischaemic skeletal muscle *in-vivo*.

Russell, K, Brown, F. J., Warwick, P., *et al.* (1993). *Bioorg. Med. Chem. Lett.*, **3**, 2727-2728.
Wesselcouch, E, O., Sargent, C., Wilde, M. W. and Smith, M. A. (1993). *J. Exp. Pharmacol. Ther.*, **267**, 410-416.
Wickenden, A. D. and Prior, H. (1994). *Br. J. Pharmacol.*, **112**, P20.

30P INHIBITION BY OUABAIN OF K^+-INDUCED DILATION IN THE RABBIT PRESSURISED RENAL ARCUATE ARTERY *IN VITRO*

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Modest elevations in extracellular K^+ concentrations have been reported to evoke vasodilation in rat posterior cerebral artery by stimulation of Na^+/K^+ATPase and inward rectifier K channels (McCarron & Halpern, 1990). The aim of the current study was to examine whether a similar phenomenon occurs in a renal artery.

Sections of artery were pressurised at 60mmHg and superfused with Krebs' bicarbonate solution (composition in mM: NaCl 119, $NaHCO_3$ 24, KCl 4.7, KH_2PO_4 1.17, $MgSO_4.7H_2O$ 1.17, EDTA 0.023, D-glucose 5.5 and $CaCl_2$ 1.6) at 37°C, gassed with 95% O_2/5% CO_2. Vessel lumen diameter was measured using video microscopy. In a Ca^{2+} free, 0.5mM EGTA solution, vessels had a lumen diameter of 226 ± 14μm (mean ± s.e. mean, n=22). During an equilibration period of 2h, vessels developed myogenic tone such that the diameter decreased to 181 ± 15μm (n=22). The response to an elevation in K^+ from 4.7 to 10mM K^+ was examined in vessels with either myogenic tone alone or additional constriction produced by phenylephrine (3×10^{-7}- 10^{-6} M).

A dilator response to 10mM K^+ was observed in 11 out of 22 vessels investigated whilst no change in diameter occurred in the remaining vessels. The dilator response to 10mM K^+ in vessels with myogenic tone only was a reduction in tone of 60 ± 15% (n=3), and in vessels with additional phenylephrine-induced tone was 51 ± 8% (n=8). The dilator response was sustained for a period of 5 min in 6 vessels that responded to 10mM K^+ whilst in the remaining 5 vessels the dilator response was transient. The role of Na^+/K^+ATPase in the dilator response was investigated by inhibition of the pump with ouabain. Superfusion with ouabain (10μM) caused an increase in tone of 28 ± 10% in 6 out of 7 vessels and had no effect in the remaining vessel. Administration of ouabain markedly inhibited the dilator response to 10mM K^+ from 58 ± 9% reduction in tone to 1 ± 2% (n=7). Superfusion with 10μM $BaCl_2$ (an inhibitor of inward rectifier K channels) caused a 15 ± 5% decrease in tone in 4 vessels and an increase in tone of 55% in a single vessel. In the presence of $BaCl_2$ there was a reduction in the vasodilator response to 10mM K^+ from 56 ± 8% to 37 ± 6% (n=5) although this difference did not attain statistical significance ($P>0.05$, paired *t*-test).

The results indicate that modest elevations in the external K^+ concentration dilate the rabbit renal arcuate artery, and this effect occurs primarily by stimulation of Na^+/K^+ATPase.

The support of the Wellcome Trust is gratefully acknowledged.

McCarron, J.G. & Halpern, W. (1990). *Am. J. Physiol.* **259**, H902-H908.

31P IDENTIFICATION OF VOLTAGE-ACTIVATED PROTON CURRENTS IN HUMAN EOSINOPHILS

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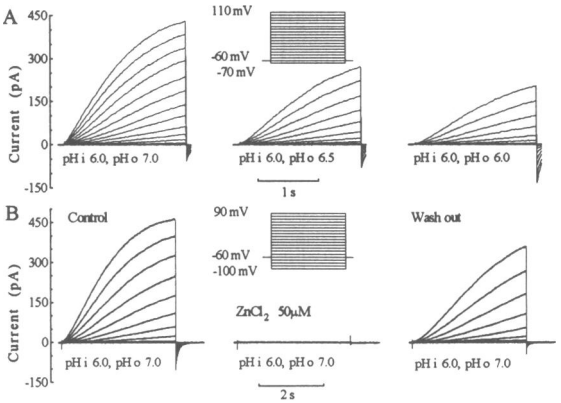
Eosinophils are thought to play important roles in numerous human inflammatory, parasitic and malignant diseases. Indirect evidence indicates that stimulation of these cells may be associated with changes in membrane conductance. Secretagogue activation of the respiratory burst oxidase results in the formation of intracellular protons for which cellular efflux pathways are presumed to exist. The aim of this study was to investigate the membrane currents that could be involved in this process.

Normodense human eosinophils were freshly prepared from the blood of healthy donors using density gradient flotation and immunomagnetic separation to remove neutrophils. Whole cell currents were recorded at room temperature using conventional patch clamp technique. Patch pipettes with resistances of around 5MΩ were used. Pipette and external solutions were free of potassium and sodium and contained mainly impermeant ions (tetraethylammonium, methanesulphonate), and 20mM or 100mM pH buffer (selected appropriately according to pK_a for pH values in the range 7.2 to 5.5). External solutions contained 2mM $CaCl_2$ and 1mM $MgCl_2$. All pipette solutions included 0.3mM $CaCl_2$, 50μM MgATP and 5mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA).

At a holding potential of −60mV, depolarizing voltage steps evoked outward currents which activated over 1-5 seconds. These currents were sensitive to changes of pH in the pipette and the external solutions. Lowering of internal pH (pHi) below 7.2 produced large increases in the amplitude of the voltage dependent outward current. Conversely at a fixed pHi, lowering of external pH (pHo) was associated with a reduction in current amplitude (Figure 1A). The reversal potential of the current was shifted with the change of the driving force for hydrogen ions which is indicative of a proton conductance. These currents were all but abolished by exposure to heavy metal cations (Zn^{2+}, 50-100μM) (n=8)(Figure 1B) which have been shown to block proton currents in a variety of cell types including neutrophils and granulocyte-differentiated HL-60 cells.

The proton current might prevent the intracellular accumulation of protons formed during the respiratory burst, or provide a means of rapid acidification of developing phagosomes.

Figure 1. Single cell voltage-clamp recordings.
A. Effects of reducing external pH on voltage activated currents. B. Inhibition of the currents by $ZnCl_2$. Inserts show schematic voltage protocols.



Supported by Pfizer Research and the MRC

32P PROLONGED INCREASES IN EFFECTS OF, AND SENSITISATION TO, AMPHETAMINE AND COCAINE AFTER CHRONIC ETHANOL TREATMENT

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Recent evidence has suggested that there may be commonalties in the mechanisms of dependence on drugs of different types. Repeated administration of the psychostimulants, amphetamine and cocaine, causes prolonged sensitisation, which has been suggested to be of importance in dependence on these drugs. We have investigated the effects of chronic ethanol administration on the stimulation of locomotor activity by amphetamine and cocaine, and sensitisation to this effect.

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. Control groups were pair-fed control diet. Locomotor activity was measured for 10 min, in separate groups of mice (n=7), 20 min after administration of either amphetamine, 3 mg/kg, cocaine, 10 mg/kg or saline. Initial measurements were made, in different groups of animals, either 24h, 6 days or 2 months after cessation of the ethanol treatment, followed by injections of amphetamine, 3 mg/kg, cocaine, 20 mg/kg or saline once daily. The effects on locomotor activity were again measured, 24h after the last injections, after 8 and after 15 days treatment.

When measurements were started 24h after the end of the ethanol treatment, there were no significant differences on the first administration of the psychostimulants. However, after 8 days of repeated injections, the effects of cocaine were greater in the animals given ethanol, compared with the controls and sensitisation to amphetamine was seen only in the ethanol-treated mice. When tested for the first time on the sixth day after cessation of ethanol treatment, amphetamine had a significantly greater effect on locomotor activity in ethanol-treated mice. Cocaine did not show differences in initial effects but the effects were increased after 15 days treatment, compared with controls. When tested for the first time 2 months after ethanol withdrawal, amphetamine had a significantly greater effect on locomotor activity in animals which had received ethanol. No significant differences were seen in the locomotor activity of controls and ethanol-treated mice after repeated administration of saline. A shorter ethanol treatment (7 days) had no effect on the locomotor stimulant action of amphetamine when tested 6 days after withdrawal, or after eight or fifteen daily injections.

The results show that chronic ethanol intake can cause prolonged increases in the effects of, and sensitisation to psychostimulant drugs. Changes in mesolimbic dopamine transmission may account for the results.

Table 1. Locomotor activity measurements, mean ± s.e.m., comparisons by Student's t-test. Con = controls; Eth. = chronic ethanol; Am = amphetamine; Coc = cocaine; In = first administration; Rp = measurements after 8 (24h and 2 months) or 15 (6 days) daily injections

	Con/Sal	Eth/Sal	Con/Am In	Eth/Am In	Con/Coc In	Eth/Coc In	Con/Am Rp	Eth/Am Rp	Con/Coc Rp	Eth/Coc Rp
24h	1997±228	2230±215	4877±686	4157±253	3326±363	2865±215	4988±1091	6399±441¶	2406±407	4102±232*
6 days	2298±122	2661±205	3672±526	6333±519†	3468±251	3973±257	3600±337	6342±518**	2475±295	4036±455*
2 month	2323±194	2196±125	3233±457	5127±896††			3442±341	6808±617**		

*P<0.05 cf. Con/Coc Rp; **P<0.01 cf. Con/Am Rp; †P<0.01 cf. Con/Am In; ††P<0.01 cf. Eth/Sal; ¶ P<0.01 cf. Eth/Am In

33P GABAPENTIN REDUCES SIGNS OF ANXIETY-RELATED BEHAVIOUR MEASURED ON THE ELEVATED PLUS MAZE DURING WITHDRAWAL FROM CHRONIC ETHANOL TREATMENT

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The ethanol withdrawal syndrome in humans includes anxiety, tremor, and convulsions. We have previously demonstrated that the novel anticonvulsant drug, gabapentin, reduced the convulsive aspects of the syndrome in mice, but showed no ataxic or sedative actions at doses up to 200 mg/kg (Watson and Little, 1995). The present study examines the effect of gabapentin on anxiety-related behaviour in the elevated plus maze during ethanol withdrawal.

Male TO mice (25-30g) were made physically dependent on ethanol over 7 days by a liquid diet schedule; ethanol intake was 22-30 g/kg/day. Controls were pair-fed an isocalorific diet containing no ethanol. Groups of 10-12 mice were withdrawn from the chronic treatment between 7 and 9 am, then 8 h later injected i.p. with gabapentin, 50 or 100 mg/kg, or saline vehicle. Controls were given saline or gabapentin, 100 mg/kg. After 60 min (9h after cessation of ethanol treatment) the mice were placed on an elevated plus-maze for 5 min. Videotapes were analysed by a trained observer, blind to the drug treatment. Results are shown in table 1 as mean ± s.e.m.
The most prominent effect of ethanol withdrawal was a decrease in the percentage time spent on the open arms of the maze. Gabapentin decreased this effect ($P < 0.01$ for the 100 mg/kg dose). At 100 mg/kg, gabapentin did not significantly alter the percentage time spent on the open arms by control mice, although the mean value was increased. Mice undergoing ethanol withdrawal showed a significant increase in head dips from the closed arms (protected head dips); this effect was significantly reduced by gabapentin, 100 mg/kg ($P < 0.01$).The decreased general activity (number of line crossings) and increased stretch attend postures during ethanol withdrawal were not significantly altered by gabapentin.

Other drugs used to treat the ethanol withdrawal syndrome, such as benzodiazepines, cause sedation and ataxia, while dihydropyridine calcium channel antagonists decrease the convulsive, but not the anxiety-related signs (File *et al.*, 1989). Gabapentin therefore may be valuable in the treatment of withdrawal and study of its effects may provide information about the mechanisms involved in the syndrome.

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Chronic/ Acute treatment	% time on open arms	total head dips	% protected head dips	stretch-attend postures	general activity
Control/Saline	36.1±3.4	32.8±4.1	39.5±7.4	0.7±0.2	53.7±5.0
Control/GP 100 mg/kg	44.8±4.6	32.2±3.2	20.0±4.5*	0.5±0.2	47.5±4.4
Ethanol/Saline	23.5±2.7*	26.4±3.2	56.9±3.9*	3.4±0.7*	34.9±2.8*
Ethanol/GP 50 mg/kg	34.3±4.8	25.7±2.7	45.5±5.3	3.7±0.6	37.3±
Ethanol/GP100 mg/kg	39.2±4.4†	24.9±1.8	27.1±6.7†	2.5±0.4	34.9±2.1*

GP = gabapentin, * $P < 0.05$ compared with control/saline, † $P < 0.01$ compared with ethanol/saline (Student's t-test)

34P CHARACTERISATION OF ENDOTHELIN RECEPTORS IN THE HUMAN PULMONARY VASCULATURE USING BOSENTAN, SB209670 AND 97-139

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Endothelin-1 (ET-1) causes potent ET_A receptor mediated constriction of isolated human pulmonary arteries (Hay *et al.*, 1993; Maguire & Davenport, 1995), and may contribute to increased tone in pulmonary hypertension. Non-peptide ET antagonists could be important in the treatment of this disease where the choice of antagonist used will in part be influenced by receptor affinity, distribution, and ligand selectivity. The aims of the present study were to determine the affinity of ET receptor selective ligands in human pulmonary artery (HPA) and to determine the localisation of ET receptors in human lung using subtype selective radioligands.

Human lung tissue obtained from patients undergoing cardiac transplantation was frozen immediately in liquid nitrogen and cut in a cryostat at -22°C. Sections of HPA (10 µm) were labelled with ^{125}I-ET-1 (0.1 nM, 2h, 22°C) in the presence of increasing concentrations of bosentan, (±)SB209670, BQ788, BMS182874 (Shiosaki, 1994), 97-139 (Mihara *et al.*, 1994) or sarafotoxin S6c, washed in Tris buffer (pH 7.4, 3x5 min, 4°C), scraped from the slide and counted. Data (n=5) were analysed using RADLIG 4.0 (Biosoft) with a K_D=0.85 nM for ^{125}I-ET-1 in HPA (Davenport *et al.*, 1993). Sections of human lung and HPA (30 µm) were labelled for autoradiography using 0.1 nM ^{125}I-PD151242 (ET_A) or ^{125}I-BQ3020 (ET_B) (2h, 22°C), washed, dried and exposed to hyperfilm for 7 days.

Our studies show that HPA contains ET_A and ET_B receptors with a predominance of the ET_A subtype (90%). ET_A receptors had high affinity for bosentan (K_DET_A=12.5±4.7 nM, K_DET_B=1.1±0.6 µM), (±)SB209670 (K_DET_A=14.3±3.5 nM, K_DET_B=5.0±1.5 µM) and 97-139 (K_DET_A=5.3±0.7 nM, K_DET_B=19.6±13.2 µM). These compounds showed 90, 350 and 3800 fold selectivity for ET_A over ET_B receptors, respectively. BMS182874 competed with low affinity (50% inhibition at 1 µM). Sarafotoxin S6c labelled ET_B receptors with high affinity (K_DET_A=0.16±0.06 µM, K_DET_B=2.7±1.2 nM) while BQ788 bound with low affinity to a single population of ET_A receptors (K_DET_A=1.0±0.2 µM). ^{125}I-PD151242 labelled lung parenchyma, airway smooth muscle, submucosal glands and epithelium as well as resistance vessels (40-90 µm diam.) and small (0.5-2.5 mm diam.) and large conduit (2-2.5 cm diam.) pulmonary arteries. ^{125}I-BQ3020 showed more intense labelling to airway smooth muscle than to the parenchyma. A low density of ET_B receptors was detected in the airway submucosal glands and in small conduit arteries but not in resistance vessels. The localisation of ET_A receptors in pulmonary arteries and ET_B receptors in airway smooth muscle is consistent with ET_A receptor mediated contraction in pulmonary artery and ET_B receptor mediated bronchoconstriction (2). The receptor distribution in human lung together with the high proportion of ET_A receptors in HPA indicates that an ET_A selective antagonist may be useful in reversing vasoconstriction associated with pulmonary hypertension without affecting ET_B mediated contractile effects in airway smooth muscle or ET_B mediated release of endothelial derived vasodilators.

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35P EVIDENCE FOR ENDOTHELIN INVOLVEMENT IN HYPOXIC VASOCONSTRICTION IN THE RAT ISOLATED PERFUSED LUNG

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Increased levels of endothelin-1 (ET-1) have been reported in plasma and lungs from rats exposed to hypoxia (Oparil *et al.*, 1995). Furthermore, ET receptor antagonists can prevent pulmonary hypertension induced by hypoxia *in vivo* (DiCarlo *et al.*, 1994). We have examined a) the effects of hypoxic perfusion and b) the effect of endothelin receptor antagonists and an endothelin converting enzyme (ECE) inhibitor on such hypoxic perfusion in the isolated rat lung.

The perfused lung model used allows simultaneous measurement of pulmonary perfusion pressure (PPP), pulmonary inflation pressure (PIP) and lung weight (LW), as a measure of fluid accumulation (Lal *et al.*, 1994). Lungs were isolated, ventilated with room air and perfused with Krebs solution gassed with 95%O_2/5%CO_2 (pO_2 = 469 ± 4.1 mmHg, n=3) and allowed to stabilise for 30 min prior to the onset of hypoxia (HYP, Krebs solution gassed with 95%N_2/5%CO_2, pO_2 = 5.5 ± 1.9 mmHg, n=4). In the studies involving antagonists / inhibitors, the lungs were perfused with the relevant agent for 15 min prior to and for the duration of the hypoxic period (90 min).

Systemic hypoxia led to steady increases in PPP, which peaked at 70 min, and LW. Table 1 shows that the hypoxia-induced increases in PPP and LW were significantly attenuated by the selective endothelin-A (ET_A) receptor antagonist BQ123 (Ihara *et* al., 1992) and by bosentan, the non-selective ET_A / ET_B antagonist (Clozel *et* al., 1994). However the effect of bosentan on PPP was not dose related. The ECE inhibitor phosphoramidon also attenuated the increases in LW and PPP. Hypoxia and the drugs used did not affect PIP.

Table 1. The effects of 70 min hypoxic perfusion on pulmonary vascular resistance and oedema formation in the isolated rat lung.

Treatment	PPP ± SEM (mmHg)	ΔLW ± SEM (g)
Normoxic Control	9.7 ± 0.7	0.53 ± 0.15
Hypoxic Control	15.8 ± 3.2 *	3.9 ± 1.0 *
HYP + 3μM BQ123	7.3 ± 0.67 #	2.3 ± 0.47
HYP + 10μM BQ123	8.8 ± 0.63 #	0.85 ± 0.21 #
HYP + 1.5μM Bosentan	9.0 ± 1.2 #	0.78 ± 0.33 #
HYP + 5μM Bosentan	12.1 ± 2.3	0.57 ± 0.07 #
HYP + 1μM Phosphoramidon	8.9 ± 0.77 #	1.08 ± 0.29 #

* p<0.05 vs. Normoxic Control. #p<0.05 vs. Hypoxic Control

These data suggest that the production and/or release of endogenous endothelin(s) contributes to the hypoxia induced increases in pulmonary vascular resistance and oedema formation in this lung model

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36P CHRONIC β BLOCKER TREATMENT SENSITISES THE HISTAMINE H_2 AND H_1 RECEPTOR SYSTEMS IN HUMAN ATRIUM: ROLE OF CYCLIC NUCLEOTIDES

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When patients are chronically treated with β blockers their atria become more sensitive to effects mediated through β_1- and β_2-adrenoceptors as well as through 5-HT_4 receptors suggesting hyperfunction of receptors coupled to Gs protein (Kaumann, 1994). We now report the H_2 receptor-mediated effects of histamine in paced right atrial preparations (0.5 Hz, 37°C) obtained from patients chronically treated (βB) or not treated (non βB) with β blockers. cAMP levels and PKA activity were measured as in Kaumann *et al.* (1989) after 5 min exposure to histamine. We also measured cGMP levels by enzymeimmunoassay. Histamine induced increases in contractile force, cAMP and cGMP levels and PKA activity which were larger in atria from βB than from non βB. Mepyramine (1 μM) abolished the histamine-evoked cGMP signal and blunted other effects of histamine in tissues from βB. Sodium nitroprusside (SNP) 10 μM increased cGMP levels from 0.28 ± 0.04 to 1.35 ± 0.48 pmol mg^{-1} but did not change cAMP levels or PKA activity. SNP reversed the reduction of the maximum inotropic effect of histamine caused by mepyramine in atria from βB patients. H_1 receptor-mediated increases in cGMP levels, enhanced through an as yet unknown mechanism by chronic β blocker treatment, may inhibit the activity of phosphodiesterase 3 thereby causing enhanced histamine-evoked increases in cAMP levels, PKA activity and contractile force, thus accounting in part for the H_2 receptor-mediated hyperresponsiveness.

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Table 1. Comparison of effects of histamine (H) in tissues from βB and non βB patients (*P < 0.05). Data are mean ± s.e.mean from 5 -16 patients per group. (ISO = isoprenaline 100 μM.)

	Positive inotropic effects			Biochemical effects (pmol mg^{-1} protein)		
Condition	-$LogEC_{50}$	Max % ISO		cAMP	cGMP	PKA ratio
Non βB	5.0 ± 0.1	33 ± 6	Basal	22 ± 2	0.20 ± 0.03	0.31 ± 0.02
			H 100 μM	64 ± 15	2.21 ± 0.47	0.44 ± 0.04
βB	5.4 ± 0.1*	69 ± 5*	Basal	30 ± 4	0.31 ± 0.04	0.28 ± 0.02
			H 100 μM	110 ± 24*	5.00 ± 1.40*	0.54 ± 0.03*
βB + mepyramine	5.2 ± 0.1	56 ± 4*	Basal	17 ± 4	0.25 ± 0.06	0.29 ± 0.04
			H 100 μM	56 ± 20	0.45 ± 0.14	0.44 ± 0.02
βB + mepyramine + SNP	5.6 ± 0.1*	81 ± 3*			Not determined	

37P PHARMACOLOGY OF GR203040, A POTENT AND SELECTIVE NON-PEPTIDE TACHYKININ NK_1 RECEPTOR ANTAGONIST

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The proposed role of substance P in the pathogenesis of migraine headache (Moskowitz *et al.*, 1989) suggests that selective tachykinin NK_1 receptor antagonists may provide a novel approach in the treatment of the disorder. In the present study, the pharmacology of GR203040 ((2S, 3S)-2-methoxy-5-tetrazol-1-yl-benzyl-(2-phenyl-piperidin-3-yl)-amine), a novel, highly potent and selective non-peptide NK_1 receptor antagonist, was investigated.

GR203040 potently inhibited [^{3}H]-substance P binding to human NK_1 receptors expressed in Chinese hamster ovary (CHO) and U373 MG astrocytoma cells, and NK_1 receptors in ferret and gerbil cortex (pK_i values of 10.3±0.1 (n=5), 10.5± 0.2 (n=4), 10.1±0.1 (n=4) and 10.1±0.2 (n=4) respectively). GR203040 had lower affinity at rat NK_1 receptors (pK_i=8.6± 0.2; n=3) and little affinity for human NK_2 receptors (pK_i<5.0) in CHO cells and NK_3 receptors in guinea-pig cortex (pK_i<6.0). In dog isolated middle cerebral and basilar arteries (Stubbs *et al.*, 1992), GR203040 (0.03nM) produced a rightward displacement of the concentration-effect curves to substance P methyl ester (SPOMe) (apparent pK_B values of 11.2±0.2 and 11.1±0.3 (both n=3) respectively). At a higher concentration (0.1nM), GR203040 caused a suppression of the maximum agonist response. In anaesthetised rabbits (Perren *et al.*, 1994), GR203040 (1-100μgkg^{-1}) antagonised reductions in carotid arterial vascular resistance evoked by SPOMe, injected via the lingual artery (DR_{10} (*i.e.* the dose producing a 10-fold shift in the SPOMe dose-response curve)=1.1μgkg^{-1} (95% confidence limits: 0.9-1.4) i.v., n=3). At a dose 20-fold greater than its DR_{10} value (i.e. 22μgkg^{-1}), antagonism was evident more than 2h after GR203040 administration. In anaesthetised rats, GR203040 (1-10mgkg^{-1}, i.v.) produced a dose-dependent inhibition of plasma protein extravasation (Buzzi & Moskowitz, 1990) in dura mater and extracranial tissues evoked by electrical stimulation of the trigeminal ganglion. It is concluded that GR203040 is one of the most potent and selective NK_1 receptor antagonists yet described, and as such, is a useful pharmacological tool to characterise the physiological and pathological roles of substance P and NK_1 receptors. The ability of GR203040 to inhibit NK_1 receptor mediated responses in the cranial vasculature suggests that the compound may possess anti-migraine activity.

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38P FURTHER STUDIES ON THE RESPONSE OF HUMAN CORONARY ARTERIES TO THE 5-HT_{1D} RECEPTOR AGONISTS SUMATRIPTAN AND MK-462

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It is established that contractile responses of isolated human coronary artery can be evoked by $5HT_{1D}$-receptor agonists. Sumatriptan is a $5HT_{1D}$-receptor agonist which is in clinical use as an antimigraine treatment, and it has been shown in angiographic studies to cause coronary artery constriction. Previous studies performed on separate segments of human isolated coronary arteries have shown that MK-462 (N,N-dimethyl-2-[5-(1,2,4-triazol-1-ylmethyl)-1H indol3yl]ethylamine), a novel selective 5-HT_{1D} receptor agonist effective in the treatment of migraine headache (Ferrari et al., 1994), also caused contractions but produced a smaller mean maximum response than did sumatriptan and 5-hydroxytryptamine (5-HT or serotonin, Ferro *et al.*, 1995). Since the response of segments of human coronary artery can vary according to location along the artery and is influenced by the presence of atherosclerotic lesions, experiments were designed to investigate the response of the same arterial segment to MK-462, sumatriptan and 5-HT.

Ring segments (without endothelium) of human coronary arteries were obtained from seven patients undergoing cardiac transplantation and were mounted in organ chambers for the recording of changes in isometric tension. The preparations were first exposed to KCl (45mM) and then to increasing concentrations of 5-HT. Finally, concentration-response curves to either sumatriptan or MK-462 were performed in a consecutive and random manner. A control for repeated application of 5-HT was obtained in separate preparations.

All preparations responded to the initial exposure to 5-HT and there was no significant difference between responses of segments from the same artery (ANOVA, $F = 0.942$, d.f.= 7,38, $P = 0.47$). However, the level of response to 5-HT was different from patient to patient (ANOVA, $F = 9.60$, d.f. = 6,101, $P < 0.001$). Desensitisation was seen consistently across all agonists between the last two concentration-response curves but there was no significant interaction between agonists. For all seven arteries tested, the mean responses to 5-HT, sumatriptan and MK-462 were obtained. Concentration-response curves were fitted to the data (using non-linear regression analysis) to provide estimates of the EC_{50} and E_{max} values. The EC_{50} values for 5-HT, sumatriptan and MK-462 (i.e. 0.2, 0.6 and 1.0μM respectively) did not differ significantly. The E_{max} values (mean ± s.e.) for 5-HT, sumatriptan and MK-462 (as % 45mM KCl) were 102.0 ± 1.5, 43.7 ± 1.8, 22.2 ± 1.8 respectively. The E_{max} values for sumatriptan and MK-462 were significantly less than that for 5-HT ($P < 0.05$, asymptotic t-test) and the E_{max} value for MK-462 was significantly less than that for sumatriptan ($t = 8.59$, d.f. = 15, $P < 0.001$).

The present results (using a 'crossover' experimental protocol) confirm suggestions made from previous data obtained on separate segments of human coronary arteries and show that the lower maximum response to MK-462 than to sumatriptan and 5-HT cannot merely be the consequence of variability of the response to 5-HT receptor agonists between patients or between segments from the same artery.

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39P [^{125}I]-SB 207710 LABELS SMALL 5-HT$_4$ RECEPTOR POPULATIONS IN PORCINE AND HUMAN ATRIUM

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5-HT causes tachycardia in piglet atria (Kaumann, 1990) and increases contractile force in human atria (Kaumann et al., 1990) through 5-HT$_4$ receptors but is less efficacious than (-)-isoprenaline in both systems. One possible explanation is that 5-HT$_4$ receptor populations in both tissues are smaller than the populations of β-adrenoceptors. We have now labelled the 5-HT$_4$ receptor populations of both systems with [^{125}I]-SB 207710, a radioligand of high specific activity, selective for 5-HT$_4$ receptors (Brown et al., 1993). We have also labelled β-adrenoceptors with (-)-[^{125}I]-cyanopindolol, using CGP 20712A to define β_1- and β_2-adrenoceptors (Kaumann & Lemoine, 1987). Membrane particles were prepared by homogenisation (Polytron) and centrifugation (twice at 14000 x g) of right atria from 25 new-born piglets and right atrial appendages from 7 patients (all chronically treated with β blockers) without heart failure, undergoing open heart surgery. Non-specific binding was defined with 100 μM 5-HT (piglet) or 1 mM 5-HT (man). [^{125}I]-SB 207710 labelled saturable sites in both porcine (0.58 ± 0.09 fmol.mg^{-1} protein) and human (5.9 ± 1.5 fmol.mg^{-1}) membranes with pK$_D$s of 9.8 and 9.7. The densities of these saturable binding sites in porcine membranes were 174 and 22 times lower respectively than those of β_1- and β_2-adrenoceptors; in human membranes the equivalent ratios were 6 and 3. Unlabelled ligands competed with binding of [^{125}I-SB 207710 with affinities and rank order potencies consistent with binding to 5-HT$_4$ receptors (Table 1).

Table 1 Mean pK$_D$ values of ligands competing with binding of [^{125}I]-SB 207710 (s.e.mean always < 0.2 log units; 5-CT = 5-carboxyamidotryptamine).

	SB 207710	5-HT	5-CT	renzapride	cisapride
Piglet	9.5	6.6	4.0	7.1	4.0
Man	10.2	6.1	5.2	6.4	5.9

The small sizes of the populations of receptors with 5-HT$_4$ characteristics in both piglet and man may, in part, explain why the maximal effects of 5-HT are smaller than those of (-)-isoprenaline mediated through both β_1- and β_2-adrenoceptors.

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40P EFFECT OF LEXIPAFANT, A SELECTIVE PAF RECEPTOR ANTAGONIST, ON ISCHAEMIA-INDUCED CARDIAC ARRHYTHMIA AND MORTALITY IN THE ANAESTHETISED RABBIT

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Evidence for the involvement of platelet activating factor (PAF) in myocardial ischaemia, comes from studies reporting a protective effect of PAF antagonists against ischaemia induced damage (Stahl et al. 1988). The aim of the present study was to examine the effect of lexipafant (N-methyl-N-4-(1H-2-methylimidazo[4,5-c]pyridin-1-yl methyl)phenyl-sulphonyl-L-leucine ethyl ester) a novel selective antagonist at the PAF receptor (Whittaker et al., 1994), on mortality and arrhythmogenesis in a rabbit model of cardiac ischaemia.

Male New Zealand White rabbits (2.0-2.6 kg) were anaesthetised with Saffan (Alphaxalone 0.9%, Alphadalone 0.3% $^{w}/_{v}$,12mg.kg^{-1} i.v.) and α-chloralose (100 mg.kg^{-1} i.v.) was given to maintain surgical anaesthesia. A carotid artery and a jugular vein were cannulated for measurement of blood pressure and administration of compound respectively. The chest was opened at the fourth intercostal space exposing the heart. A ligature was placed under the circumflex coronary artery. Animals received either lexipafant at 1 mg.kg^{-1}.hr^{-1} or vehicle (10 ml.hr^{-1}) starting 10 minutes before ischaemia was induced by tying the ligature. Lead II ECG, arterial blood pressure and heart rate, were monitored throughout the experiment. Ischaemia was maintained for 20 minutes. Results are expressed as number of ventricular ectopic beats (VEBs) and mortality. In all cases mortality was as a result of irreversible ventricular fibrillo-flutter. The area at risk was determined by Langendorff perfusion with Evans blue dye.

Table 1. The effect of infusion of lexipafant (1mg.kg^{-1}.hr^{-1} starting 10 minutes prior to occlusion) on the incidence of ventricular ectopic beats (vebs), mortality & area at risk

Treatment	N	VEB's (mean ± s.e.m.)	Mortality (%)	Area at risk (% ± s.e.m.)
Vehicle	10	97 ± 40	80	47 ± 6
Lexipafant	10	70 ± 26	20 *	51 ± 5

The incidence of mortality was significantly reduced in the lexipafant group compared with control(*p=0.012 as determined by analysis using Fishers exact probability test).

The antidysrhythmic action observed in this study may be due to the antagonism of endogenously released PAF or PAF-like agents, which could induce tissue damage to the ischaemic myocardiums of these animals (Lucchesi, et al. 1990). From this study it might be concluded that lexipafant would have a utility in ameliorating the effect of an ischaemic insult in the heart.

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41P CONTRACTION CHARACTERISTICS OF VENTRICULAR CARDIOMYOCYTES FROM DOGS WITH PACING-INDUCED HEART FAILURE

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Six mongrel dogs were subjected to pacing-induced tachycardia (250 pulses min^{-1}) for 6 weeks, with haemodynamic and echocardiographic measurements made at end of the pacing period. Left ventricular dp/dt had dropped to 1612 ± 150 $mmHg.s^{-1}$ (mean ± sem) compared with 4713 ± 304 $mmHg.s^{-1}$ in 6 sham-operated controls ($P<0.001$), and the left ventricular end-diastolic volume had risen to 60.5 ± 6.2 ml (normal range 30-35ml). A portion of the left ventricular free wall (5-10g) was dissected away under terminal anaesthesia. Myocytes were isolated from the tissue by perfusion through a surface artery or vein. Lengths, widths and areas were measured from pictures of 30 or more cells from each preparation using a digitising tablet. Cells from paced dogs (P) were found to be longer and thinner than those from sham-operated animals (S), with a form factor reduced from 0.47 ± 0.01 to 0.40 ± 0.01 ($P<0.001$). Myocytes were initially perfused with Krebs-Henseleit solution containing 2mM Ca^{2+} at 32°C and field stimulated at 0.5Hz. Contraction was followed using a video-based edge detection system.

At the basal Ca^{2+} and stimulation frequency there was no significant difference in contraction amplitude (% cell shortening) between myocytes from S and P, although some depression was evident in P (S, 3.9 ± 0.4%, n=6 animals/8 cells: P, 2.4 ± 0.5%, (5/10)). Statistics were performed using data pooled by animal, i.e. n=dogs. At higher Ca^{2+} levels (4, 6 and 8mM) the difference between S and P was statistically significant. For example, contraction amplitudes in 6mM Ca^{2+} (0.5Hz) were 10.0 ± 0.8% for S (5/8) and 6.6 ± 0.9% for P (4/9), $P<0.02$. Increasing the frequency of stimulation also accentuated the difference between S and P. At 6mM Ca^{2+} and 1Hz, the contraction amplitude was 9.4 ± 1.1 for S (4/5) and 4.5 ± 0.4% for P (4/4) ($P<0.02$). Conversely, rested state beats were similar in both (3 min post-rest beat: S, 13.6 ± 2.3%, (6/7); P, 14.1 ± 2.6%, (6/8)).

Although contraction and relaxation velocities were decreased in parallel with decreased amplitude in myocytes from P, times to peak contraction (TTP) and to 50% (R50) relaxation were not altered (TTP:S, 0.22 ± 0.02s, (6/36); P, 0.20 ± 0.03s (6/40) and R50: S, 0.10 ± 0.02s, (6/36); P, 0.12 ± 0.03s, (6/40)).

Thapsigargin, the SR Ca^{2+}-uptake inhibitor, had concentration-dependent negative inotropic effects on the dog myocytes, as well as slowing contraction and relaxation. There was little difference between S and P with respect to their sensitivity to thapsigargin. Amplitude in the presence of 1μM thapsigargin was was reduced by 44.6 ± 8.5% in S (6/6) compared with 56.1 ± 6.9% in P (6/8).

There are points of similarity between the contractile responses of myocytes from the hearts of paced dogs and those from failing human heart, where the depression of contraction amplitude is also frequency dependent. However, the slowing of contraction and relaxation and the reduced sensitivity to thapsigargin, which are key features of human heart failure, are not reproduced in this animal model.

42P Ro31-8220 INHIBITS DIHYDROPYRIDINE-SENSITIVE ISOMETRIC FORCE AND WHOLE CELL Ba^{2+} CURRENT IN RAT ISOLATED BASILAR ARTERY

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The recently developed bisindolymaleimide compound Ro 31-8220 (Davies *et al.*, 1989) is thought to be a specific inhibitor of protein kinase C (PKC).

Roberts *et al.* (1995) recently reported that both 75 mM K^+-induced contraction and the first phase of hypoxia-induced, Ca^{2+}-dependent contraction of intrapulmonary arteries, precontracted by prostaglandin F(2α) or KCl, could be inhibited by Ro 31-8220, suggesting a link between PKC and intracellular Ca^{2+} handling.

Whether Ro 31-8220 influences release of internal Ca^{2+} stores, or activation of plasmalemmal voltage sensitive Ca^{2+} channels has not been fully examined. We have investigated the latter possibility by examining the effect of Ro 31-8220 on dihydropyridine- (DHP) sensitive isometric force and whole cell Ba^{2+} current in rat isolated basilar artery. Basilar arteries isolated from wistar strain rats were either mounted in a Mulvany design small vessel myograph for measurement of isometric force or were dissociated using papain according to the method of Quayle *et al.* (1995). Isolated cells were kept on ice prior to experimentation for up to 8 hours.

Established 40 mM K^+-induced isometric contractions were concentration-dependently inhibited by cummulative addition of Ro 31-8220 with a mean IC_{50} of 3.0 ± 0.2 μM and a slope of 1.5 ± 0.17 (mean ± sem, n=4). This inhibition was only partly reversible after 1hr.

In patch clamp experiments inward currents carried by Ba^{2+} (10 mM) were recorded using the conventional variant of the whole-cell patch clamp technique. Outward currents carried by K^+ were minimised by replacing K^+ with either CsCl or NMDG in the pipette solution. Individual cells were superfused using a purpose made micro superfusion system (Langton, 1993) which allowed the superfusate to be changed in about 1 second. Cells were stepped from -80 mV to a series of positive potentials in the absence and presence of increasing concentrations of Ro 31-8220 (0.3-10 μM). Ro31-8220 inhibited inward barium current in a concentration dependent manner with a mean IC_{50} of 3.94 ± 0.79 μM and slope of 0.9 ± 0.1 at +10 mV (n = 4 cells). Current recovered to more than 90% of control on washout. The mechanism of action of Ro 31-8220 on DHP-sensitive force and Ba^{2+} current remains unclear although a direct block of Ca^{2+} channels is consistent with these data.

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43P REGULATION OF β-ADRENOCEPTOR AND L-TYPE CALCIUM CHANNEL NUMBER IN RAT HEART

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The effect of β–adrenoreceptor stimulation on the heart is in part mediated by activation of L-type calcium channels in the cardiac muscle cells. Exposure to isoprenaline or forskolin results in down-regulation of L-type calcium channels in cultured myocytes from chick heart (Marsh, 1989) and it is possible that the loss of this effector molecule may contribute to the functional effects of β–adrenoreceptor desensitization in these cells. This study investigates whether a similar down-regulation of L-type channels accompanies β–adrenoreceptor down-regulation in the rat heart.

Hearts from 2-3 day old Wistar rat pups were removed and chopped with crossed scalpels into rough cubes <0.5mm in any dimension and maintained for 17h in Dulbecco's modified Eagle's medium supplemented with penicillin and streptomycin, which contained 10μM forskolin, 10μM isoprenaline with 1mM ascorbate or the appropriate vehicle as control. At the end of the incubation period the tissue was washed three times in phosphate buffered saline and frozen at -80°C until use. Heart tissue was thawed and homogenized in 50mM TrisHCl, pH7.4, containing 1mM ascorbate. Binding assays were performed using the same buffer in a final volume of 250μl with 150-350μg protein per sample; 0.03-3nM [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) ± 50μM isoprenaline was used to detect β–adrenoreceptors and 0.02-7nM [^{3}H]-PN200-110 ± 1μM nimodipine was used to detect L-type channels. Incubations were carried out for 40 min at room temperature in the dark and then samples were filtered onto GF/B glass fibre filters and washed with 3 x 3.5ml assay buffer using a Brandel cell harvester. Data were analyzed by non-linear regression analysis using a single site model.

Both [^{3}H]-DHA and [^{3}H]-PN200-110 appeared to recognize single populations of sites in control homogenates; for [^{3}H]-DHA, pK_d=8.783±0.013 and B_{max}=74.6±8.5 fmol.mg protein^{-1} and for [^{3}H]-PN200-110, pK_d=9.141±0.118 and B_{max}=148.1±45.2 fmol.mg protein^{-1} (all values mean±s.e.mean, n=3). Pretreatment with both forskolin and isoprenaline reduced the density of [^{3}H]-DHA binding sites by 45% and and 52%, respectively, with no apparent effect on affinity. Similarly, neither pretreatment produced any apparent effect on the binding affinity of [^{3}H]-PN200-110. However, while forskolin pretreatment reduced the density of specific binding sites for [^{3}H]-PN200-110 by 55%, isoprenaline pretreatment had no effect on [^{3}H]-PN200-110 binding, the K_d values obtained being 0.48nM and 0.42nM and the B_{max} values being 100.8 and 102.9 fmol.mg protein^{-1}, in control and treated hearts respectively (values from a single experiment, representative of two).

Thus exposure to isoprenaline and forskolin both produced a similar reduction in β–adrenoreceptor number. In the case of forskolin pretreatment this was accompanied by a loss of L-type calcium channels, which seems likely to be mediated by a cAMP-dependent process. However, L-type channel down-regulation appears not to accompany agonist-mediated β–adrenoreceptor down-regulation in the mammalian heart.

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44P BIPHASIC REGULATION OF HEPATIC INOSITOL 1,4,5-TRISPHOSPHATE RECEPTORS BY CYTOSOLIC Ca^{2+} IS NOT MEDIATED BY PHOSPHORYLATION

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In many cells, the release of Ca^{2+} from inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive stores is regulated by cytosolic Ca^{2+}. The mechanism(s) underlying this effect is unknown, although studies of fibroblasts have implicated Ca^{2+}-dependent phosphorylation (Zhang *et al.*, 1993). In the present study, we have investigated the regulation of $InsP_3$-induced Ca^{2+} mobilisation by cytosolic Ca^{2+} in hepatocytes.

The intracellular stores of permeabilised rat hepatocytes were loaded to steady state with $^{45}Ca^{2+}$ in a cytosol-like medium with the free $[Ca^{2+}]$ ($[Ca^{2+}]_m$) buffered at 200 nM. The $[Ca^{2+}]_m$ was then changed simultaneously with the inhibition of further Ca^{2+} uptake by diluting cells into a similar medium at 37°C or 2°C containing thapsigargin (1 μM) and various amounts of $CaCl_2$ and either EGTA (for $[Ca^{2+}]_m$ < 1.4 μM) or HEDTA (for $[Ca^{2+}]_m$ > 1.4 μM). After 30 s, $InsP_3$ was added, and after a further 30 s the Ca^{2+} contents of the intracellular stores were determined by rapid filtration. $[Ca^{2+}]_m$ was determined fluorimetrically at 37°C.

At 37°C, modest increases in $[Ca^{2+}]_m$ did not affect the response to a maximal $InsP_3$ concentration. $InsP_3$ (10 μM) mobilised 40 ± 2 % (n = 17) of the ATP-dependent $^{45}Ca^{2+}$ uptake when $[Ca^{2+}]_m$ was ≤ 2 nM and 41 ± 2 % (n = 8) when $[Ca^{2+}]_m$ was 1.4 μM. However, these changes in $[Ca^{2+}]_m$ significantly increased the sensitivity of the stores to $InsP_3$. The EC_{50} for $InsP_3$-induced Ca^{2+} mobilisation decreased from 309 ± 33 nM (n = 18) when $[Ca^{2+}]_m$ was ≤ 2 nM to 94 ± 8 nM (n = 8) when $[Ca^{2+}]_m$ was 1.4 μM. This sensitising effect of cytosolic Ca^{2+} was half maximal when $[Ca^{2+}]_m$ was ~ 200 nM. Further increasing $[Ca^{2+}]_m$ caused an inhibition in the maximal amount of Ca^{2+} mobilised by $InsP_3$. When $[Ca^{2+}]_m$ was 30 μM, $InsP_3$ (10 μM) mobilised only 9.5 ± 3.5 % (n = 9) of that observed when $[Ca^{2+}]_m$ was 50 nM. Half maximal inhibition was achieved when $[Ca^{2+}]_m$ was 4.4 ± 0.4 μM (n = 5). These results confirm that $InsP_3$-induced Ca^{2+} mobilisation is biphasically regulated by cytosolic Ca^{2+}.

Removal of ATP by addition of glucose (10 mM) and hexokinase (10 units/ml) 30 s prior to changing $[Ca^{2+}]_m$ did not effect regulation of the $InsP_3$ receptor by cytosolic Ca^{2+}. The EC_{50} for Ca^{2+} mobilisation decreased from 289 ± 51 nM (n = 4) to 94 ± 10 nM (n = 4) when $[Ca^{2+}]_m$ was increased from ≤ 2 nM to 1.4 μM. The response to 10 μM $InsP_3$ was inhibited by 84 ± 9 % (n = 4) when $[Ca^{2+}]_m$ was 30 μM.

At 2°C, Ca^{2+} mobilisation was more sensitive to $InsP_3$ than at 37°C. In cells that were pre-chilled for 60 s prior to changing $[Ca^{2+}]_m$ to ~ 2 nM, the EC_{50} for $InsP_3$-induced Ca^{2+} mobilisation was 67 ± 2 nM (n = 18), and it decreased further to 35 ± 2 nM (n = 7) when $[Ca^{2+}]_m$ was increased to ~ 1.4 μM. When $[Ca^{2+}]_m$ was further increased to ~ 30 μM, the response to a maximal $InsP_3$ concentration was inhibited by 88 ± 4 % (n = 7).

We conclude that in hepatocytes, biphasic regulation of $InsP_3$ receptors by cytosolic Ca^{2+} ocurs at 2°C and does not require ATP. Neither phosphorylation nor other enzyme-catalysed steps are therefore likely to mediate the effects of cytosolic Ca^{2+} on $InsP_3$ receptors.

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45P DISCORDANT EFFECTS OF TWO STRUCTURALLY DIFFERENT ALDOSE REDUCTASE INHIBITORS ON VASCULAR FUNCTION IN CONTROL AND DIABETIC RATS

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We have previously reported that some diabetes-induced changes in vascular function are prevented by treatment with the aldose reductase inhibitor ponalrestat (Otter & Chess-Williams, 1994)). The present study investigates the effect of a structurally different aldose reductase inhibitor, sorbinil (Pfizer, Inc.), on vascular function in control and diabetic rats.

Diabetes was induced by a single i.p. injection of streptozotocin ($65mgkg^{-1}$) dissolved in citrate buffer. Control rats received citrate buffer alone. Animals to be treated were administered a daily oral dose ($25mgkg^{-1}$) of either ponalrestat (Zeneca) or sorbinil (Pfizer, Inc.). Fourteen days later thoracic aortae were isolated from each group of rats, mounted in gassed Krebs solution and then concentration-response curves constructed to phenylephrine and carbachol (after precontraction with 1μM phenylephrine).

Maximum contractions to phenylephrine were enhanced ($P<0.01$) in aortae from diabetic rats (0.70±0.05g) compared with untreated controls (0.47±0.06g). Ponalrestat treatment of diabetic rats prevented this enhancement (0.52±0.04g). However sorbinil treatment of diabetic rats failed to do so (0.75±0.06g). Ponalrestat and sorbinil treatment of controls did not affect maximum responses to phenylephrine. EC_{50} values to phenylephrine were reduced ($P<0.001$) in diabetics when compared to controls (10 (4-29) nM and 401 (161-995) nM respectively. Both ponalrestat and sorbinil treatment failed to prevent this reduction in diabetics (45.9 (35.1-60.2) nM and 44.14 (28.53-68.29) nM respectively). In addition sorbinil treatment of controls reduced EC_{50} values to 2.59 (0.96-6.99) nM ($P<0.001$), whereas ponalrestat treatment had no effect on control values. Endothelium-dependent relaxation was impaired ($P<0.0.5$) in aortae from diabetic rats when compared to controls (60.2±4.3% and 78.2±4.4% of the precontraction respectively). Ponalrestat treatment of diabetic rats prevented this impairment (76.3±5.6%), whereas sorbinil treatment did not (49.2±5.9%). EC_{50} values to carbachol were not affected by diabetes or ponalrestat treatment. However sorbinil treatment of controls increased EC_{50} values from 0.89 (0.61-1.29) μM in untreated controls to 1.98 (1.35-2.9) μM ($P<0.05$). Sorbinil treatment of diabetics also increased EC_{50} values from 1.31 (1.05-1.64) μM in untreated diabetics to 2.94 (2.19-3.95) ($P<0.05$).

In conclusion ponalrestat, but not sorbinil, prevented some diabetes-induced changes in vascular responsiveness. The effects of sorbinil suggest that it has actions in addition to it's reported effects on the polyol pathway or that polyol pathway flux is involved in vascular function.

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46P EVIDENCE THAT GUINEA-PIG SMALL INTESTINE CONTAINS CB1 CANNABINOID RECEPTORS AND POSSIBLY ALSO A CANNABINOID RECEPTOR LIGAND

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Previous results (Pertwee *et al.*, 1992) suggest that cannabinoid-induced inhibition of electrically-evoked contractions of the guinea-pig myenteric plexus-longitudinal muscle preparation is receptor-mediated. In the present experiments, we have investigated this possibility further by establishing whether this effect of cannabinoids can be prevented by SR141716A, a CB1 cannabinoid receptor antagonist (Rinaldi-Carmona *et al.*, 1994).

Strips of myenteric plexus-longitudinal muscle dissected from the small intestines of male albino Dunkin-Hartley guinea-pigs (343-659 g) were mounted in 4 ml organ baths and stimulated supramaximally at 0.1 Hz as described by Pertwee *et al.* (1992). Percentage changes in the amplitude of electrically evoked isometric twitches were calculated by comparing the amplitude immediately before exposure to an agonist with the amplitude when the drug response was complete. Cumulative concentration-response curves were constructed. Cannabinoids and SR141716A were mixed with two parts of Tween 80 by weight and dispersed in saline. Other drugs were dissolved in saline.

CP 55,940, WIN 55,212-2 and delta-9-tetrahydrocannabinol (THC), all of which are cannabinoid receptor agonists (Pertwee, 1993), inhibited the twitch response in a dose-related manner. Concentrations producing half-maximal inhibition were 5.3, 9.7 and 181.9 nM respectively (n=5 or 6). SR141716A, injected 25 min before these agonists, produced concentration-related parallel rightward shifts in their log concentration-inhibitory response curves. Tween 80 had no such effect. When the log concentration-response curve of CP55,940, constructed in the presence of Tween 80, was compared with curves constructed in the presence of 31.62, 100, 316.2 or 1000 nM SR141716A, the rightward shifts (dose ratios) were 6.2, 34.5, 40.2 and 96.9 respectively (n=5). SR141716A (316.2 nM) induced 64.9 and 32.9 fold shifts respectively in the log concentration-response curves of THC and WIN 55,212-2 (n=6 to 8).

By itself, SR141716A induced small but significant increases in twitch amplitude. For example, 10, 40 and 160 nM produced increases of 8.91 ± 3.23%, 13.03 ± 3.99% and 15.83 ± 6.65% respectively (mean ± s.e.; n=12). Each of these increases was significantly greater ($P < 0.05$; unpaired t test) than the change in amplitude observed after injection of the corresponding dose of Tween 80 (0.11 ± 1.23%, -1.62 ± 2.63% and 0.88 ± 1.58% respectively; n=11). The twitch amplitude was decreased by a concentration of Tween 80 (17.4 µg/ml) far higher than any concentration of Tween present during the construction of cannabinoid dose-response curves in the absence of SR141716A (up to 0.63 µg/ml). This inhibition by Tween 80 (51.75 ± 8.45%) was completely reversed by 100 nM SR141716A ($P < 0.001$; paired t test; n=7) although not by naloxone (1 µM) or yohimbine (1 µM). The inhibitory effects on the twitch response of 50 nM normorphine and of 200 nM clonidine were not reversed by 1 µM SR141716A. Our findings support the hypothesis that the guinea-pig small intestine contains CB1 cannabinoid receptors and also suggest that this tissue can release cannabinoid receptor ligands(s). Whether Tween 80, at high concentrations, inhibits the twitch response by enhancing such release or by acting through some other mechanism remains to be established.

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47P CYCLOSPORIN A NEPHROTOXICITY IN ESTABLISHED RENAL CELL LINES: THE ROLE OF THE IMMUNOPHILIN CYCLOPHILIN A

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Cyclophilin A (CYP-A) is a member of the immunophilin family (Handschumacher *et al.*, 1984) required to mediate the immunosuppressive activity of cyclosporin A (CsA). The possible role of CYP-A in the nephrotoxicity of CsA is largely unknown. We have previously shown that CsA is directly toxic to renal tubular cells with a relative site selective action to the proximal tubule (Healy *et al.*, 1994). The aims of the present study were to investigate the hypothesis that different expression of CYP-A may be related to the site selectivity of CsA toxicity. Therefore in this study, CYP-A was localized and quantified within established renal cell lines and the effects of CsA on these levels were examined.

The established renal epithelial cell lines LLC-PK$_1$ (proximal tubular origin) and MDCK (distal tubule/collecting duct origin) were used as the model system. CYP-A was detected using a polyclonal (rabbit) anti-CYP-A antibody. The protein was localised within the cells using immunocytochemistry and a streptavidin-biotin-HRP detection system. SDS-PAGE and western blot techniques were used to confirm the presence of a protein of the correct molecular weight.. Flow cytometric analysis and a swine-anti-rabbit FITC secondary antibody were employed to quantify the basal and CsA treated levels of CYP-A within the cells.

CYP-A was localised to the cytoplasm and perinuclear areas of both cell types. Western Blot analysis indicated a molecular weight of 18kDa which is similar to that reported in the literature (Handschumacher *et al.*, 1984). The intensity of staining for CYP-A in both cell types appeared to be very similar. This suggested similar expression of the protein in both cell types. Flow cytometric analysis of CYP-A expression confirmed these observations with mean channel fluoresence (M.C.F.) values of 110 ± 3 and 98 ± 2 for LLC-PK$_1$ and MDCK cells respectively. CsA (4.2μM) treatment for 48 hours increased the M.C.F. by 47.3% within LLC-PK$_1$ cells.

In conclusion, CYP-A was found in similar amounts in both cell types. This indicates that different expression of CYP-A in LLC-PK$_1$ and MDCK cells is unlikely to account for the previously observed relative selectivity of the LLC-PK$_1$ cells from the proximal tubule to CsA toxicity (Healy *et al.*, 1994). However, increased expression of CYP-A after CsA treatment in LLC-PK$_1$ cells suggests that CYP-A may be involved in some way in the mediation of CsA induced nephrotoxic damage.

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48P THE COUPLING OF ADENOSINE A_1, A_{2A} RECEPTORS AND AN A_1/A_{2A} CHIMERIC RECEPTOR TO AN INWARDLY RECTIFYING K^+ CHANNEL

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The G protein-gated K^+ channel from rat atrium (GIRK1 or KGA) is a member of a gene family encoding inwardly rectifying K^+ channels (Kubo *et al.*, 1993, Dascal *et al.*, 1993). This family of channels is coupled to seven-helix receptors via the Gi/Go family of heterotrimeric G proteins. The adenosine A_1 receptor is thought to couple to G_i. Following coinjection of A_1 receptor cRNA and GIRK1 cRNA into xenopus oocytes, bath application of 1nM adenosine induced an inwardly rectifying current in 90mM K^+ solution under two electrode voltage clamp. In the range of concentrations tested, the inwardly rectifying K^+ current could only be activated when both GIRK1 and the receptor were heterologously expressed. The activation of GIRK1 was presumably mediated by endogenous oocyte G proteins. From current-voltage relationships, conductance values were calculated for a range of adenosine concentrations. The EC_{50} for adenosine was 1.8nM (Log EC_{50} = -8.74± 0.11, n= 18).

The A_{2A} adenosine receptor couples to a G_s subtype of G protein and therefore might not be expected to activate GIRK1. However this receptor also gave a functional response when expressed in oocytes. The EC_{50} value for the A_{2A} receptor was 200nM (Log EC_{50} = -6.78± 0.10, n = 3). The coupling of a G_s linked receptor to GIRK1/KGA has also been observed recently for the β_2 receptor (Lim *et al.*, 1995).

To determine regions of adenosine receptors involved in selective G protein recognition, chimeric receptors composed of the human A_1 and human A_{2A} sequence were constructed using the PCR technique of gene splicing by overlap extension (Horton *et al.*, 1993) and were fully sequenced. One chimera (CH4) consisted mainly of A_1 receptor with the third extracellular loop, the seventh transmembrane domain and the -COOH terminus tail of the A_{2A} sequence. This chimera coupled to GIRK1 when expressed in xenopus ooctyes. The EC_{50} value of adenosine for the chimera was 8nM (Log EC_{50} = -8.09± 0.21, n=11). However the Hill coefficient was 0.46 ± 0.11 in contrast to the A_1 and A_{2A} receptors where the Hill Coefficients were unity. This may indicate that CH4 activates GIRK1 by two mechanisms. The dose-response curve could be fitted to a two component model having an EC_{50} of 2.4nM (Log EC_{50} = -8.62 ± 0.17 (64 ± 7%) and 170nM (Log EC_{50} = -6.78 ± 0.33 (36 ± 7%). Both EC_{50} values are close to the values observed for the A_1 and the A_{2A} receptors.

The ligand binding properties were also examined following transient expression in COS M6 cells. Competition experiments were performed as described in Cohen *et al.*, (1995). 8-[Dipropyl-2,3-^{3}H(N)]cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX), an A_1 selective antagonist, and the agonists cyclohexyladenosine (CHA) and 5'-N-ethylcarboxamido adenosine (NECA) had a higher affinity for CH4 than for the A_1 receptors (2 -3 fold). Thus it appears that this chimeric receptor has an A_1 receptor pharmacology. However it is possible that it has both A_1 and A_{2A} functional characteristics by being able to interact with G_i as well as G_S G proteins.

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49P CONSTRUCTION AND USE OF ADENOSINE A_1/A_{2A} RECEPTOR CHIMERAS AS PROBES FOR THE LOCATION OF THE ALLOSTERIC SITE ON THE A_1 RECEPTOR

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Allosteric enhancers of adenosine receptor function may provide more selective therapeutic effects than direct-acting agonists. Their action would be limited to times and locations of significant release of adenosine, so that systemic side effects could largely be avoided. The aminothiophene derivative (2-amino-4,5-dimethylthien-3-yl)[3-trifluoromethyl) phenyl]-methanone (PD81, 723) acts as an allosteric enhancer at the cloned human A_1 receptor expressed in CHO cells, but not the A_{2A} receptor (Cohen *et al*.,1994).

To study the structural basis for the selectivity of PD81,723 for the A_1 subtype, segments of the human A_1 receptor were replaced with the corresponding human A_{2A} sequence. The chimeric receptors were constructed using the PCR technique of gene splicing by overlap extension (Horton *et al.*, 1989) and fully sequenced prior to transfection into COS M6 cells. Chimeras included substitution by the corresponding A_{2A} sequence of a) the -NH_2 terminus domain of the A_1 receptor (CH1), b) the 3rd extracellular loop (CH4a) and c) the 3rd extracellular loop, the seventh transmembrane domain and the -COOH terminus domain (CH4).

Competition studies between 8-[dipropyl-2,3-^{3}H(N)]-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, 0.5nM) and either cyclohexyladenosine (CHA) or 5'-N-ethylcarboxamido adenosine (NECA) were performed with cell membranes (80mg/ml) in binding buffer (NaCl 100mM, HEPES 20mM, $MgCl_2$ 10mM, GTP 1mM) for 1 hour at 25°C with PD81,723 (0 - 10μM). The Table shows the fold change in potency of CHA and NECA (range, n=2) caused by 10μM PD81,723. Positive cooperativity was retained in CH1 and CH4a but was changed to a negative cooperative interaction in CH4.

	A1	CH1	CH4a	CH4
CHA	+ 7-13 fold	+ 2 fold	+ 2-4 fold	- 5-15 fold
NECA	+ 5 fold	+ 3-8 fold	+ 2 fold	- 6 fold

Competition studies between [^{3}H]DPCPX and PD81,723 were performed as above for the A_1 receptor and the 3 chimeric receptors. At the A_1 receptor the K_d of PD81,723 was 24 ± 2μM (n=3). The K_d values of PD81,723 at CH1 and CH4a were similar to that for the A_1 receptor (27 ± 2μM, n=3, 17 ± 5μM, n=3) respectively. However PD81,723 was 12 fold more potent (2.1 ± 0.2μM, n=3) in binding to CH4 than to the A_1 receptor.

The data suggest that the amino acids in the -NH_2 terminus and the 3rd extracellular loop of the A_1 receptor are not essential for the binding of PD81,723. Furthermore, a region of the A_1 receptor including transmembrane domain 7 and the -COOH terminus tail, although not a determinant of PD81,723 binding, is important for the manifestation of the positive cooperative interaction between the agonist binding site and the allosteric site.

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50P TWO P_2 PURINOCEPTOR SUBTYPES ACTIVATE INWARD CURRENT IN FOLLICULATED OOCYTES OF *XENOPUS LAEVIS*

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Mature folliculated oocytes (stages V/VI) from *Xenopus laevis* frogs respond with fast (D_1) and slow (D_2) depolarising currents to superfusion of ATP or ADP, but not adenosine (Lotan *et al.*, 1982, 1986). We have re-examined these depolarising currents to identify the subtypes of P_2 purinoceptors involved and to determine the nature of the currents activated, information of some importance where *Xenopus* oocytes are used for expression cloning of P_2 purinoceptors.

Under voltage-clamp conditions (V_h, -40mV), 118 of 285 follic-ulated oocytes responded with depolarising inward currents (range: -1 to -95nA; mean: -16±2nA) to ATP (10μM), whereas none of 136 defolliculated oocytes were activated. Thus, endogenous P_2-purinoceptors appeared to be located solely on the enveloping follicular cell layer which is known to be electrotonically coupled to the oocyte via intercellular gap junctions (Greenfield *et al.*, 1990). Both fast and slow inward currents to ATP could occur alone (D_1 response: 31 of 118 oocytes; D_2 response: 17 of 118 oocytes) or together (D_1 and D_2 responses: 70 of 118 oocytes).

The rank order of potency of agonists (at 10μM) was (for both D_1 and D_2 responses): 2-MeSATP~UTP>2-chloroATP>ATP> ADP>α,β–meATP>adenosine>ITP, with β,γ-meATP, CTP and GTP inactive. EC_{50} values were (for D_1 response): ATP, 5.6±2.1μM; UTP, 1.0±0.3μM; 2-MeSATP, 0.6±0.2μM (n=3): (for D_2 response): ATP, 6.7±3μM; UTP, 0.9±0.3μM; 2-MeSATP, 9.2±7.4μM (n=3). The equipotency of 2-MeSATP and UTP, and lower potency of ATP, suggested that both P2Y (or P2Y1) and P2U (or P2Y2) subtypes activated inward current. Inward current to ATP (10μM) was weakly inhibited by suramin. IC_{50} values were: 65±20μM (D_1 response) and 57±10μM (D_2 response) (n=3). Suramin (100μM) inhibited by 50±12% (n=3) inward current to UTP (10μM) but did not inhibit inward current to 2-MeSATP.

Inward current to ATP (10μM) reversed to outward current at holding potentials in the region of -10mV (E_{rev}=-10±4mV; n=5). Inward current to UTP (10μM) also reversed at holding potentials in the region -10mV while inward current to 2-MeS-ATP (10μM) reversed at -30mV. Inward currents to ATP and UTP were inhibited by the Na^+ channel blocker amiloride (10-100μM), whereas inward current to 2-MeSATP was inhibited by the Cl^- channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, 10μM). Substituting Na^+ with choline diminished inward current to ATP and UTP and shifted E_{rev} to -30mV but barely affected inward current to 2-MeSATP.

In conclusion, two subtypes (P2Y and P2U) of endogenous P_2 purinoceptors are located on the follicle cell layer that envelops *Xenopus* oocytes. 2-MeSATP activated fast and slow inward Cl^- currents via a suramin-insensitive P2Y purinoceptor while UTP and ATP activated fast and slow inward Na^+ currents via a suramin-sensitive P2U purinoceptor. Expression cloning of purinoceptor subtypes should be carried out on defolliculated oocytes which are devoid of these endogenous P_2 purinoceptors.

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51P A NOVEL P_1 PURINOCEPTOR ACTIVATES AN OUTWARD K^+ CURRENT IN FOLLICULATED OOCYTES OF *XENOPUS LAEVIS*

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Folliculated oocytes (Stages V/VI) from *Xenopus laevis* frogs possess P_1 purinoceptors where either adenosine or ATP activates a hyperpolarising outward K^+ current (Lotan *et al.*, 1982). The basis of ATP agonism at the oocyte P_1 purinoceptor was investigated using electrophysiological and biochemical procedures.

Adenosine and ATP (10μM) evoked outward current which reversed at -90mV and was inhibited by TEA (20mM), confirming the involvement of K^+ channels. Outward K^+ current also was inhibited by theophylline (100μM) and 8-sulphophenyl-theophylline (8-SPT, 10μM), suggesting purinoceptor blockade reduces the number of K^+ channels activated. Outward K^+ current was not inhibited by suramin (100μM) but was inhibited by α,β-meATP (10μM) which was as effective as 8-SPT.

The potency order for agonists of outward K^+ current was (at 10μM): NECA > adenosine > AMP = CGS-21680 > β,γ-meATP = ATP > ADP > R-PIA, with 2-MeSATP, UTP, ATP-γ-S, and α,β-meATP inactive. EC_{50} values were: adenosine, 1.9±0.3μM (n=6) and ATP, 1.7±0.3μM (n=3); adenosine was a full agonist but ATP a partial agonist. An equal affinity for adenosine and ATP, and the above potency order, has not been reported for known (A_1, $A_{2a/b}$, A_3 and A_4) adenosine receptors. However, adenosine and ATP agonism (with equipotency of ATP and β,γ-meATP) and receptor blockade by α,β-meATP and 8-SPT has been described for prejunctional purinoceptors on sympathetic nerves (Shinozuka *et al.*, 1988, 1990). Adenosine deaminase (0.1U/ml), used to degrade adenosine to inosine, abolished outward K^+ current evoked by adenosine but did not affect outward K^+ currents evoked by ATP, NECA, CGS-21680 and R-PIA. Adenosine deaminase (2U/ml) did abolish outward K^+ currents to ATP and adenosine but also affected outward K^+ current to isoprenaline (300nM), suggesting the enzyme exerted non-specific effects at this higher concentration.

With ATP (100μM) as a substrate, oocyte ecto-ATPase showed a velocity of 234±6 pmol P_i min^{-1} $oocyte^{-1}$ (colorimetric analysis; n=4) or a rate of breakdown of 114±4pmol ATP, 87±6pmol ADP and 65±5pmol AMP min^{-1} $oocyte^{-1}$ (HPLC analysis; n=14). Also, ATP (10μM, for 1 minute) was degraded by a single oocyte to ATP (9.55±0.05μM), ADP (0.14± 0.04 μM), AMP (0.067± 0.033μM) and adenosine (0.021±0.012μM) in the electrophysiological chamber, (HPLC analysis). Super-fusing a mixture of AMP (0.067μM) and adenosine (0.021μM) did not mimic the response to ATP (10μM) nor desensitised ATP and adenosine mediated outward K^+ current.

In conclusion, outward K^+ current to ATP does not appear to require the breakdown of ATP to adenosine nor involves the activation of a known subtype of P_1 purinoceptor. Instead, agonism by adenosine, ATP and β,γ-meATP, plus antagonism by α,β-meATP and 8-SPT (with suramin ineffective), suggest the involvement of a novel P_1 purinoceptor similar to a prejunctional purinoceptor found on some sympathetic nerves.

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52P DEGRADATION OF ATP AND ADP BY ECTO-ENZYMES IN FOLLICULATED OOCYTES OF *XENOPUS LAEVIS*

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Xenopus oocytes are used extensively to study the pharmacological properties of cloned receptors for extracellular ATP (P_2-purinoceptors). Previously, we have shown that ecto-ATPase is present on the enveloping follicule cell layer of these cells (Ziganshin *et al.*, 1995). In the present study, we investigated whether or not ATP and ADP are handled by the same ecto-enzyme in these cells. Ovarian lobes were removed surgically from *Xenopus laevis* frogs anaesthetised with tricaine, and oocytes (stages V and VI) were separated mechanically. Folliculated oocytes were incubated at 20±1°C in a Ringer's solution containing a given concentration of either ATP or ADP as the enzyme substrate. The amount of adenine nucleotides remaining after 30 min incubation time was measured by high performance liquid chromatography (HPLC).

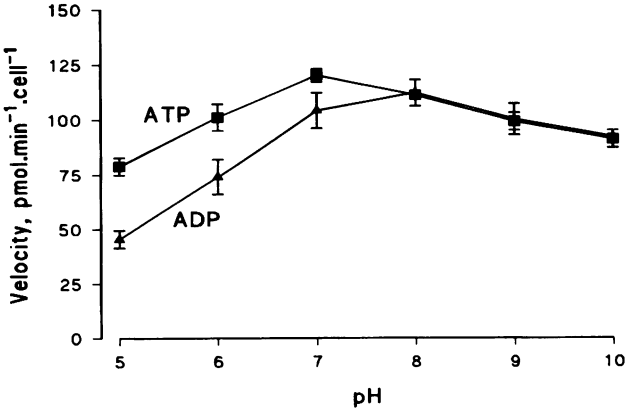

Fig.1. pH-dependency of degradation of adenine nucleotides (n=8).

At a concentration of 100 μM, the rates of degradation of ATP and ADP were similar at pH 7-10, but at pH 5-6 the velocity of breakdown of ATP was significantly higher than that of ADP (Fig.1).

In the presence of extracellular Ca^{2+} and Mg^{2+} ions, each at a concentration of 1.8 mM (normal Ringer's solution), the velocities of degradation of ATP and ADP (100 μM) were 128 ± 4 pmol.min^{-1} $oocyte^{-1}$ and 129 ± 4 pmol .min^{-1}.$oocyte^{-1}$ respectively (n=4). Where cations were added seperately, Ca^{2+} provided higher rate of degradation of ATP than Mg^{2+}, while in case of ADP both cations were equipotent (Table 1).

Table 1. Cation-dependency of velocity of degradation of ATP and ADP (at 100 μM) by oocytes , pmol . min^{-1}.$oocyte^{-1}$ (M ± S.E.M., n=4).

	Concentration of cations, mM				
	0	*0.1*	*0.5*	*1.0*	*2.5*
ATP + Ca^{2+}	117 ± 5	165 ± 5	172 ± 13	161 ± 13	161 ± 2
ATP + Mg^{2+}	117 ± 5	116 ± 6	131 ± 6	141 ± 9	123 ± 8
ADP + Ca^{2+}	97 ± 3	114 ± 5	134 ± 9	125 ± 5	126 ± 11
ADP + Mg^{2+}	97 ± 3	127 ± 9	117 ± 6	138 ± 5	125 ± 9

At ATP concentrations of 10-1000 μM, K_m and V_{max} values were estimated as 186 μM and 280 pmol. min^{-1}.$oocyte^{-1}$. The presence of 50 μM of ADP in the buffer did not significantly changed these kinetic constants, which became 204 μM and 285 pmol.min^{-1}. $oocyte^{-1}$, respectively. This indicates that ATP and ADP do not compete for the same binding site.

Thus, different pH- and cation-dependency and the absence of substrate competition support the hypothesis that ATP and ADP are handled by either different ecto-enzymes or one ecto-enzyme having at least two binding sites on *Xenopus* oocytes.

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53P CHARACTERIZATION OF P_2-PURINOCEPTORS IN THE ISOLATED MESENTERIC ARTERIAL BED OF THE GOLDEN HAMSTER

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Three distinct subtypes of P_2-purinoceptors, namely P_{2X}-, P_{2Y}- and P_{2U}-purinoceptors have been described on many blood vessels including rat mesenteric arteries (Ralevic & Burnstock, 1988). In general, P_{2X}-purinoceptors mediate endothelium-independent vasoconstriction and P_{2Y}- and P_{2U}-purinoceptors mediate endothelium-dependent vasodilatation. The aim of the present study was to characterize pharmacologically P_2-purinoceptors in the mesenteric arterial vasculature of the Golden hamster.

Mesenteric arterial beds from adult male Golden hamsters were perfused via the superior mesenteric artery at 3 ml/min with warmed (37°C), gassed (95% O_2/5% CO_2) Krebs solution. Responses to bolus injections (50 μl) of agonists were measured as changes in perfusion pressure (mmHg). Vasoconstrictor responses to the purines α,β-meATP, 2meSATP (50 pmol-0.5 μmol), ADP, ATP and UTP (5 nmol -5 μmol) were examined at basal tone. Vasodilator responses to α,β-meATP, 2meSATP, ATP, ADP, UTP and adenosine (5 pmol-0.5 μmol) were examined in preconstricted preparations with tone raised with methoxamine (10-80 μM). Endothelium removal was achieved by perfusion of 1 ml of sodium deoxycholate solution (2 mg/ml). Antagonists were allowed to equilibrate for 30 min.

At basal tone purines elicited dose-dependent vasoconstriction with a potency order of α,β-meATP >> 2meSATP > ATP = ADP > UTP (n = 4 - 10). Responses were blocked following desensitization with a single dose of α,β-meATP (0.5 μmol). The P_2-purinoceptor antagonist suramin (100 μM) did not affect vasoconstrictor responses to ATP. The selective P_{2X}-purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 3 μM) (Winscheif *et al.*, 1994) blocked responses to ATP. Responses to ATP were augmented by removal of the endothelium. At raised-tone, purines elicited vasodilatation with a potency order of ATP = UTP >> ADP >> adenosine (n = 5 - 17). 2meSATP was virtually without vasodilator effects. α,β-meATP elicited only constriction. Suramin (100 μM) attenuated vasodilator responses to ATP and UTP (pA_2 values 4.60 and 4.89 respectively) and abolished those to 2meSATP. PPADS (3 μM) did not affect responses to ATP and UTP, but blocked responses to 2meSATP. Reactive blue 2 (30 μM) produced a non-specific antagonism of dilator responses to ATP, UTP, 2meSATP and ACh; RB2 was without effect at 3 μM. Removal of the endothelium abolished vasodilator responses to the purines, except for those to adenosine.

In conclusion, mesenteric arteries of the Golden hamster possess smooth muscle P_{2X}-purinoceptors which mediate vasoconstriction and endothelial P_{2U}-purinoceptors which mediate vasodilatation. In contrast to many other vessels, including mesenteric arteries of the rat, P_{2Y}-purinoceptors comprise a relatively small proportion of the endothelial P_2-purinoceptor population. This study of the Golden hamster is intended as a baseline for future studies of purinoceptors in hibernation.

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54P CHARACTERISATION OF THE ELECTROPHYSIOLOGICAL ACTIONS OF P_2-PURINOCEPTOR AGONISTS IN CULTURED NEURONES IN THE RAT DORSAL ROOT GANGLIA

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When adenosine 5'-triphosphate (ATP) is released as a cotransmitter from postganglionic autonomic nerves, it acts via P_2-purinoceptors to activate a non-selective cation conductance and evoke smooth muscle contraction. Recently, ATP was also shown to be a neurotransmitter at central and autonomic synapses, but how ATP acts in neurones is less clear. The aim of this study was to characterise the actions of P_2-purinoceptor agonists in cultured neurones of rat dorsal root ganglia.

Neurones of rat pup dorsal root ganglia were isolated by acute enzymatic dissociation. The whole-cell mode of the patch-clamp technique was used to record currents from cells held at -65 mV. Agonists were applied for 500 ms at 10 min intervals, using a solenoid valve-controlled U-tube system (equilibration time <10 ms) to minimise desensitisation.

Isolated neurones had a resting membrane potential of -55.2 ± 0.6 mV (n=88). 95.5 % of these cells responded to ATP, 2-methylthioATP (2-meSATP) or α,β-methyleneATP (α,β-meATP). ATP (1nM - 100 μM) evoked concentration-dependent inward currents, with an EC_{50} of 719 nM and Hill slope of 1.5. The time to onset of the response was in the order of a few ms, suggesting that a ligand-gated channel had been activated. At 300 nM, ATP and 2-meSATP induced inward currents of 477 ± 85 pA (n=23) and 611 ± 85 pA (n=22) respectively, which were significantly greater ($P<0.05$) than the current evoked by 300 nM α,β-meATP (174 ± 34 pA, n=20).

In 3/3 cells, 1 μM uridine 5'-triphosphate (UTP) had no effect on holding current, but at 10 μM, UTP induced an inward current in 8/10 cells with mean peak amplitude of 526 ± 104 pA, which was significantly smaller than that induced by 10 μM ATP ($P < 0.05$).

Suramin (100 μM) applied in the superfusate reversibly abolished the currents evoked by 300 nM ATP (n=10), 2-meSATP (n=11) and α,β-meATP (n=4). In 3/4 cells, suramin (100 μM) also reversibly abolished the currents induced by UTP (10 μM), while in the fourth cell a 90% decrease in current was observed. To further investigate the site of action of UTP, cross-desensitisation studies with ATP were performed. In four cells tested, prior administration of ATP (10 μM), 2 min beforehand, reversibly reduced the response to UTP (10 μM) by 80 ± 10%.

The results of this study show that P_2-purinoceptor agonists rapidly activate an inward current in neurones of the rat dorsal root ganglia. Their relative potencies are consistent with an action at the P_{2X}-purinoceptor, when studied in the absence of agonist breakdown (Kennedy & Leff, 1995). UTP also activated an inward current, but at present it is unclear whether this was via the same receptor as the P_2-purinoceptor agonists or via a separate pyrimidoceptor.

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55P COMPARISON OF THE ELECTROPHYSIOLOGICAL ACTIONS OF ATP AND UTP IN SINGLE DISSOCIATED CELLS OF THE RAT TAIL ARTERY

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It has previously been shown that adenosine 5'-triphosphate (ATP) is able to evoke inward currents in single cells of the rat tail artery by activating P_{2X}-purinoceptors (Evans & Kennedy, 1994). It is unclear if uridine 5'-triphosphate (UTP) is able to evoke ionic currents in this tissue. The aim of this study was to compare the actions of ATP and UTP in single cells acutely dissociated from the rat tail artery.

Smooth muscle cells were isolated from the artery by enzymatic dissociation. The whole-cell mode of the patch-clamp technique was used to record currents from cells clamped at -60mV. Agonists were applied for 1 s at 10 min intervals, using a solenoid valve-controlled U-tube system. This rapid application system (equilibration time <5 ms) was used so that desensitisation could be minimised.

ATP (30nM – 10μM) and UTP (1μM – 500μM) evoked concentration-dependent inward currents (Table 1). The time to onset for responses to ATP and UTP was a few ms in each case, suggesting that both agents are activating ligand-gated channels. Rise times to peak response and half decay times (t50%, Table 1) for responses to both ATP and UTP decreased with increasing agonist concentration. Preliminary studies indicate that the inward currents evoked by both ATP (300nM) and UTP (30μM) are abolished by the P_{2X}-purinoceptor antagonists PPADS (10μM) and suramin (100μM).

Table 1

	n	Peak(pA)	Rise time(ms)	t50%(ms)
ATP				
30nM	1	-27	-	-
100nM	5	-515±155	71±7	390±63
300nM	6	-1122±354	74±16	214±33
1μM	6	-2103±301	21±2	94±26
3μM	4	-5564±1388	19±10	68±13
5μM	3	-6188±573	9±1	71±7
10μM	2	-5066	6	44
UTP				
1μM	4	-38±3	-	-
3μM	4	-84±12	149±30	1454±139
10μM	5	-301±84	51±8	730±333
30μM	5	-881±214	48±8	165±21
100μM	6	-1164±152	42±15	107±35
300μM	6	-3442±746	16±8	105±22
500μM	2	-3004	12	56

These results suggest that UTP as well as ATP is able to elicit inward currents in single vascular smooth muscle cells, either by activation of P_{2X}-purinoceptors or a separate pyrimidoceptor.

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56P P_{2u} PURINOCEPTOR ACTIVATION OF CHLORIDE TRANSPORT IN CYSTIC FIBROSIS AND CFTR-TRANSFECTED PANCREATIC CELL LINES

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Cystic Fibrosis (CF) is a disease of the secretory epithelia, attributed to loss of cAMP regulation of chloride transport across epithelial cells. Pancreatic adenocarcinoma cells derived from a patient with CF (CFPAC cells) thereby carrying the basic CF defect, and CFPAC cells transfected with wild-type CFTR (TPAC cells) and therefore corrected for the basic CF defect, (Cliff *et al.*, 1992) were used in this study as models to investigate possible activation of chloride (Cl) transport in CF and normal epithelia by a range of nucleotides and nucleotide analogues.

Cells were loaded with ^{36}Cl for 2 hours at 37°C. Efflux was measured at 25°C over a 10 minute time course using a modification of the method of Rugolo *et al.* (1992). The effects of forskolin and thapsigargin (TGN) were investigated. The dose dependent effects of nucleotides and analogues on ^{36}Cl efflux were determined in both cell types. Efflux was expressed as a fraction of total ^{36}Cl present in the cell at the time the drugs were added.

cAMP dependent Cl transport in the two cell types was studied using forskolin (10μM), which significantly ($p \leq 0.05$) stimulated Cl efflux from TPAC cells increasing efflux from 0.465 ± 0.023 to 0.693 ± 0.016. No difference was observed in the CFPAC cells when basal efflux (0.282 ± 0.023) was compared to efflux in the presence of forskolin (0.299 ± 0.013). The presence of an intracellular calcium-dependent component of Cl transport was investigated using TGN. In both cell types, TGN (0.5μM) significantly increased Cl efflux, to 0.516±0.016 in the CFPAC cells and to 0.758±0.018 in the TPAC cells.

Nucleotides and analogues were used to examine the possibility of increasing Cl transport in CF via activation of Cl channels other than the CFTR. All agents were studied in the concentration range 0-100μM. ATP, UTP and ATPγS stimulated Cl efflux in both cell types in a dose dependent manner. At a concentration of 10μM, ATP increased efflux from 0.229±0.012 to 0.319±0.018 in the CFPAC cells and from 0.246±0.015 to 0.400±0.022 in the TPAC cells. UTP at the same concentration stimulated CFPAC Cl efflux from 0.203±0.018 to 0.298±0.004 and Cl efflux from TPAC cells from 0.253±0.009 to 0.339±0.033. ATPγS was less potent than both ATP and UTP, 100μM increasing efflux in CFPAC cells from 0.246±0.018 to 0.312±0.021 and in the TPAC cells from 0.288±0.021 to 0.316±0.011. None of the other nucleotides or analogues tested, namely 2 methylthio-ATP, αβmethylene-ATP, βγmethylene-ATP, ADP, adenosine-5'-O-(2-thiodiphosphate), increased Cl efflux from either cell type. The order of potency of these agents suggests that they are acting via a P_{2U} purinoceptor (Lustig *et al.*, 1994).

These results indicate that in these experimental cell models, it is possible to bypass the basic Cl transport defect and activate Cl transport via alternative pathways. Activation of the P_{2U} receptor may provide additional useful therapies in the treatment of CF.

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57P EFFECTS OF DIADENOSINE POLYPHOSPHATES ON CAPSAICIN-SENSITIVE SENSORY-MOTOR NEUROTRANSMISSION IN ISOLATED GUINEA-PIG ATRIA

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Capsaicin-sensitive sensory-motor neurotransmission in the heart is mediated by calcitonin gene-related peptide (CGRP), which produces positive inotropic and chronotropic actions, and is modulated by several autacoids, including adenosine and ATP (Rubino, 1993). Cardiovascular responses to diadenosine polyphosphates have been recently investigated (Schluter *et al.*, 1994; Ralevic *et al.*, 1995). However, no information has been reported about the neuromodulatory actions of these nucleotides in the heart. This work aimed to investigate the activity of a series of diadenosine polyphosphates (Ap_n; n=2-6) on cardiac sensory-motor neurotransmission. Guinea-pig atria were used for this study. The tissue was isolated and driven at 4 Hz, in the presence of guanethidine 5 μM, propranolol and atropine 1 μM; isometric contractile tension was recorded. Sensory-motor neurotransmission, evaluated as increase in contractile tension following electrical field stimulation (EFS; 0.1 ms, 20 Hz, at maximal voltage for 5 s), was examined in the absence and in the presence of increasing concentrations of the adenosine polyphosphates tested. The effect of diadenosine phosphates was evaluated as percent inhibition of control responses to EFS and their potency was evaluated from -log concentrations that gave 30% inhibition of control responses ($p[A]_{30}$). Basal contractile tension (234.8±14.3 mg; n=25) increased by 136.2±10.3 mg (n=25) following EFS. At concentrations ranging from 0.3 to 30 μM, all diadenosine polyphosphates tested reduced cardiac responses to EFS. $p[A]_{30}$ values did not significantly differ, being 5.97±0.11 (n=6), 5.82±0.16 (n=5), 5.64±0.11 (n=5), 5.68±0.12 (n=6), 6.07±0.13 (n=3), for Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A, respectively. In the same concentration range, adenosine inhibited sensory-motor neurotransmission, showing a $p[A]_{30}$ value of 5.72±0.08 (n=4). In the presence of the A_1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 nM), dose-response curves to Ap_3A and adenosine were similarly shifted to the right and $p[A]_{30}$ values were 5.22±0.12 (n=4) and 5.16±0.08 (n=4), respectively. All diadenosine polyphosphates mimicked the negative inotropism of adenosine, which consisted of about 30% reduction of basal contractility, at the highest concentration tested (30 μM). However, in the presence of 30 μM Ap_4A, cardiac responses to the neurotransmitter CGRP (1-30 nM) applied exogenously did not significantly differ from controls, thus indicating that inhibition of sensory-motor neurotransmission by diadenosine polyphosphates is prejunctional.

In summary, this study demonstrates that diadenosine polyphosphates modulate cardiac sensory-motor neurotransmission via prejunctional A_1 adenosine receptors.

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58P DIADENOSINE POLYPHOSPHATES EVOKE CALCIUM SIGNALS IN GUINEA-PIG BRAIN SYNAPTOSOMES VIA RECEPTORS DISTINCT FROM THOSE FOR ATP

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Diadenosine polyphosphates can stimulate calcium entry into guinea-pig brain synaptosomes, but the type of receptor that they activate is not yet known. In order to determine if the dinucleotides act on typical P_2-purinoceptors, the non-selective P_2-purinoceptor antagonist, suramin was tested against diadenosine tetraphosphate (Ap_4A) and diadenosine pentaphosphate (Ap_5A). Additionally, they were tested for additive effects with P_2-purinoceptor agonists.

Guinea-pigs were killed by cervical dislocation and exsanguination. The paleocortex and cerebellum were removed and synaptosomes were prepared from the remaining diencephalon and brain stem. The synaptosomes were incubated with FURA 2-acetoxymethylester (5 μM) at 37°C for 30 min at a protein concentration of 1 $mg.ml^{-1}$ then washed in Elliott's buffer and placed in a cuvette in a fluorimeter set to measure fluorescence at 510 nm. After 1 min an agonist (100 μM) was added to the cuvette, and 1 min later either KCl (60 mM) or another dose of an agonist was added. The signal for each preparation was calibrated by adding EGTA (25 mM) and triton-X100 (20% v/v) (zero Ca^{2+}-signal) followed by $CaCl_2$ (10 mM). The mean resting calcium concentration was 115 ± 3 nM. The selective P_{2X} agonist, α,β-methylene ATP (α,β-meATP) was more effective than either of the P_{2Y}-selective agonists, ADP-β-S and 2-methylthio ATP (2-MeSATP), but was equieffective with ATP. Both Ap_4A and Ap_5A were equieffective with ATP. (Table 1). In the presence of suramin (100 μM) responses to ATP were blocked, while Ap_4A was unaffected. Following and initial dose of α,β-mATP a second dose had no effect, while a dose of Ap_5A was fully effective. Similarly, when Ap_5A had been applied first, a subsequent dose of Ap_5A was ineffective, while α,β-mATP was fully effective (Table 1). These results indicate that the population of receptors activated by Ap_4A or Ap_5A is distinct from that activated by ATP.

Table 1. Increase in synaptosomal Ca^{2+}-concentration (nM) induced by agonists. Mean ± s.e. mean (n=3). (Statistical tests: ANOVA and post hoc Student's *t*-tests, * $P < 0.05$)

	ATP	α,β-mATP	ADP-β-S	2-MeSATP	Ap_4A	Ap_5A	Ap_5A
Control	24.5±0.2	23.0±0.6	19.1±1.7*	13.1±0.8*	29.6±2.3	20.7±5.1	22.5±1.3
+ Suramin	3.4±0.6*				28.4±3.1		
2° α,β-mATP		0*				22.4±0.8	
2° Ap_5A		25.7±4.2					0*

59P HISTAMINE-INDUCED H_1-RECEPTOR INTERNALISATION IN HUMAN U373 MG ASTROCYTOMA CELLS

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Receptor internalisation is well established as a mechanism for the control of cellular response to agonists at G protein-coupled receptors. However, there has been no direct demonstration of the internalisation of histamine H_1-receptors in the presence of histamine. We have set out to develop a protocol for the measurement of the binding of [^{3}H]-mepyramine to H_1-receptors on intact human U373 MG astrocytoma cells at 37°C and to determine the effect of pre-exposure to histamine on the relative amounts of intracellular and plasma membrane binding sites.

U373 MG cells were cultured and dissociated as described previously (Arias-Montaño *et al.*, 1994). Measurements of [^{3}H]-mepyramine binding were made in a medium containing (in mM): NaCl 120, KCl 5.4, $MgCl_2$ 1.6, $CaCl_2$ 1.8, D-glucose 11 and HEPES 25, adjusted to pH 7.4 with NaOH (final concentration Na^+ 133 mM) in the presence or absence of 2 μM pirdonium or 2 μM temelastine. Cell-bound [^{3}H]-mepyramine was separated by filtration, after dilution of the incubation mixture with ice-cold medium and, in most experiments, washing of the cells by centrifugation and resuspension in ice-cold medium.

At 6°C dissociation of the pirdonium-sensitive binding of [^{3}H]-mepyramine from the cells was negligible over a 2 h period after dilution of the incubation mixture and addition of 1 μM mepyramine. The introduction of a washing step before filtration reduced the level of pirdonium-insensitive binding from 56 ± 5% (6) to 39 ± 5% (3). The pirdonium- or temelastine-sensitive binding of [^{3}H]-mepyramine measured after 10 min equilibration at 37°C failed to saturate and the parameters associated with the high-affinity site were obtained by fitting an hyperbola + a linear component. The K_d for [^{3}H]-mepyramine for the saturable site, 2.1 ± 0.3 nM (7), was in good agreement with the value, 2.5 nM, from inhibition of histamine-induced inositol phosphate formation in U373 MG cells (Arias-Montaño *et al.*, 1994). Values of B_{max} were the same whether 2 μM pirdonium or 2 μM temelastine was used to define non-specific binding (11 ± 1 and 11 ± 2 fmol.mg cell protein^{-1}). Curves of the inhibition of 5 nM [^{3}H]-mepyramine binding by H_1-antagonists were biphasic, but the K_d values derived from the IC_{50}s for the high-affinity site were consistent with those expected for binding to the H_1-receptor. However, the amount of the high-affinity site, as a percentage of the total binding of [^{3}H]-mepyramine, determined from inhibition curves for two cell-penetrant tertiary amines, 4-methyldiphenhydramine, 63 ± 4% (3) and *nor*pirdonium, 68 ± 3% (3), were significantly greater than for the corresponding quaternary derivatives, 45 ± 3 and 50 ± 1% ($P<0.001$, two-way analysis of variance). The significance was not altered when the percentages of the high-affinity sites in the curves for the tertiary amine mepyramine, 65 ± 7% (3), and the non-penetrant antagonist temelastine, 42 ± 5% (3), were included in the analysis. The differences within groups (cell penetrant/non-penetrant) were not significant. This suggests strongly that the pirdonium-sensitive binding of [^{3}H]-mepyramine is to plasma membrane receptors. After pretreatment of the cells with 100 μM histamine for 5, 10 or 60 min at 37°C, followed by washing, the total binding of [^{3}H]-mepyramine measured after 120 min incubation at 4°C was not altered, but the pirdonium-sensitive binding had declined to 59 ± 6 (3), 44 ± 6 (3) and 33 ± 8% (3) of the control, respectively. The decrease after 1 min exposure to 100 μM histamine, 84 ± 8% (3) was not statistically significant. The evidence is consistent with a time-dependent internalisation of the H_1-receptor induced by 100 μM histamine.

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60P NITRIC OXIDE DONORS INHIBIT RELEASE OF ENDOGENOUS DOPAMINE IN THE RABBIT RETINA

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Vertebrate retinal neurones can function optimally in response to visual stimuli under illumination conditions that may vary in intensity by several orders of magnitude. This is made possible in part by modulatory synaptic effects, and both nitric oxide (NO) and dopamine (DA) have been suggested to be light adaptive transmitters (Djamgoz & Wagner, 1992; Greenstreet & Djamgoz, 1994). However, it is not known if these signals interact, therefore in this study, we have investigated the effects of NO donor compounds on endogenous DA release in the isolated rabbit retina.

Experiments were carried out on isolated retinae of New Zealand white rabbits. Light-adapted retinae were incubated with Krebs bicarbonate medium, containing pargyline (50μ M), in a small (1ml) chamber at room temperature and the medium changed every 10 min. The retinae were exposed to three separate periods of depolarisation with high K^+ (50mM) (S1, S2 and S3). Samples (100μl) were collected into 1M perchloric acid (10μl) and analysed for DA content by HPLC with electro-chemical detection (Hutson et al, 1991).

The effect of a given NO donor on K^+ evoked DA release was determined by including it during the second K^+ stimulation period (S2).

In the absence of any drug, K^+ evoked DA release (S2/S1) was 0.64±0.09 (n=5). This value was significantly reduced to 0.10±0.03 (n=3) ($P<0.01$, students t test) following the inclusion of 300μM sodium nitroprusside during the second stimulation (S2), corresponding to an 85% reduction in the amount of DA released. A similar effect was seen using other NO donors; thus at a concentration of 300μM, hydroxylamine and s-nitroso-n-acetyl penicillamine decreased K^+ evoked DA release by 64% (n=3, $P<0.05$) and 89% (n=3, $P<0.01$) respectively.

Results suggest that NO exerts an inhibitory control upon depolarization-induced DA release in the rabbit retina. Such an effect suggests a complex, interactive involvement of NO and DA in the mammalian retina.

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61P VASORELAXATION BY NITRIC OXIDE DONOR DRUGS ON PULMONARY ARTERIES FROM PULMONARY HYPERTENSIVE RATS

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Nitric oxide (NO) donor drugs, as well as inhaled NO, are of potential value as pulmonary vasodilators in pulmonary hypertension (PH). The NO donor, nitroprusside (NP), is less potent in pulmonary arteries (conduit and resistance vessels) from PH rats than in arteries from normotensive control rats (Wanstall & O'Donnell, 1992; Wanstall *et al.*, 1993). However NP may not act solely via generation of NO and, therefore, may not be representative of all NO donor drugs (Feelisch, 1991). This study aimed to determine if a reduction in potency in pulmonary arteries from PH rats is seen with NO donors other than NP.

Male Wistar rats were housed in hypoxic chambers (10% O_2) for 7 days to induce PH. These rats developed right ventricular hypertrophy confirming the presence of PH (R.Ventricle/[L.Ventricle + septum] 0.40±0.02 (n=18) compared with control rats housed in room air: 0.28±0.01 (n=24); $P<0.05$). Concentration-response (relaxation) curves were obtained for the NO donor drugs, sodium nitrite ($NaNO_2$) and linsidomine (SIN-1), and for the combined NO donor and K^+ channel opener, KRN2391 (KRN; N-cyano-*N'*-(2-nitroxyethyl)-3-pyridine-carboximidamide) on isolated ring preparations of main pulmonary artery (endothelium intact) contracted with 0.1μM noradrenaline. The effects of KRN were attenuated by 10μM glibenclamide (glib) and by 10μM methylene blue (MB) (Table 1; control rats), confirming an action via K^+ channels as well as via NO in rat pulmonary artery. The potencies (neg log EC50) of $NaNO_2$, SIN-1 and KRN (glib present) were all significantly less in preparations from PH rats than in controls but, in the absence of glib, KRN was equipotent in arteries from PH and control rats (Table 1).

Table 1 Neg log EC50 of NO donor drugs (mean ± s.e.m.; n in parentheses)

	Control rats	PH rats
$NaNO_2$	3.62 ± 0.06 (5)	3.27 ± 0.09 (4)*
SIN-1	6.47 ± 0.05 (6)	5.57 ± 0.07 (6)*
KRN	6.36 ± 0.06 (5)	6.21 ± 0.04 (4)
KRN (+ glib)	5.68 ± 0.05 (4)#	4.86 ± 0.11 (4)*
KRN (+ MB)	5.93 ± 0.13 (4)#	Not tested

# $P<0.05$ KRN (+ glib or MB) vs. KRN alone
* $P<0.05$ PH rats vs. Control rats

We conclude that a reduction in potency in pulmonary arteries from PH rats may be a feature of all drugs that act by generating NO. For KRN the reduction in potency was not seen because KRN has the properties of a K^+ channel opener (potency of these drugs not reduced in PH; Wanstall & O'Donnell, 1992) in addition to those of an NO donor.

KRN2391 was a gift from Kirin Brewery Co Ltd, Gunma, Japan.

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62P EFFECT OF SAPONIN VS L-NAME ON THE RELATIONSHIP BETWEEN CORONARY FLOW AND NITRIC OXIDE PRODUCTION UNDER BASAL AND AGONIST-STIMULATED CONDITIONS IN GUINEA-PIG ISOLATED HEART

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L-NAME reduces endothelium-dependent nitric oxide (NO) release and basal coronary flow (CF) (Smith *et al.*, 1992). Saponin induces endothelial injury, yet it does not alter basal CF (Mankad *et al.*, 1991). To examine this anomaly we compared saponin with L-NAME for effects on basal CF and NO release and responses to the agonists ACh, 5-HT and substance P (SP).

Male Dunkin Hartley guinea-pigs (350-400 g) were terminally anaesthetised with pentobarbitone (60 mg kg^{-1}, i.p.) and heparinised (250 iu sodium heparin, i.p.). Excised hearts were perfused with modified Krebs solution containing (mM) KCl 4.0 and $CaCl_2$ 1.4 (pH 7.4, 37°C) and paced at 275 beats min^{-1}. CF was measured by timed collection of coronary effluent. After 30 min, ACh (1 μM), 5-HT (1 μM) or SP (1 nM) were delivered for 5 min, followed by either 100 μM L-NAME for 30 min or a 30 μg ml^{-1} saponin protocol (Mankad *et al.*, 1991), then agonists were reassessed. Coronary effluent was collected during the last min of drug perfusion; NO content was measured by chemiluminescence (Menon *et al.*, 1991). Values are mean ± s.e. mean (*$P < 0.05$ vs pre-L-NAME or pre-saponin; paired t-test).

Saponin did not reduce basal CF even though it reduced basal NO; L-NAME reduced both variables (Table 1). The responses (ΔCF and ΔNO) to ACh, 5-HT and SP were inhibited by both L-NAME and saponin (Table 1). For each agonist, ΔCF correlated ($P<0.01$) with logΔNO (data in the absence and presence of L-NAME and saponin combined). The best correlation occurred with SP: ΔCF=1.2(logΔNO)-1.8, r=0.92. The relationship for ACh was similar: ΔCF=1.4(logΔNO)-1.33, r=0.79. However, 5-HT produced a disproportionately greater ΔCF per ΔNO: ΔCF=3.19(logΔNO)-2.72, r=0.79.

In conclusion, the apparently anomalous lack of effect of saponin on basal CF (compared with the effect of L-NAME) appears to be related to its limited effect on basal NO production. However, there was little difference between saponin and L-NAME in terms of effects on agonist-stimulated responses; both reduced ΔCF and this correlated with ΔNO. This indicates that saponin is an appropriate tool for assessing endothelium-dependent vasodilatation. The relationship between ΔNO and ΔCF differed between 5-HT, ACh and SP. The vasodilatation elicited by SP appears to be largely attributable to NO release, whereas that elicited by 5-HT and ACh may involve additional NO-independent, mechanisms.

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Table 1

		ΔCF ml min^{-1} g^{-1}				ΔNO pmol min^{-1} g^{-1}			
		pre-L-NAME	post-L-NAME	pre-saponin	post-saponin	pre-L-NAME	post-L-NAME	pre-saponin	post-saponin
basal	*(n=16)*	*0*	*-3.9 ± 0.5**	*0*	*-0.2 ± 0.5*	*0*	*-416 ± 28**	*0*	*-198 ± 36**
ACh	(n=5)	+3.4 ± 0.3	+1.2 ± 0.4*	+3.5 ± 0.3	+1.3 ± 0.3*	+849 ± 166	+93 ± 22*	+989 ± 105	+84 ± 29*
5-HT	(n=5)	+2.9 ± 0.2	+1.2 ± 0.3*	+4.3 ± 0.3	+2.1 ± 0.3*	+698 ± 140	+91 ± 62*	+732 ± 108	+86 ± 78*
SP	(n=6)	+1.7 ± 0.2	+0.3 ± 0.3*	+1.8 ± 0.1	+0.4 ± 0.2*	+1038 ± 160	+84 ± 25*	+999 ± 89	+42 ± 53*

63P DEFICIENT NITRIC OXIDE IS RESPONSIBLE FOR REDUCED NERVE BLOOD FLOW IN DIABETIC RATS: PREVENTION BY ESSENTIAL FATTY ACIDS

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Deficient nerve blood flow contributes to early conduction defects in experimental diabetic neuropathy and both anomalies are prevented by essential fatty acids (Stevens et al., 1994). This study examined involvement of deficient nitric oxide via the response of the system to L-NAME. Drugs were delivered into the sciatic nerve endoneurium via a glass micropipette (5 μm tip) attached to a Drummond microinjector, whilst monitoring sciatic nerve laser Doppler flux (LDF) a few mm distal to the injection site. Rats were anaesthetised (Na pentobarbitone 45 mg/kg, diazepam 2 mg/kg) and systemic arterial pressure recorded as described elsewhere (Stevens et al., 1994). Of two groups of diabetic (streptozotocin 60 mg/kg i.p.; duration 8 weeks) rats, one received evening primrose oil (EPO) by dietary admixture (5% diet w:w); age-matched non-diabetic rats were controls. LDF values are in arbitrary units as mean ± 1SD. Significance levels are derived by paired t tests, except where stated otherwise.

Diabetic rats had approximately half the resting LDF values (90.1±34.7; n=8) of control (212±95.5; n=10) or EPO-diabetic (203.1±65.2; n=9; both $p<0.005$ by unpaired t). Endoneurial L-NAME (1 nmole in 1μl) reduced LDF over 20 min in control (to 105.5±40.6; $p<0.002$) and EPO-diabetic rats (to 100.8±52.7; $p<0.002$), but was without significant effect in untreated diabetic rats (to 80.1±33.8). Thirty min after L-NAME sub-groups representing each condition were given L-arginine (100 nmoles in 1μl) by endoneurial injection. In all three groups this increased LDF, reaching 183.2±64.7 in controls ($p<0.01$; n=5), 128.8±27.7 in diabetics ($p<0.02$; n=4) and 216.6±45.2 ($p<0.05$; n=5) in EPO-diabetics. In controls and EPO-diabetics L-arginine gave LDF values which were not significantly different from those before L-NAME (i.e. giving full reversal of the latter). In untreated diabetics L-arginine increased ($p<0.05$) LDF above the starting value, indicating a deficit in substrate for nitric oxide synthase under resting conditions. The remaining rats were given sodium nitroprusside (10 nmoles in 1μl) instead of L-arginine. This produced similar changes to L-arginine (LDF values - controls 218.5±83.4, untreated diabetics 147.7±55.4 and EPO-diabetics 224.3±113.7), indicating an additional impairment in the response to nitric oxide, with prevention thereof by EPO, in diabetic rats. There were no differences between groups in resting systemic arterial pressure and the drug-induced changes in LDF were not accompanied by changes in systemic arterial pressure. These findings indicate defects in the endoneurial production of and response to nitric oxide in endoneurial ischaemia in short-term streptozotocin-diabetic rats.

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64P EVIDENCE THAT L-2-CHLOROPROPIONIC ACID-INDUCED NEUROTOXICITY IS MEDIATED BY NITRIC OXIDE GENERATION

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The chemical intermediate, L-2-chloropropionic acid (L-CPA), used in the manufacture of pharmaceutical and agrochemical products produces selective neuronal cell necrosis in the rat cerebellum when administered in large doses. The cerebellar damage is characterised by extensive loss in granule cell numbers, some loss in Purkinje cells, severe oedema and changes in cerebellar excitatory amino acid concentrations. N-methyl-D-aspartate receptors play an important role in the neurotoxicity since complete neuroprotection can be acheived when NMDA antagonists, such as MK-801 and CGP40116 are administered with the L-CPA. We examined the nitric oxide synthase (NOS) pathway in L-CPA-induced neurotoxicity which may be enhanced following L-CPA administration to rats. The ability of NOS inhibitors (N^G-nitro-arginine, NOARG, 50 mg/kg/i.p. twice daily; its methyl ester, NAME, 50 mg/kg.i.p./ twice daily; N^G-iminoethyl-L-ornithine, NIO 25 or 100 mg/kg/i.p. twice daily; or 3-bromo-7-nitroindazole, BNI, 50 mg/kg/i.p., twice daily) to prevent the L-CPA-induced neurotoxicity was investigated *in vivo*. In addition, experiments were performed to determine whether L-CPA or either of its two proposed metabolites L-CPA-glutathione and L-CPA-cysteine were able to alter NOS activity *in vitro*. Finally the NOS activity was measured in rat cerebellum 48h following L-CPA administration, a time when there is extensive granule cell necrosis and compared to the NOS activity at 6h when there is no neuropathology. L-CPA was administered orally to rats (750 mg/kg, neutralised to pH 7.0 with NaOH) and the rats killed by CO_2 anaesthesia. Control rats were orally dosed with water. The water content in one half of the cerebellum was estimated by drying in an oven at 105 °C to constant weight. Concentrations of amino acids, [^{3}H]glutamate binding to glutamate receptors and neuropathology were estimated in the other half of the brain. Both NAME and BNI provided complete neuroprotection against L-CPA-induced neurotoxicity. NOARG was partially effective but NIO administered at 25 or 100 mg/kg was ineffective. Both NAME and BNI treatment also prevented the oedema (cerebellar water content, control = 3.62 ± 0.02, L-CPA = 4.06 ± 0.09*, L-CPA + NAME = 3.63 ± 0.05, L-CPA + NOARG =3.97 ±0.08*, L-CPA + NIO (100mg/kg) = 3.93 ± 0.14*, L-CPA + BNI = 3.68 ± 0.05 g water/g dry wt, n = 5-10 rats/group, * $P< 0.05$ as compared to controls) changes in cerebellar excitatory amino acid concentrations and alterations in [^{3}H]glutamate binding to cerebellar cortex (Widdowson et al., 1995). L-CPA and its two metabolites did not alter NOS activity *in vitro*, nor was NOS activity changed in cerebellum measured 6 or 48h following L-CPA dosing. The lack of efficacy with NIO *in vivo* was possibly due to low blood-brain barrier penetration since NIO potently inhibited NOS activity in cerebellar homogenates *in vitro* (IC_{50} = 1 .8 μM). In conclusion, the NOS inhibitors, NAME and BNI were able to prevent L-CPA-induced neuropathology, as measured by a loss in cerebellar granule cell number and prevent the appearance of cerebellar oedema. L-CPA mediated changes in excitatory amino acid concentrations and changes in glutamate receptor densities in the cerebellar cortex were prevented by NAME and BNI. NIO was ineffective in this model whilst NOARG was only partially effective. L-CPA treatment did not alter the activity of NOS in the cerebellum *ex vivo* or *in vitro*. Thus, L-CPA mediated neurotoxicity probably involves the synthesis of excessive amounts of nitric oxide leading to the formation of cytotoxic free radicals.

Widdowson, P.S. et al., (1995) *Toxicol. Appl. Pharmacol.* (in press).

65P STRETCH OF CULTURED BOVINE AORTIC ENDOTHELIAL CELLS INCREASES THE PRODUCTION OF NITRIC OXIDE AND SO REDUCES STIMULATED PLATELET ADHESION

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Cyclic stretch of cultured bovine aortic endothelial cells (BAEC) increases the activity of endothelial nitric oxide synthase (eNOS) and so the production of nitric oxide (NO) (Awolesi et al., 1994). As NO inhibits platelet function, we have examined the effects of stretch on the adhesion of platelets to BAECs.

Platelets were isolated from rat blood and labelled with [^{3}H]-adenine, as described previously (Sneddon and Vane, 1987). BAEC (up to passage 10) were seeded onto 6-well culture plates with flexible bottoms (Flex-1, Flexcell USA) and grown to confluence. The plates were then placed on a Flexcell strain unit and stretched by 20 % at a frequency of 0.5 Hz for 6 h. As a control, some plates were left unstretched. After 6 h, the medium was replaced with 1 ml of warm (37 °C) Locke's buffer containing 10 μM indomethacin and 10 U/ml of superoxide dismutase. Cells were then left static or stretching continued, while 100 μl of platelet suspension was added to the wells of the culture plates plus thrombin (0, 0.1, 0.3 or 1 U/ml). The incubation was then continued for a further 15 min. The plates were then gently washed (3x) with warm Locke's buffer before the addition of 2 ml of hot (70 °C) Triton-X 100. The extracted radioactivity associated with each well was then assessed by scintillation counting as a measure of platelet adhesion.

Thrombin caused a concentration-dependent increase in platelet adhesion to unstretched BAEC which was unaffected by 6 h stretch plus no stretch after platelet addition (Table 1). However, platelet adhesion was significantly reduced by continuing the stretch after platelet addition. This inhibition of platelet adhesion was prevented by addition of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 1 mM) to the BAEC prior to adding the platelets. Platelet adhesion was not inhibited by stretching of previously static BAEC (data not shown).

Thus, 6 h of stretch increases the ability of BAEC to produce NO, an effect which is only revealed by the further stimulation of the cells with stretch.

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Awolesi, M.A., Widmann, M.D., Sessa, B.C. *et al.* (1994) *Surgery* 116, 439-445.
Sneddon, J.M. and Vane, J.R. (1987) *Proc. Natl. Acad. Sci.* 85, 2800-2804.

Table 1. Data expressed as % increase in platelet adhesion above that to control plates (mean ± s.e.m). * $p < 0.05$; significantly different from control as assessed by unpaired t-test.

Treatment (n)	platelet adhesion (% above basal)			
	Thrombin (U/ml)			
	0	0.1	0.3	1
control (n=6)	0	31±9	47±7	70±8
6 h str. + 15 min unstr. (n=3)	13±2	44±6	37±1	74±18
6 h str. + 15 min str. (n=6)	3±9	16±4 *	13±5 *	21±7 *
6 h str. + 15 min str. + L-NAME (n=4)	7±11	25±8	41±5	47±12

66P POLYCLONAL ANTIBODIES AGAINST TNF-α AND IL-1β PREVENT THE CIRCULATORY FAILURE ELICITED BY LIPOTEICHOIC ACID IN ANAESTHETISED RATS

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In anaesthetised rats, lipoteichoic acid (LTA), from the cell wall of *Staphylococcus aureus* (a gram-positive organism without endotoxin), causes circulatory failure (hypotension and vascular hyporeactivity to noradrenaline) due to an enhanced formation of nitric oxide following the induction of nitric oxide synthase (iNOS; De Kimpe *et al*, 1995). Here, we investigate the role of TNFα and IL-1β in these effects.

Male Wistar rats (250-350g) were anaesthetised with thiopentobarbitone sodium (120mg/kg, ip). The carotid artery was cannulated for the measurement of blood pressure and the jugular vein for administration of compounds. Sheep polyclonal antibodies (PAb) against human TNFα (3mg/kg, iv) or a mixture of PAb against human TNFα and IL-1β (both 3mg/kg, iv) was administered 30 min prior to injection of LTA (10mg/kg, iv, time 0). At 90 min, a plasma sample was collected to measure TNFα using an ELISA. The pressor response to noradrenaline (1μg/kg, iv) was assessed prior to and every 60 min after the injection of LTA. At 360 min, rats were killed and lungs removed to determine iNOS activity via the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (De Kimpe *et al*, 1995).

Injection of LTA resulted in a sustained hypotension, an attenuated pressor response to noradrenaline and induction of iNOS activity (Table 1). These effects of LTA were substantially reduced by treatment of rats with the PAb mixture to TNFα and IL-1β, but not with PAb to TNFα alone. The increase in plasma TNFα level elicited by LTA was significantly inhibited by the treatment with PAb against TNFα, either when given alone or in combination with PAb to IL-1β.

Thus, inhibition of both TNFα and IL-1β by PAb is more effective in reducing the induction of iNOS and delayed circulatory failure elicited by LTA than inhibition of TNFα alone. We suggest that both TNFα and IL-1β are important mediators in the pathogenesis of Gram-positive sepsis.

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De Kimpe, S.J., Hunter, M.L., Bryant, C.E., Thiemermann, C. and Vane, J.R. (1995). *Br. J. Pharmacol.* 114, 1317-1323.

Table 1. Effect of PAb to TNFα and IL-1β on iNOS induction and circulatory failure elicited by LTA in the anaesthetised rat.

Treatment	TNFα (90min) (ng/ml)	Mean arterial pressure (360min) (mmHg)	Pressor response to noradrenaline (360min) (mmHg·min)	iNOS activity (360 min) (nmol/30min/g tissue)
sham control	0.2 ± 0.04	113 ± 3	48 ± 4	2.2 ± 1.0
LTA	3.5 ± 0.60#	78 ± 3#	23 ± 4#	12.7 ± 1.5#
+ PAb TNFα	0.4 ± 0.10*	87 ± 7	32 ± 5	8.9 ± 4.3
+ PAb TNFα + PAb IL-1β	1.0 ± 0.21*	100 ± 5*	48 ± 8*	5.6 ± 1.2*

Values are given as mean ± s.e.mean (n=4-6); # $P<0.05$ compared to sham control and * $P<0.05$ compared to LTA by ANOVA (Bonferroni's test).

67P THE EFFECT OF NITROVASODILATOR DRUGS ON THE cAMP RESPONSE TO ISOPRENALINE IN CULTURED SMOOTH MUSCLE CELLS

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Nitrovasodilator drugs are known to relax smooth muscle through elevation of cGMP levels. In this study we have examined how exposure to the three nitrovasodilator drugs glyceryl trinitrate (GTN), sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1) and the cell-permeable guanosine-3',5'-cyclic monophosphate (cGMP) analogue, 8-bromo-cGMP may influence the cAMP response to another vasodilator, isoprenaline in cultured smooth muscle cells.

A10 cells (passage 14-19) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum and 1.5mM glutamine and plated out onto 35mm multiwells. Confluent cells were washed and preincubated with Tris-HCl pH7.4 (50mM) - IBMX (0.3mM) for 20-30min at 37°C. Cells were then incubated for 30min at 37°C in Tris-IBMX containing either saline, 30nM or 300nM isoprenaline in the presence or absence of GTN (500μM), SNP (500μM), SIN-1 (500μM) or 8-bromo-cGMP (500μM). In chronic studies, confluent cells were treated with SNP (500μM) or GTN (500μM) for 18h and treated as described above. Parallel studies were conducted in each case with appropriate vehicle. Cells were broken with liquid N_2 and stored at -80°C for subsequent assay of cAMP based on the method of Brown *et al.* (1971). Protein was determined according to the method of Lowry. The statistical significance ($p \leq 0.05$) of the observed change in the levels of cAMP was assessed according to Student's t-test for paired data.

In control cells (n=33), 30nM and 300nM isoprenaline stimulated a 5-fold and 9-fold increase respectively over basal cAMP production of 26±7 pmol/mg protein. Exposure to each of the nitrovasodilator drugs appeared to have no effect on basal production of cAMP. Acute (n=5) or chronic (n=4) exposure to SNP had no effect on isoprenaline-stimulated cAMP levels. Similarly, GTN had no effect on isoprenaline-stimulated cAMP levels following acute exposure (n=6), however chronic exposure to GTN (n=5) significantly reduced the cAMP response to isoprenaline at 30nM and 300nM by 47±7% and 26±10% respectively. Whilst acute exposure to SIN-1 (n=4) significantly reduced the cAMP response to 30nM isoprenaline by 77± 12%, little or no inhibition of the effect at 300nM isoprenaline was observed in two of the four experiments resulting in a cAMP level not significantly different from untreated cells. The effect of chronic exposure to SIN-1 on cAMP levels remains to be determined.

The differential effect of SNP, GTN and SIN-1 on the cAMP response to isoprenaline in smooth muscle cells may be due at least in part to the amount of NO produced by these nitrovasodilator drugs. The order as determined by chemiluminescence was SIN-1 >> GTN > SNP. In addition, acute exposure to 8-bromo-cGMP significantly reduced the isoprenaline-stimulated cAMP response at 30nM and 300nM by 87±5% and 90±3% respectively, further suggesting a role for the NO/guanylate cyclase pathway.

These results suggest the nitrovasodilator drugs GTN and SIN-1 inhibit the cAMP response to isoprenaline in smooth muscle cells, possibly through NO increasing levels of cGMP. The mechanism(s) mediating this effect remain to be determined.

Our thanks to Cassella for the generous gift of SIN-1 and to the Wellcome Trust for financial support (035151/Z/92/MP/SRD).

Brown, B.L., Albano, J.D.M., Ekins, R.P. *et al.* (1971) *Biochem. J.*, **121**, 561-562.

68P INTERACTION BETWEEN SYMPATHETIC AND SENSORY NERVES IN RAT SMALL ARTERIES

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Activation of capsaicin sensitive primary afferent neurones (CSPANs) causes an inflammatory response characterised by vasodilation and oedema formation. It has been suggested CSPANs are located in small arteries and that released calcitonin gene-related peptide (CGRP) is responsible for arteriolar dilatation whereas released substance P (SP) increases venular permeability. However few experiments have examined the reactivity of arterioles directly to determine the mediators of the dilator response. We have now investigated the activity of CSPANs in small arteries of the rat mesentery microcirculation using the tension myograph.

Male Wistar rats (240-290g) were sacrificed by cervical dislocation. The mesentery was removed and placed in cold (4°C) Krebs solution. Small arteries (131-350 μm diameter) were dissected free and 1-2 mm lengths were cut and mounted in a myograph for the measurement of isometric tension in Krebs solution at 37°C bubbled with 5 % CO_2 in O_2. Vessels were left to equilibrate for 45 min prior to stretching to determine the relationship between the passive tension and internal circumference of each vessel using the Laplace equation. From this the internal diameter was determined (Mulvany & Halpern, 1977). These vessels were stretched to an internal circumference 90 % of that when under a transmural pressure of 100 mmHg.

Vessels were maximally contracted with the thromboxane A_2-mimetic, U-46619 (11α,9 α-epoxymethano-PGH_2) (1 μM) until the contraction was constant. Vessels were then subjected to electrical field stimulation of 16 Hz, 28 Vfor 5 s at 0.01 trains/s. The contractions produced by this stimulation were due to activation of sympathetic nerve fibres since guanethidine (5 μM, n=4) or tetrodotoxin (1 μM, n=4) completely abolished the response. Capsaicin (1 μM) inhibited the contractile response to EFS to give a response of 30.9 ± 7.3 % of the control EFS-induced contraction (n=6). In segments of the same vessels the response to capsaicin was significantly ($p < 0.01$) attenuated by the NO synthase inhibitor L-NMMA (100 μM); the response to capsaicin (1μM) in the presence of L-NMMA was 63.0 ± 7.6 % of control contraction to EFS (n=6). The inhibitory effect of capsaicin was also partially attenuated ($p < 0.05$) by CGRP receptor antagonist $CGRP_{8\text{-}37}$ (1μM) (the response to capsaicin in the presence of $CGRP_{8\text{-}37}$ was 78.5 ± 4.1% (n=5) of the control EFS contraction and in the absence was 57.5 ± 6.3% (n=5). CGRP (0.1-100nM) (n=4) caused a concentration related inhibition of the EFS contraction which was unaltered in the presence of L-NMMA (100μM) (n=4). SP (1 μM) had no effect on the EFS induced contraction (n=3).

In vessels sub-maximally contracted with U-46619 capsaicin (1μM) caused a relaxation ranging from 28-83 % of the maximum (n=10), additionally CGRP (0.01 -30 nM) (n=3) caused a concentration-related relaxation which was shifted to the right 3-fold by $CGRP_{8\text{-}37}$ (1 μM) (n=3) whilst SP (1 μM) had no effect (n=3). In vessels with no tone capsaicin (1 μM) had no contractile activity (n=4).

These results suggest that small arteries in the mesentery possess CSPANs capable of regulating their reactivity, stimulation of which leads to relaxation and modulation of sympathetic activity. The response is dependent on both CGRP and NO.

Mulvany, M.J. & Halpern, W. (1977). *Circ. Res.* **41**, 19-25.

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69P THE MULTIPLE ORGAN FAILURE SYNDROME (MOFS) CAUSED BY ENDOTOXIN IN THE RAT: ATTENUATION OF LIVER DYSFUNCTION BY INHIBITORS OF NITRIC OXIDE SYNTHASE

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Circulatory shock is characterized by severe hypotension, vascular hyporeactivity, maldistribution in organ blood flow, and reduced tissue oxygen extraction, which ultimately lead to multiple organ failure (MOF) and death (Altura et al., 1983). This study compares the effects of aminoethyl-isothiourea (AE-ITU), a relative selective inhibitor of inducible nitric oxide synthase (iNOS) activity, with those of N^G-methyl-L-arginine (L-NMMA), a non-selective inhibitor of NOS activity on the MOF caused by endotoxaemia in the anaesthetised rat.

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The carotid artery was cannulated for the measurement of mean arterial pressure (MAP) and the femoral vein for the administration of compounds. After stabilisation of hemodynamic parameters, at time 0, rats received saline (0.9% NaCl; 1 ml kg^{-1} i.v., n=6) or *E.coli* 0127:B8 lipopolysaccharide (LPS; 10 mg kg^{-1} i.v., n=21). The pressor response to noradrenaline (NA; 1 μg kg^{-1} i.v.) was assessed 10 min prior to and every hour after LPS injection. At 2 h, a continuous infusion of vehicle (0.6 ml kg^{-1} h^{-1} saline, i.v., n=15), AE-ITU (1 mg kg^{-1} h^{-1} i.v., n=10) or L-NMMA (3 mg kg^{-1} h^{-1} i.v., n=5) was started until the end of the experiment 6 h after the administration of LPS. At 6 h, plasma samples were taken and analysed for alanine aminotransferase (ALT) and bilirubin for liver function, and creatinine for renal function by a contract laboratory for veterinary, clinical chemistry (Vetlab Services, Sussex, UK). Nitrite in the plasma was measured by the Griess reaction.

In the anaesthetised rat, LPS caused hypotension, vascular hyporeactivity, renal and liver dysfunction ($p<0.05$, Table 1). Treatment of LPS-rats with L-NMMA caused a sustained rise in MAP. L-NMMA and AE-ITU enhanced the pressor response to noradrenaline and reduced plasma nitrite levels. AE-ITU or L-NMMA also inhibited the increase in bilirubin concentration elicited by LPS. Interestingly, only AE-ITU attenuated the increase in plasma ALT activity (Table 1).

AE-ITU or L-NMMA attenuate the delayed circulatory failure (hypotension, vascular hyporeactivity), and liver failure caused by endotoxin in the anaesthetised rat. Thus, an increased release of NO contributes to the liver dysfunction in endotoxaemia.

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Altura, B.M. *et al.* (1983). *Handbook of Shock and Trauma*, Vol. 1: *Basic Science*. New York: Raven Press.

Table 1.

Treatment	MAP (mmHg)	Hyporeactivity (% time 0)	ALT (iu l^{-1})	Bilirubin (μM)	Creatinine (μM)	Nitrite (μM)
Sham	117 ± 4*	115 ± 17*	64 ± 4*	2.6 ± 0.3*	31 ± 5*	2.1 ± 0.2*
LPS + saline	84 ± 4	65 ± 5	510 ± 94	6.2 ± 1.0	79 ± 6	8.9 ± 0.7
LPS + AE-ITU	94 ± 3	110 ± 12*	395 ± 78*	3.6 ± 0.6*	71 ± 9	6.0 ± 0.5*
LPS + L-NMMA	100 ± 6*	115 ± 21*	703 ± 208	3.8 ± 0.6*	62 ± 9	5.2 ± 0.4*

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

70P NITRIC OXIDE, RELEASED FROM ROUSSIN'S BLACK SALT, INCREASES THE RELEASE OF ACETYLCHOLINE FROM THE RAT STRIATUM *IN VITRO*

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We have previously demonstrated that nitric oxide (NO), released from the photosensitive NO-donor Roussin's Black Salt (RBS), increases spontaneous dopamine (DA) release from the rat striatum (Stewart *et al*, 1995; Black *et al*, 1994) in a Ca^{2+}-independent manner. In order to determine whether this release mechanism was specific to DA, we extended the study to examine the effect of NO on the release of acetylcholine from the rat striatum *in vitro*.

Cross chopped striatal slices (350 x 350μm) were prepared from male AHA Sprague Dawley rats (200-300g). Slices were incubated with 20 mM KCl for 15 min and washed prior to loading with 0.1μM [^{3}H]choline. The slices were than placed in a superfusion chamber maintained at 37°C and superfused (0.5ml min^{-1}) with a modified Krebs containing 10μM EDTA and 10μM hemicholinium-3. The experiment was performed under darkened conditions. RBS (30μM) was added to the superfusion medium for 20 min and allowed to wash off 30 min prior to the start of collection of superfusate. The brain slices were illuminated using a tungsten-halogen light source for a 4 min period. Release of tritium (>87% acetylcholine) in response to illumination was measured in 3 x 4min fractions over a 12 min period from the start of illumination. Data are expressed as fractional release i.e. % of total tritium remaining in the tissue. Results were analysed by one-way ANOVA.

Basal tritium efflux in untreated slices (8.79 ± 0.6%; n=4) was not significantly different from that in RBS-pretreated slices (8.57 ± 0.6%; n=4). Illumination of untreated slices had no significant effect on tritium release (9.10 ± 0.7%; n=4). However, in slices that were pretreated with RBS, illumination significantly increased the spontaneous efflux of tritium (18.05 ± 0.4%; n=4). The release peaked in the first 4 mins and subsided to basal levels within 12-16 min. Inclusion of oxyhaemoglobin (10μM) 20 min prior to and during illumination, did not affect basal tritium efflux but markedly decreased the stimulated release by 82 ± 1% (10.07 ± 0.8%; n=4). Superfusion with nominally calcium-free Krebs slightly elevated the basal efflux of tritium (10.96 ± 0.5%; n=4) and markedly decreased the light stimulated release (13.05 ± 1.1%; n=4). Chelation of intracellular calcium with sufficient BAPTA-AM (10μM) to prevent ionomycin (1μM) stimulated release, abolished the light-stimulated release (9.34 ± 0.7%; n=4). Superfusion with methylene blue (10μM) slightly reduced the stimulated release (15.84 ± 0.9%) without affecting the basal release (8.64 ± 0.8%; n=4). Inclusion of zaprinast (10μM) enhanced both the basal (10.62 ± 1.2%; n=4) and the stimulated release of tritium (22.88 ± 2.2%; n=4).

The present study demonstrates that illumination of RBS pretreated slices evokes an increase in tritium release from [^{3}H]choline prelabelled slices, presumed to reflect the release of acetylcholine. This release, like that of DA, was due to NO since it was inhibited by 82% by haemoglobin. However, unlike the release of DA (Stewart *et al*, 1995), the release of tritium from [^{3}H]choline prelabelled slices was calcium-dependent, being reduced markedly by removal of extracellular calcium, and completely inhibited by removal of both extra- and intra-cellular calcium. Furthermore, in contrast to studies on DA release, the tritium release was affected by agents known to modify cGMP levels. Methylene blue, an inhibitor of guanylate cyclase, reduced the response by 23%, and zaprinast, a cGMP phosphodiesterase inhibitor, enhanced the NO stimulated release. Our findings suggest that NO from RBS causes calcium-dependent acetylcholine release from striatal slices and that this release may involve activation of guanylate cyclase.

Black, M.D., *et al.* (1994) *Neuropharm.* **33**, 1357-1365.
Stewart, T.L., *et al.* (1995) *Br. J. Pharmacol*, 114, 113P.

71P AREA-SPECIFIC DISTRIBUTION OF $GABA_A$ RECEPTOR α1-SUBUNIT IN PRENATAL RAT AND MARMOSET MONKEY NEOCORTEX

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The $GABA_A$-receptor α1-subunit, one of the most abundant subunits in the adult mammalian cerebral cortex, has a delayed onset during ontogeny. In rat, it is largely absent from neonatal neocortex, being restricted to layers III-IV of primary somatosensory (S1) and visual (V1) areas (Fritschy *et al.*, 1994). These observations suggest that the maturation of the α1-subunit is influenced by the ingrowing thalamo-cortical projection. In this study, we have characterized further the factors regulating the early expression of the α1-subunit in neocortex. To this end, we have investigated immunohistochemically with a subunit-specific antibody at which age the α1-subunit first reveals areal boundaries in the rat neocortex. In addition, we have taken advantage of the prolonged gestation (about 142 days) of the marmoset monkey (*Callithrix jacchus*) to analyze the maturation of the α1-subunit at defined stages of cortical development.

Immunohistochemical staining of perfusion-fixed sections from foetal rat brain (see Paysan *et al.*, 1994, for a detailed procedure) revealed that the boundaries of S1 and V1 were first discernible at embryonic day (ED) 20 (i.e. 36-48h before birth) with the selective laminar distribution of the α1-subunit in the cortical plate. In S1 and V1 a uniform staining of the cortical plate was observed, whereas in adjacent areas α1-subunit staining was detected only in layer V neurons. Thus, the area-specific distribution of the α1 subunit reveals a parcellation of the rat neocortex into distinct areas at least 2-3 days before the ingrowth of thalamic afferents. This finding indicates that functional connections between thalamic axons and their target neurons in layer IV of S1 and V1 are not required for the onset of α1-subunit expression. Rather, factors intrinsic to the developing cortical anlage are likely to contribute to the emergence of cortical areas and to the maturation of $GABA_A$-receptors.

In the marmoset, cortical layers differentiate between ED 100-120, when the thalamo-cortical projection is established. At ED 120, the six layers are formed and the boundaries of V1 (area 17) are visible in Nissl-stained sections. However, the α1-subunit could not be detected immunohistochemically in the neocortex before ED 130 and was restricted initially to layers IV and VI of both V1 and S1. Thus, while the onset of α1-subunit expression exhibits the same area specificity as in the rat, it occurs at a very different stage of brain maturation. This suggests that the signals triggering α1-subunit expression are operative shortly before birth in both species, independently of the stage of brain development. Also, the delayed onset of the α1-subunit rules out the possibility that thalamic afferents, at the time they reach layer IV, provide a signal triggering the α1-subunit expression.

These results underscore the need for comparative studies to unravel the regulation of neurotransmitter receptor expression in developing brain. The different timing of expression of the $GABA_A$-receptor α1-subunit in the rat and marmoset does not imply that different mechanisms are operative in rodents and primates. Rather, they reflect the fact that cortical maturation takes place within very different time-frames in these animals. $GABA_A$-receptors are thus tailor-made to fulfil specific functional requirements which appear at different stages of ontogeny.

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72P ANTICONVULSANT EFFECT OF 7-NITROINDAZOLE IN DBA/2 MICE AND IN GENETICALLY EPILEPSY-PRONE RATS

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The role of nitric oxide in epilepsy is equivocal. We evaluated the preferred substrate, L-arginine (L-arg) versus D-arginine (D-arg), and the neuronal selective inhibitor, 7-nitroindazole (7-NI; Babbedge et al., 1993), of nitric oxide synthase as anticonvulsants in DBA/2 mice and in genetically epilepsy-prone (GEP) rats. These animals, after exposure to loud intermittent sound, exhibit wild running, clonic, and tonic seizures, serving as a model of reflex epilepsy (Faingold, 1988).
Seizures were induced with sound of 100-110 dB at 12-16 kHz for up to 60s. Locomotor deficit was tested in GEP rats with a rod (9 cm diameter with 1.5 mm deep grooves at 10° intervals) rotating at 12 r.p.m. for 60s. GEP rats (200-400 g) received compounds or vehicle (i.p.) and were tested repeatedly at times after administration detailed in table 1A. Results are shown as ED_{50} values in mg/kg for the dose of compound which protected 50% of the rats from clonic seizure (EPI) or induced ataxia (RR). Since inhibitors of vascular nitric oxide synthase increase blood pressure (B.P.), the effect of 7-NI (80 mg/kg i.p.) was studied on mean arterial B.P. in mm Hg and heart rate (H.R.) in b.p.m. from the femoral artery of conscious GEP rats for 4h (see table 1B).

In DBA/2 mice, ED_{50} values (95% confidence limits) (mg/kg i.p.) against clonic seizure for 7-NI were 72 (61-86) at 15 min and 125 (108-145) at 1h and for L-arg was 1954 (639-5977) at 15 min. The incidence of clonic seizure was reduced in DBA/2 mice pretreated (-15 min) with L-arg 300ug (1/10) but not D-arg (10/10) compared with vehicle i.c.v. 10/10 ($P<0.05$). After 1h, L-arg but not D-arg (50 mg/kg i.p.) increased seizure incidence in GEP rats given 7-NI (25 mg/kg i.p.) from 25/32 to 20/20 ($P<0.05$) (Fisher's Exact test).

Table 1A: Anticonvulsant and ataxia ED_{50} values (mg/kg)

	+15'	+30'	+1h	+2h	+4h	+8h
7-NI EPI	56	42	35	28	38	92
7-NI RR	31	31	40	41	135	NH
7-NI T.I.	0.6	0.7	1.1	1.5	3.6	--
L-ARG EPI	6300	2433	2836	2006	936	1524
D-ARG EPI	NH	NH	NH	2500	2075	2000

Table 1B: Cardiovascular measures in GEP rats

	0h	+15'	+30'	+1h	+2h	+4h
Veh B.P.	117±12	121±12	118±12	117±10	126±11	127±12
7-NI B.P.	118±2	122±2	121±3	122±1	126±1	130±3
Veh H.R.	408±12	408±13	408±18	411±14	402±22	404±18
7-NI H.R.	416±15	375±23	321±15*	289±9*	297±20*	297±19*

(T.I.: therapeutic index RR/EPI, NH: not high enough doses tested, --: not calculable, *: $P<0.001$ ANOVA)

7-NI (10-80 mg/kg i.p.) is anticonvulsant in two animal models of reflex epilepsy. 7-NI (80 mg/kg i.p.) has no effect on B.P. in the conscious GEP rat but induces sedation and ataxia. L-Arg has enantioselective anticonvulsant effects in DBA/2 mice or GEP rats after i.c.v. or i.p. administration. In GEP rats, low dose L-arg (50 mg/kg) enantioselectively reversed the anticonvulsant effect of low dose 7-NI (25 mg/kg).

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73P THE ACTION OF PENTOBARBITONE AT KAINATE, $GABA_A$ AND GLYCINE RECEPTORS EXPRESSED IN *XENOPUS* OOCYTES

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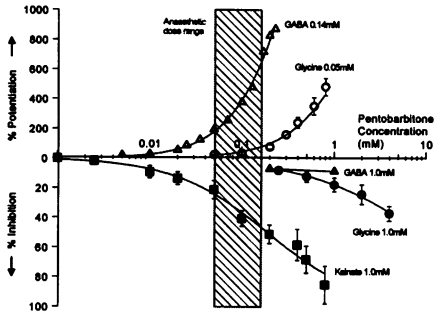
Potential molecular targets for general anaesthetics are the neurotransmitter gated ion channels. Many studies have implicated the inhibitory $GABA_A$ receptor in barbiturate anaesthesia and more recently effects on non-NMDA glutamate receptors and inhibitory glycine receptors have been demonstrated. We now report the effects of pentobarbitone on mammalian postsynaptic kainate-sensitive glutamate, $GABA_A$ and glycine receptors expressed in oocytes from *Xenopus laevis* following injection of mRNA.

Oocytes (>1mm diameter, stage 5 & 6), obtained from extra large female *Xenopus laevis* were maintained in modified Barths solution containing streptomycin and penicillin. The outer follicular layer was removed by treatment with collagenase and 50nl of mRNA was injected into each oocyte at a concentration of $1\mu g.mm^{-3}$. Injected oocytes were cultured at 18° C for up to three weeks. Poly(A^+) mRNA for kainate and $GABA_A$ receptors was extracted from whole brain and for glycine receptors from spinal cord of male PVG rats. In addition, homomeric human α1 receptors were expressed following injection of cRNA. cDNA encoding the human α1 glycine receptor subunit inserted in the Bluescript SK plasmid was cloned using Epicurian Coli® XL 1-Blue electroporation-competent cells and the plasmid linearised and used to generate capped mRNA transcripts *in vitro*. Oocytes were used two days after injection. They were perfused with frog ringers at room temperature (20-21°C) and the membrane potential was controlled by the two microelectrode voltage clamp technique. Recordings were made at a holding potential of -60mV.

Membrane currents were measured in the pressure of various concentrations of pentobarbitone in response to applied agonist concentrations of 1mM kainate, GABA and glycine (saturating concentrations) and of 0.14mM GABA and 0.05mM glycine. The



results are shown below:

At anaesthetic concentrations of pentobarbitone, the kainate receptor is inhibited by 50%, the glycine receptor is little affected and the effect on the $GABA_A$ receptor is highly dependent on the concentration. At a low GABA concentration there was 400% potentiation but at a saturating concentration there was no effect. It has been suggested recently that the physiological activation of synaptic receptors occurs at saturating concentrations of agonist (Clements *et al.* 1992). If this is the case then it would seem that pentobarbitone does not produce its anaesthetic action by an effect at the $GABA_A$ receptor.

We wish to thank Professor H. Betz for the α1 glycine cDNA.

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74P $GABA_B$ RECEPTORS MODULATE FAST EXCITATORY SYNAPTIC TRANSMISSION IN THE SUPRAOPTIC NUCLEUS *IN VITRO*

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The Supraoptic nucleus (SON) is a hypothalamic nucleus that contains both vasopressin and oxytocin releasing cells. SON cells have been shown to receive glutamatergic (Gribkoff and Dudek, 1990) and GABAergic (Jhamandas and Renaud, 1986) innervation. Although the intrinsic properties of these cells have been extensively investigated, relatively few studies have examined the role and interactions of these transmitters and their receptors on synaptic transmission. This study was therefore performed to examine the role of $GABA_B$ receptors on synaptic transmission *in vitro*.

Sprague-Dawley rats (25-40 days old) were anaesthetized with halothane and decapitated. 400 μm thick coronal slices were prepared and placed in a submersion chamber and perfused at 2-3 ml/min with oxygenated CSF at 27-29°C. Glass patch pipettes (5-10 MΩ) were filled with a solution containing (in mM) K-Acetate, 120; HEPES, 40; $MgCl_2$, 5; EGTA, 10; and nystatin (450 μg/ml in DMSO and pluronic acid). Whole cell nystatin patch recordings were made using an Axopatch 1D amplifier. 1-5 GΩ seals were formed and access (resistance 10-30 MΩ) was attained in 1-30 min after seal formation. Stimulus intensities of 5-50V, 100-500 μs duration were used to evoke synaptic responses through a bipolar electrode placed dorso-medial to the SON.

The results reported in the present study were obtained from stable recordings (45-150 min) made from 35 SON neurones [mean resting membrane potential of -84.6 ±2.1mV, SEM) and input resistances ranged from 350-1000 MΩ]. All cells were voltage clamped at -80 mV (V_h). Evoked, intensity-dependent inward currents (I_{in}) could be recorded from >80% of cells. The evoked I_{in} were mediated by both non-NMDA and $GABA_A$ receptors at this V_h as CNQX (10 μM), a non-NMDA receptor antagonist, decreased the response by 46.7 ±14.2% ($p< 0.05$; n=3;paired t-test) while picrotoxin (25 μM), a $GABA_A$ chloride channel blocker, reduced it by 25.4 ±7.3% ($p<0.05$; n=3). A combination of both antagonists reduced the I_{in} by 94.1 ±3% ($p<0.001$; n=3). To examine the pure glutamate-mediated I_{in}, picrotoxin (25μM) was present throughout the remaining experiments. The $GABA_B$ agonist baclofen, reversibly and dose-dependently (.03-20μM) reduced (10-95%) the isolated EPSC with an apparent IC_{50} of about 275nM. A presynaptic locus of action was indicated by: 1) no effect of baclofen (up to 10μM) on AMPA (5μM)-induced inward currents (100.5 ±18 pA, n=4 control and 84.3 ±10 pA, n=4 in the presence of baclofen; $p>0.2$); 2) a concentration-dependent enhancement by baclofen of paired pulse ratio (interstimulus interval of 50ms)[paired pulse facilitation of 1.6 ±0.12, n=8 in control and in the presence of baclofen 10μM, 4.0 ±.5, n=3; .3μM, 2.4 ± .6, n=3; .1μM, 2.9, n=1 and .03μM 1.8 n=1]. Further examination of the pharmacology of this effect showed that the potent $GABA_B$ receptor antagonist CGP36742 at 50 μM blocked the effect of baclofen on the EPSC. In control, baclofen (300nM) reduced the EPSC by 51.9 ±4.5%, n=12 and by -4.2 ±9.6.4%, n=4 in the presence of CGP36742 (50 μM; $p<0.001$). In addition, CGP36742 at this concentration caused an enhancement of the EPSC by 23.8 ±8.6%, n=8 ($p<.05$).

These results indicate that: 1. Stable, relatively long lasting recordings can be made from SON neurons *in vitro* using the nystatin patch technique. 2. Activation of presynaptic $GABA_B$ receptors reduce fast excitatory synaptic transmission. 3. These receptors may be tonically activated *in vitro*. Thus, $GABA_B$ receptors may influence the level of activity and excitation of SON neurones and modulate the function of this nucleus.

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75P INTRATHALAMIC PERTUSSIS TOXIN DECREASES THE DURATION OF SPIKE WAVE DISCHARGES AND $GABA_B$ RECEPTOR BINDING IN RATS WITH SPONTANEOUS GENERALISED NONCONVULSIVE EPILEPSY

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$GABA_B$ receptor mechanisms have been implicated in the generation of spike and wave discharges (SWD) in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) (Marescaux et al 1992). These receptors, unlike $GABA_A$ receptors are G-protein coupled (Hill et al 1984). In previous studies pertussis toxin was shown to decrease the duration of seizures in two pharmacological models of absence epilepsy (Snead 1992), suggesting that G-protein mediated mechanisms may be involved in the pathogenesis underlying the SWD of absence seizures. In the present study we have investigated the effects of pertussis toxin on the SWD recorded from GAERS rats and determined the effects of this treatment on autoradiographic $GABA_A$ and $GABA_B$ receptor binding.

Male GAERS rats (350-400g) were implanted with a guide cannula into the relay nuclei of the thalamus. Four stainless steel electrodes were placed bilaterally over the frontal and parietal cortices to record left and right EEG's for 40 min periods. 9 days after surgery pertussis toxin, denatured pertussis toxin (0.4µg/ 0.4µl/ side) or saline (0.4µl/ side) were injected bilaterally into the thalamus. EEG was recorded for up to 6 days after treatment. Rats were subsequently anaesthetized with sodium pentobarbital and perfused-fixed (intracardiac) with 0.1% paraformaldehyde in phosphate buffered saline. Binding to $GABA_A$ and $GABA_B$ receptors in thalamus, hippocampus and caudate putamen was determined autoradiographically by the method of Bowery et al (1987) using 50nM [^{3}H]-GABA.

Total SWD duration in the pertussis toxin treated animals decreased from 520 ± 63 s/ 40 min (n=7) before injection to 22 ± 12 s/ 40 min (n=6) 6 days after injection. This decrease was time dependent. There was no decrease in total SWD duration following treatment with denatured toxin or saline.

$GABA_B$ receptor binding density was significantly decreased (>60%) in the ventrolateral and ventral posterolateral thalamic nuclei but not other brain regions of pertussis toxin treated animals compared with control treated animals. By contrast, no significant difference in $GABA_A$ site binding was detected. These results support the involvment of G-protein coupled $GABA_B$ receptor mechanisms in the pathogenesis underlying the SWD of rats with spontaneous generalised nonconvulsive epilepsy.

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76P ANTIDROMIC ACTIVATION OF BURST-FIRING DORSAL RAPHE NEURONES BY STIMULATION OF THE MEDIAL FOREBRAIN BUNDLE

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Previous electrophysiological studies have demonstrated that in the rat dorsal raphe nucleus (DRN), 5-hydroxytryptamine (5-HT)-containing neurones fire broad action potentials in a slow and regular pattern and are inhibited by 5-HT_{1A} agonists (Aghajanian et al., 1978). In a recent electrophysiological study, we reported evidence of a sub-population of presumed 5-HT neurones which are located in the DRN and display characteristics of classical 5-HT neurones but in addition have some unusual properties (Hajós et al., 1995a). Specifically, these neurones during an otherwise regular firing pattern, fire spike doublets or triplets of spikes when only single spikes are expected. Spikes in this burst-like firing mode (spikes in doublets or triplets) show a very short interspike interval (typically < 10 ms), and occur with a diminishing spike amplitude. Furthermore, these neurones are inhibited by administration of direct and indirect acting 5-HT_{1A} agonists (Hajós et al., 1995a). Here we have investigated whether these atypical, presumed 5-HT neurones, like classical 5-HT neurones, project to the forebrain. To this end we have tested whether the neurones could be activated antidromically by stimulation of the medial forebrain bundle (MFB).

Extracellular recordings were performed from male Sprague-Dawley rats (260-280 g) anaesthetised with chloral hydrate or Saffan using glass microelectrodes and conventional electrophysiological methods (Hajós et al., 1995b). The signals were discriminated, computed on-line and stored on a DAT recorder for off-line analysis (Spike2). Antidromic stimulations were performed from the MFB, using concentric bipolar electrodes (0.1-3 mA, 0.05-0.1 ms duration). The position of the stimulating and recording electrode was marked and verified histologically.

Electrical stimulation of the MFB evoked antidromic spikes in both single spiking, classical 5-HT neurones (n=40) and in burst-firing 5-HT neurones (n=17). In both types of neurones antidromic spikes showed a biphasic positive-negative full spike or triphasic positive-negative-small positive full spike. Often the full spike had an inflection on the rising phase of the initial positive rise, which is characteristic of an initial segment-somatodendritic brake. Antidromic spikes followed a high frequency of stimulation (up to 300 Hz) and had a constant latency. Although there was some overlap in the antidromic spike latency of the two populations of neurones, burst-firing cells showed significantly shorter mean (± s.e.mean) latency (6.9 ± 0.32 ms) than solitary firing cells (9.41 ±0.54 ms; $p < 0.005$, unpaired t-test). Collision tests were also carried out, for two main reasons: (i) to validate the antidromic nature of the activation of the recorded neurone, and (ii) to analyse whether spikes fired in doublets progessed along the axon. For both classical 5-HT neurones and burst-firing neurones, antidromic spikes made collision with spontaneously occurring spikes. Furthermore, for burst-firing neurones collision occurred either with the first or second spike of the doublets. Interestingly, in a small number of burst-firing neurones (n=3), antidromic stimulation evoked spike doublets, similar to those recorded spontaneously.

In summary, the present study demonstrates that burst-firing neurones in the DRN can be activated antidromically by electrical stimulation of the MFB, indicating that these neurones project to the forebrain via the MFB, as has previously been observed for classical 5-HT neurones (Sawyer et al., 1985). Furthermore, the collision test demonstrate that each spike generated by the burst propagates along the axon and could thereby modify transmitter (5-HT?) release.

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77P EFFECTS OF ISOQUINOLINE DERIVATIVES ON [^{3}H]DOPAMINE UPTAKE BY STRIATAL SYNAPTOSOMES

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Isoquinoline derivatives structurally related to the selective nigral toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are possible endogenous neurotoxins causing Parkinson's disease. We and others have shown that these compounds exert 1-methyl-4-phenylpyridinium (MPP^+)-like activity as potent inhibitors of mitochondrial function (McNaught *et al.*, 1994; 1995a,b). However, the dopaminergic toxicity of MPP^+ depends on its high affinity for the dopamine re-uptake system. So we have examined the substrate affinity of isoquinoline derivatives for the dopamine re-uptake system by studying the ability of 14 neutral and quaternary compounds from 3 classes of isoquinoline derivatives (7 isoquinolines, 2 dihydroisoquinolines and 5 1,2,3,4-tetrahydroisoquinolines) to inhibit the uptake of [^{3}H]dopamine by rat striatal synaptosomes.

Striatal synaptosomes were prepared form male Wistar rats (200-250g) as previously described (Javitch *et al.*, 1985). The uptake of [^{3}H]dopamine were measured at 37 °C in 1.0 ml modified Krebs-Ringer phosphate buffer containing 1.7 mM ascorbic acid, 80 µM pargyline and 10-1000 nM [^{3}H]dopamine, as described elsewhere (Javitch *et al.*, 1985). To determine the effects of isoquinoline derivatives or MPP^+ on [^{3}H]dopamine uptake, the compounds up-to 100 µM were added to the incubation medium with 10 nM [^{3}H]dopamine and allowed to equilibrate before freshly prepared synaptosomes (0.9 mg striata; based on original wet weight) were added. Experiments were terminated after 10 min incubation by rapid filtration, and the radioactivity accumulated by synaptosomes measured by liquid scintillation spectrometry. Data are presented as mean ± SEM (n = 6) and results analysed statistically using Student's *t*-test.

In this study, the kinetics (K_m = 0.26 µM; V_{max} = 2.02 nmol/min/g) of [^{3}H]dopamine uptake was as previously determined (Javitch *et al.*, 1985). Ten isoquinoline derivatives and MPP^+ inhibited [^{3}H]dopamine uptake in a concentration-dependent manner (Fig.1), but none of the isoquinoline derivatives were as potent as MPP^+ (IC_{50} = 0.33 µM). Only N-methyl-1,2,3,4-tetrahydroisoquinoline (IC_{50} = 8.0 µM), N-methyl-6-methoxy-1,2-dihydroisoquinoline (IC_{50} = 10.0 µM), 7-methoxyisoquinoline (IC_{50} = 10.0 µM), N-methyl-salsolinol (IC_{50} = 37.0 µM) and 1,2,3,4-tetrahydroisoquinoline (IC_{50} = 50.0 µM) produced 50% inhibition of [^{3}H]dopamine uptake (Fig. 1). At 100 µM, the isoquinoline derivatives inhibited [^{3}H]dopamine uptake by 33-73% ($p < .05$). These results suggests that isoquinoline derivatives are moderate substrates for the dopamine re-uptake system.

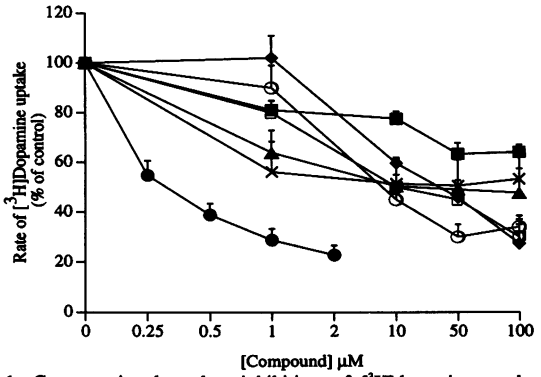

Fig. 1. Concentration-dependent inhibition of [^{3}H]dopamine uptake. ●, MPP^+; ○, N-methyl-1,2,3,4-tetrahydroisoquinoline; □, 7-methoxy-isoquinoline; ◆, N-methyl-salsolinol; ×, 1,2,3,4-tetrahydroisoquinoline; ▲, N-methyl-6-methoxy-1,2-dihydroisoquinoline; ■, isoquinoline.

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78P ALTERED STRIATAL PREPROENKEPHALIN mRNA LEVELS IN NORMAL MACAQUE MONKEYS (*MACACA FASCICULARIS*) WITH DYSKINESIAS INDUCED BY CHRONIC L-DOPA ADMINISTRATION

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L-DOPA-induced dyskinesias occur in 30-80% of patients with Parkinson's disease and are a major complication in the treatment of this neurodegenerative disorder (Nutt, 1990). It has been claimed that damage to the nigrostriatal system is necessary for the development of dyskinesias following L-DOPA administration and that the evolution of dyskinesias may involve an imbalance in the direct and indirect striatofugal pathways which occurs after dopaminergic denervation of the striatum (Crossman, 1987). We now report the occurrence of typical L-DOPA-induced dyskinesias in normal macaque monkeys receiving high dose chronic L-DOPA therapy and correlate the appearance and severity of dyskinesias with changes in neuropeptide mRNA levels in the striatum.

Eight normal macaque monkeys *(Macaca fascicularis)* 18-36 months old prior to dosing were treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) once daily for 13 weeks while a control group (n= 8) received placebo. The animals were observed by a clinical neurologist for the appearance of dyskinesias and dyskinetic movements were graded as mild, moderate or severe. At the end of the study, the animals were killed by barbiturate overdose and the brains sectioned coronally and frozen to -70°C. *In situ* hybridization study of brain sections for striatal pre-proenkephalin and pre-protachykinin and nigral tyrosine hydroxylase mRNAs was performed as *per* Young *et al.* (1986).

Four L-DOPA-treated monkeys developed severe dyskinetic movements upon L-DOPA administration, while two animals showed mild dyskinesias. No differences were found between normal monkeys, monkeys with no or mild dyskinesia and monkeys with severe dyskinesia with respect to striatal pre-protachykinin and nigral tyrosine hydroxylase mRNA, but pre-proenkephalin mRNA was significantly increased in the caudate and putamen of the severely dyskinetic monkeys (Figure 1).

These findings demonstrate that L-DOPA can cause dyskinesias in neurologically intact subhuman primates, and that altered striatal neuropeptide expression may be relevant to the expression of these abnormal movements.

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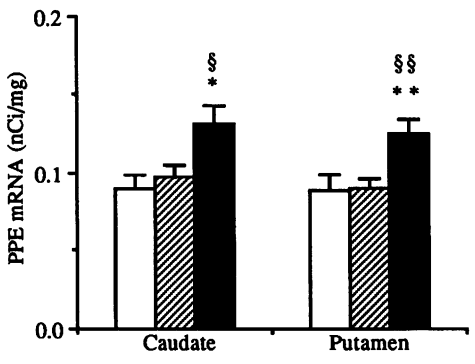
Figure 1. Pre-proenkephalin (PPE) mRNA (nCi/mg) in caudate and putamen of normal monkeys (n= 8; open bars) and in L-DOPA-treated monkeys displaying no or mild dyskinesia (n= 4; hatched bars) or severe dyskinesia (n= 4; filled bars). *P< 0.031 vs normal monkeys; §P< 0.048 vs animals displaying no or mild dyskinesia; **P< 0.0063 vs normal monkeys; §§P< 0.024 vs animals displaying no or mild dyskinesia.



79P EFFECTS OF RECEPTOR DENSITY ON PHOSPHOINOSITIDASE C ACTIVATION IN CHO-K1 CELLS EXPRESSING RECOMBINANT HUMAN m3 MUSCARINIC RECEPTORS

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It is well established that m3 muscarinic receptors couple, via the $G_{q/11}$ family of G-proteins, to the activation of phosphoinositidase C (PIC) leading to Ins(1,4,5)P_3 formation and intracellular Ca^{2+} mobilisation (Caulfield, 1993). The significance of receptor-G-protein stoichiometry in agonist-mediated inositol phosphate responses may be investigated by expressing recombinant proteins in a host cell line. By manipulating recombinant m3 muscarinic receptor expression levels in a Chinese hamster ovary (CHO-k1) cell line we have attempted to determine how changes in receptor density influence the receptor-effector coupling relationship.

Agonist-stimulated PIC activation was examined in CHO-k1 cells expressing three different densities of human m3 muscarinic receptors (CH0-m3 = 942 ± 62 fmol / mg protein CH0-vt9 = 436 ± 62 fmol / mg protein CH0-vt200 = 29 ± 4 fmol / mg protein: measured by [^{3}H]N-methyl scopolomine binding) and was determined by measuring [^{3}H]inositol phosphate ([^{3}H]-IP) accumulation, in the presence of Li^+, or by assaying the mass accumulation of Ins(1,4,5)P_3, previously described by Challiss *et al.* (1988).

Concentration response curves following a 600s exposure to methacholine or arecoline (a partial agonist) were performed in all three cell lines. CHO-vt9 cells produced a maximum response to methacholine which was similar (~10 fold higher than basal [^{3}H]-IP accumulation) to that produced by CHO-m3 cells. In CHO-m3 cells, arecoline induced a maximal response equal in size to, but less potent than that induced by methacholine (pEC_{50} = 5.73 ± 0.35 and 6.29 ± 0.12 for arecoline and methacholine respectively). In the CHO-vt9 cells, arecoline was unable to induce the same maximal response as methacholine. The CHO-vt9 cells responded to agonists in a less sensitive manner than the higher expressing CHO-m3 cells (pEC_{50} = 6.29 ± 0.12 and 5.60 ± 0.08 for methacholine stimulated [^{3}H]-IP accumulation in CHO-m3 and CHO-vt9 respectively). The response to methacholine in the vt200 cell line was much smaller than in the CHO-m3 and CHO-vt9 cell lines being only 1.6 ± 0.27 fold basal accumulation.

In all three cell lines methacholine and arecoline increased Ins(1,4,5)P_3 concentration with biphasic kinetics. The initial peak phase was maximal at 10s and was followed by a sustained plateau phase from 60s to 600s. The potency of methacholine to stimulate Ins(1,4,5)P_3 formation at 10s and 600s was investigated. The pEC_{50} for methacholine-stimulated Ins(1,4,5)P_3 production at 10s was 5.57 ± 0.05 and 5.23 ± 0.10 (for CH0-m3 and CH0-vt9 respectively).

Under conditions where Ca^{2+} was not added to the assay buffer the potency of methacholine, as a stimulus of [^{3}H]-IP formation at 600s, was dramatically reduced in all cell lines (P=0.0173 ANOVA). Methacholine could no longer induce maximal [^{3}H]-IP accumulation in CHO-vt9 cells and arecoline could not induce maximal [^{3}H]-IP accumulation in even the higher-expressing CHO-m3 cells. Decreased extracellular [Ca^{2+}] also significantly (P=0.021 ANOVA) reduced the pEC_{50} of methacholine at the plateau phase of Ins(1,4,5)P_3 production from 5.87 ± 0.06 and 5.44 ± 0.11 to 5.45 ± 0.21 and 5.17 ± 0.16 (for CHO-m3 and CHO-vt9 cells respectively). A modest reduction in basal and peak Ins(1,4,5)P_3 production was observed, however there was a dramatic reduction in plateau Ins(1,4,5)P_3 production in CH0-m3 and CH0-vt9 cell lines. Ins(1,4,5)P_3 formation at 10s appeared to be less sensitive to changes in extracellular [Ca^{2+}] than plateau phase (600s) Ins(1,4,5)P_3 formation.

Here we have demonstrated a receptor reserve for PIC activation via the m3 muscarinic receptor which is sensitive to extracellular [Ca^{2+}]. These data emphasise the importance of receptor expression density and receptor reserve and also the method used to quantitate phosphoinositide turnover in determining the characteristics of transmembrane phosphoinositidase C signalling.

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80P MUSCARINIC CHOLINOCEPTOR-STIMULATED INOSITOL 1,4,5,-TRISPHOSPHATE GENERATION AND PHASIC CONTRACTION ARE INHIBITED BY PHOSPHODIESTERASE III/IV INHIBITION IN AIRWAYS SMOOTH MUSCLE

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Previous studies have demonstrated that selective inhibition of type III phosphodiesterase (PDE III) activity does not increase cyclic AMP levels, inhibit agonist-stimulated phosphoinositide turnover or exert any significant anti-spasmogenic action in bovine tracheal smooth muscle (BTSM), however, in the presence of a PDE III inhibitor the actions of sub-maximally effective concentrations of PDE IV inhibitors are greatly enhanced (Adams *et al.*, 1993, 1994). We also noted that an inhibitor combination of ORG 9935 and rolipram (a PDE IV-selective inhibitor) could profoundly affect the phasic contraction of BTSM strips stimulated by histamine in the absence of extracellular Ca^{2+} (Adams *et al.*, 1993). Here we have investigated whether PDE III/IV inhibition in BTSM exerts effects at the level of inostiol 1,4,5-trisphosphate ($InsP_3$) generation stimulated by muscarinic cholinoceptor agonists.

BTSM slices were maintained in culture medium for 24 h in the absence or presence of [^{3}H]-inositol. Slices were washed (6 x 20 ml) into oxygenated Krebs-Henseleit buffer (KHB) before dispensing 75 µl aliquots into 400 µl KHB (± [^{3}H]-inositol ± 5 mM LiCl). Each sample was purged with O_2/CO_2 (95:5) and incubated at 37°C; additions of PDE inhibitors were made 30 min before additions of methacholine (MCh). Incubations were terminated by trichloroacetic acid addition and processed for [^{3}H]-InsP accumulation or $InsP_3$ mass assay. BTSM strips (approx. 10 x 2 x 2 mm) were incubated for assessment of phasic contraction as described previously (Adams *et al.*, 1993).

Under Ca^{2+}-free conditions, MCh (1 µM) induced a phasic contraction (T_{max} 13.7 ± 1.5 g) which rapidly declined to resting tension. Pre-addition of either ORG 9935 or rolipram did not significantly inhibit the subsequent response to MCh, however, ORG 9935 (1 µM) plus rolipram (0.1 µM) caused a 37% inhibition (T_{max} 8.6 ± 0.9 g; P<0.05) of the phasic contraction. MCh (100 µM) caused a rapid, transient 2.5-fold increase in $InsP_3$ (basal, 8.7 ± 1.9; +MCh (5 s), 22.1 ± 1.2 pmol/mg protein). Neither ORG 9935 (1 µM: 22.4 ± 1.7) nor rolipram (100 µM: 20.2 ± 1.6) had any effect on this initial increase in $InsP_3$ accumulation, however in combination a highly significant 50% inhibition was observed (+ORG/roli: basal, 7.7 ± 0.6; +MCh (5 s), 14.4 ± 0.8 pmol/mg protein; P<0.01). Qualitatively similar results were obtained in experiments where [^{3}H]-InsP accumulations were measured. The effect of PDE III/IV inhibition on the agonist-stimulated $InsP_3$ response was observed at all concentrations of MCh. Furthermore, in the presence of ORG 9935 (1 µM), rolipram concentration-dependently inhibited the MCh-stimulated $InsP_3$ response with a half-maximal inhibition being observed at 171 (95% confidence limits: 106-275) nM.

In contrast to the complete lack of effect β_2-adrenoceptor-agonists on muscarinic agonist-stimulated phosphoinositide turnover in BTSM, combined inhibition of type III and IV PDE activities can inhibit $InsP_3$ and [^{3}H]-InsP accumulations in response to MCh and inhibit the phasic component of contraction. Elevations of cyclic AMP caused by either a β_2-adrenoceptor or PDEIII/IV inhibition are similar in BTSM, therefore the ability of the latter manipulation to cause an inhibitory effect either relates to differences in the microenvironment within the cell where cyclic AMP is elevated or additional effects of PDE inhibition or the agents used to induce such inhibition.

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81P THE EFFECT OF REPEATED TREATMENT WITH PYRIDOSTIGMINE ON THE ACTIVITY OF ACETYLCHOLINESTERASE MOLECULAR FORMS IN MOUSE SKELETAL MUSCLE

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The reversible acetylcholinesterase (AChE) inhibitor pyridostigmine bromide is used in the treatment of myasthenia gravis. AChE exists in skeletal muscle as a number of molecular forms (Massoulie & Toutant, 1980), however there is little information on the effects of repeated administration of pyridostigmine bromide on the total activity of AChE or its molecular forms. The asymmetric (A12) form localised at the neuromuscular junction is probably the functional form which hydrolyses acetylcholine to terminate neuromuscular transmission.

In this work we studied the effect of repeated administration of pyridostigmine bromide on the activity of the major AChE molecular forms in the mouse diaphragm muscle. The drug (100μg/kg) was administered by subcutaneous injection, twice a day for three weeks, to adult male mice of the BKW strain. The mice were killed immediately after cessation of the treatment or one week or two weeks later. Untreated mice were used as controls. The diaphragm was carefully dissected out and snap frozen in liquid nitrogen. The muscles were then homogenised in high salt solubilizing buffer and the molecular forms were separated on a 5-20% sucrose density gradient. The AChE activity was determined using a radiometric assay (Johnson & Russel, 1975). Three major peaks of activity were seen and these corresponded to the globular monomer (G1), the globular tetramer (G4) and the functional asymmetric A12 form

The total AChE activity was significantly reduced to approximately 40% of the control value at the end of the treatment period ($P < 0.05$). This decrease in activity was not due to persistent inhibition by the drug and was therefore likely to be due to downregulation of the enzyme. However during the following week the activity recovered and moreover by one week after cessation of the treatment it had increased to a level approximately 45% higher than that of the untreated control ($P < 0.01$). By one week later the activity had declined again to within the normal range.

The activity of the individual molecular forms followed a similar pattern but the most dramatic changes were seen in the activity of the A12 form which was reduced to less than 20% of the control value at the end of the treatment period ($P < 0.01$), and had increased to 70 % above the control value ($P < 0.05$) by one week later and then decreased again.

The results are consistent with the drug acting both to inhibit the activity of the A12 molecular form and to promote its down regulation. Furthermore the findings indicate the operation of a feedback mechanism whereby the synthesis of the enzyme is controlled by the concentration of acetylcholine in the cleft at the neuromuscular junction; the high concentration resulting from AChE inhibition causing upregulation of the AChE gene and new synthesis of the G1 monomeric precursor.

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82P CHARACTERISATION OF A CLONED HUMAN 5-HT$_3$ RECEPTOR SUBUNIT STABLY EXPRESSED IN HEK 293 CELLS

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The 5-HT$_3$ receptor is a ligand-gated cation selective ion channel known to exhibit variable pharmacological properties that arise largely from inter-species heterogeneity (Peters *et al.*, 1992). A cloned murine 5-HT$_3$ receptor subunit (5-HT$_3$R-A), which functions as a homo-oligomeric complex upon heterologous expression, demonstrates the distinctive pharmacological profile of the receptor endogenous to mouse tissues (Maricq *et al.*, 1991). Thus, the cloning and characterization of species homologues of the 5-HT$_3$R-A should assist in pinpointing the structural determinants of inter-species heterogeneity within this receptor class. Here, the properties of a human 5-HT$_3$R-A subunit stably expressed in HEK 293 cells are described.

A cDNA encoding a 5-HT$_3$ receptor subunit isolated from a human amygdala cDNA library was cloned into a eukaryotic expression vector (CNOD) and introduced into HEK 293 cells by lipid-mediated transfection. Stable transformants were isolated from single geneticin resistant colonies and preliminary expression of the cloned human 5-HT$_3$ receptor subunit was evaluated by northern blot analysis of total RNA from individual cell lines. Hybridization was performed using an α^{32}P labelled human 5-HT$_3$R-A cDNA probe. The post-hybridization washes were performed twice in 0.1 x SSC, 0.1% SDS at 65°C for 15 minutes. The cell line expressing the highest level of 5-HT$_3$ receptor mRNA was studied further.

Preliminary radioligand binding assays utilising the 5-HT$_3$ receptor antagonist [^{3}H] granisetron were performed upon membrane homogenates of transfected HEK 293 cells essentially as described by Bufton *et al.* (1993). [^{3}H] granisetron bound saturably (B_{max} = 542 fmol/mg prot; n = 2) and with high affinity (K_d = 1.4 nM; n = 2) to a single population of binding sites (non-specific binding defined in the presence of 10 μM ondansetron). The order of potency of selective and non-selective 5-HT$_3$ receptor antagonists in competing for [^{3}H] granisetron (1 nM) binding (IC_{50} values in parenthesis) was: granisetron (2.5 ± 0.1 nM; n = 3) > ondansetron (9.0 ± 0.4 nM; n = 3) > metoclopramide (650 ± 10 nM, n = 3) > cocaine (7.2 ± 0.5 μM, n = 3) > (+) tubocurarine (28.0 ± 2.0 μM, n = 6). All antagonists competed for binding with Hill coefficients close to unity (range 1.06 - 1.25). The function of the expressed receptor, maintained over 24 passages, was demonstrated in whole-cell voltage-clamp recordings upon the transfected cells. Rapidly superfused 5-HT (0.3 - 30 μM) elicited a concentration-dependent inward current response (EC_{50} = 3.7 μM; Hill coefficient 1.8; n = 7) that desensitized in the continued presence of the agonist. The selective 5-HT$_3$ receptor agonists 2-methyl-5-HT (10μM, n = 5), phenylbiguanide (80μM, n = 5) and *m*-chlorophenylbiguanide (1μM, n = 5) mimicked the effects of 5-HT. Inward currents to locally applied 5-HT (10μM) were reversibly suppressed (IC_{50} values in parenthesis) by the 5-HT$_3$ receptor antagonists ondansetron (103 ± 11 pM, n = 6) and the non-selective agents metoclopramide (69 ± 5 nM, n = 5), cocaine (456 ± 9 nM, n = 5) and (+) tubocurarine (2.8 ± 0.1 μM, n = 6). Responses were unaffected by methysergide or ketanserin (1 μM; n = 3 each). Responses to 5-HT (10 μM) reversed in sign ($E_{5\text{-}HT}$) at a potential of -1.0 ± 0.4 mV (n=9). Partial replacement of extracellular and intracellular monovalent metals by poorly permeant organic monovalents produced hyperpolarizing and depolarizing shifts in $E_{5\text{-}HT}$ respectively, consistent with the known cation selectivity of 5-HT$_3$ receptors (Peters *et al.*, 1992).

The difficulty in obtaining human tissues amenable to detailed analysis of 5-HT$_3$ receptor function and pronounced species differences in pharmacology, highlight the importance of the approach adopted here.

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