1P CYTOKINES INHIBIT AIRWAY SMOOTH MUSCLE DNA SYNTHESIS BY GLUCOCORTICOID-SENSITIVE INDUCTION OF CYCLO-OXYGENASE-2

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Asthma is characterised by the presence of chronic airways inflammation and airway wall thickening which are implicated in airways hyperresponsiveness. Airway wall thickening is in part due to airway smooth muscle (ASM) hyperplasia. The glucocorticoid, dexamethasone inhibits human ASM proliferation induced by a range of stimuli (Stewart et al., 1995a). We have also shown that the cytokine tumour necrosis factor α (TNFα) has biphasic effects on ASM DNA synthesis (Stewart et al., 1995b). Since ASM *in vivo* is likely to be chronically exposed to pro-inflammatory cytokines, we have now evaluated the effects of a combination of the pro-inflammatory cytokines interleukin-1α (IL-1α) and TNFα on the inhibitory effects of dexamethasone on DNA synthesis.

Human cultured ASM was obtained by enzymatic digestion of bronchi obtained from lung resection specimens. ASM was subcultured into 24 well plates, allowed to grow to monolayer confluency and serum-deprived for 24 h to produce growth arrest. The ASM was then treated with IL-1α and TNFα for 24 h before the addition of basic fibroblast growth factor (bFGF). Dexamethasone was added 1 h before the addition of bFGF. Indomethacin or the selective cyclo-oxygenase-2 (COX-2) inhibitor L-745, 337 was added 30 to 60 min before the cytokine combination. [^{3}H]-Thymidine was added 24 - 28 h after the bFGF to measure incorporation of radiolabel into newly synthesised DNA. Levels of prostaglandin E_2 (PGE_2) were measured by radioimmunoassay. COX-2 protein levels were determined by Western Blotting.

The bFGF (300 pM)-induced increase in DNA synthesis (17.5 ± 5.5 fold, n = 15) was inhibited concentration-dependently by dexamethasone (1 - 1000 nM) with a maximum inhibition of 33 ± 6 % (n = 15). The combination of IL-1α (0.1 pM) and TNFα (30 pM) increased DNA synthesis by 3.8 ± 0.9 fold (n = 15), but decreased DNA synthesis in response to bFGF. Paradoxically, dexamethasone caused a concentration-dependent increase in DNA synthesis in response to bFGF plus the cytokine mixture. Moreover, indomethacin (3 μM) or L-745,337 (0.3 μM) enhanced DNA synthesis in response to the cytokine mixture or bFGF plus cytokine mixture, but not to bFGF alone. Neither unstimulated nor bFGF-stimulated ASM produced detectable levels of PGE_2 (levels of detection 0.05 nM). The cytokine mixture elicited readily detectable levels of PGE_2 (7.73 ± 2.72 nM, n = 3) which were further increased by bFGF (123.11 ± 38.89 nM, n = 3). Dexamethasone (0.1 μM), indomethacin (3 μM) or L-745,337 (0.3 μM) reduced PGE_2 levels of cytokine mixture-treated ASM to below the limits of detection. The cytokine mixture-induced and bFGF plus cytokine mixture-induced expression of COX-2 was attenuated by dexamethasone (0.1 μM).

These results suggest that cytokine-induced prostanoid production via COX-2 inhibits DNA synthesis. Inhibition of prostanoid production in the asthmatic airway by glucocorticoids may limit their potential beneficial effects on airway wall remodelling.

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2P A FUNCTIONAL ROLE OF ENDOGENOUS MCP-1 IN IL-1β PERITONITIS

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Chemokines selectively recruit leucocytes during inflammation and the chemokine monocyte chemoattractant protein-1 (MCP-1) is a specific chemoattractant for monocytes *in vivo* (Ajuebor *et al.*, 1998). In this study, we have investigated the interrelationship between MCP-1 and the proinflammatory cytokine interleukin-1β in a murine peritonitis model, assessing in particular, the ability of IL-1β to induce MCP-1 gene expression and protein release in the mouse peritoneal cavity.

Male Swiss Albino mice (28-32 g) were treated i.p. with 10 ng murine recombinant IL-1β (mrIL-1β) in 250 μl of sterile saline. At designated time-points, animals were euthanised by CO_2 exposure and peritoneal cavities washed with 3 ml of phosphate-buffered saline (PBS). Aliquots of the lavage fluids were then stained with Turk's solution and differential counting performed using a Neubauer hemocytometer and a light microscope. Lavage fluids were then centrifuged at 400 *g* for 10 min, the supernatants collected and endogenous MCP-1 measured by ELISA (Biosource, USA), whereas pellets were used for detecting MCP-1 mRNA by polymerase chain reaction (PCR) (Ajuebor *et al.*, 1998). In other experiments, IL-1β was co-injected i.p. with IL-1 receptor antagonist (1-10 μg). The role of the transcription factor NF-kB was investigated using calpain inhibitor I (Calbiochem, Nottingham, UK). Mice were treated i.p. with different doses of calpain inhibitor I (5-15 mg/kg) or with dexamethasone (DEX; 30 μg s.c.) 2 h prior to IL-1β. In a separate set of experiments, mice received 100 μg i.p. of specific goat anti-murine MCP-1 IgG, or the same dose of control goat IgG together with 10 ng IL-1β. In all cases peritoneal cavities were washed 2 or 4 h later as described above. Polymorphonuclear cell (PMN) influx is reported as 10^6 cells per mouse. Data are shown as mean ± s.e.mean, and statistical differences were assessed by ANOVA.

I.p. injection of mrIL-1β produced a time-dependent PMN influx into the cavity, maximal at 4 h (1.42 ± 0.15 x 10^6 cells per mouse, n= 6). Endogenous MCP-1 was detected in the lavage fluids and peaked at 2 h post-injection (0.27 ± 0.1, 1.50 ± 0.19, 3.63 ± 0.34 and 0.45 ± 0.13 ng MCP-1 per mouse at 0.5, 1, 2 and 4 h post-IL-1β, respectively; n= 5-8), with values being below detection at later time-points. This was mirrored by the expression of MCP-1 mRNA as assessed by PCR. Treatment of mice with the IL-1 receptor antagonist (10 μg) attenuated (> 80%) IL-1β-mediated MCP-1 release at 2 h (from 3.98 ± 0.32 to 0.27 ±0.16 ng MCP-1 per mouse, n= 4-6). Calpain inhibitor I (15 mg/kg) significantly reduced the amounts of MCP-1 recovered in the lavage fluids (1.38 ± 0.13 ng MCP-1 per mouse, n= 7-9, $P < 0.05$), and a 40% reduction was also obtained with 30 μg DEX (n=10, $P < 0.05$). In both cases a potent reduction in MCP-1 mRNA was also seen. Finally, we sought for a functional role of IL-1β-induced MCP-1 release. Co-injection of a specific polyclonal antibody against MCP-1 reduced PMN accumulation in response to mrIL-1β by more than 50% (n= 6, $P < 0.05$ vs. control IgG).

In conclusion, mrIL-1β induces MCP-1 gene expression and protein release in a receptor-mediated fashion, leads to PMN accumulation and this may involve the activation of the transcription factor NF-kB. The entire process is sensitive to the glucocorticoid hormone DEX.

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3P EFFECTS OF NITRIC OXIDE ON STEM CELL FACTOR-INDUCED MAST CELL ACTIVATION AND SURFACE EXPRESSION OF C-KIT

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Nitric oxide has been shown to inhibit the IgE-dependent activation of rodent tissue mast cells (Eastmond *et al.*, 1997). Mast cells can be activated independently of IgE, for example by the cytokine stem cell factor (SCF) (Taylor *et al.*, 1995). We investigated whether nitric oxide might inhibit SCF-induced mast cell degranulation, and whether this might be via control of cell surface expression of the SCF receptor, c-kit.

Cells were obtained by peritoneal lavage of adult Wistar rats (250-300 g), and cultured (10^6 cells/ml) in DMEM medium supplemented with 5% foetal calf serum for 48 h at 37°C in humidified 5% CO_2 in air, in the presence or absence of the nitric oxide synthase inhibitor *N*-monomethyl-L-arginine (L-NMMA). At the termination of cultures, supernatant medium was removed for measurement of nitrite by the Griess assay, or the cells were challenged with SCF (100 ng/ml) for 30 min at 37°C for measurement of mast cell 5-hydroxytryptamine (5-HT) release (Eastmond *et al.*, 1997). In further experiments, at the end of the cultures, mast cells were purified to >98% by density gradient fractionation through 65% isotonic Percoll (Pharmacia) and c-kit expression was measured by flow cytometry (Becton-Dickinson FACScan) using biotinylated rat SCF, followed by streptavidin-phycoerythrin.

As shown in Table 1, culture of peritoneal cells with 5-500 μM L-NMMA for 48 h led to a concentration-dependent increase in SCF-induced 5-HT release (expressed as % of cell total after subtracting spontaneous release) but did not influence mast cell surface expression of c-kit. L-NMMA at 500 μM reduced nitrite production from control values of 70.02±7.5 to 0.8±1.2 nmol of nitrite/10^6 cells.

Table 1. Effects of L-NMMA on SCF-induced net % 5-HT release and c-kit expression (median fluorescence)

	5-HT release (%)	median fluorescence
No L-NMMA (control)	3.6±0.3	4.2±0.2
L-NMMA, 5 μM	8.8±3.1 *	3.9±0.4
L-NMMA, 50 μM	19.5±1.1 *	4.4±0.4
L-NMMA, 100 μM	16.0±0.1 *	5.4±0.0
L-NMMA, 500 μM	24.1±2.1 *	4.9±0.1

Results are means ± s.e.m. for 4 separate experiments.
*$P < 0.0001$ compared to control values by one-way ANOVA with Bonferroni correction.

In conclusion, these results show that rat peritoneal cells spontaneously synthesise nitric oxide; nitric oxide inhibits SCF-induced mast cell degranulation but not c-kit expression, presumably by targeting post-receptor signalling events.

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Oxide Synthase inhibition.

4P REACTIVE OXYGEN SPECIES GENERATION BY NON-IMMUNOLOGICAL MAST CELL ACTIVATION: MODULATION BY NITRIC OXIDE SYNTHASE INHIBITION

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Reactive oxygen species (ROS) have been shown to enhance mast cell histamine (H) release (Wolfreys & Oliveira, 1997), while nitric oxide (NO) has an inhibitory effect (Mannaioni *et al.*, 1991). In the present study we have investigated non-immunological release of H and intracellular generation of ROS by rat peritoneal mast cells (RPMC). We have also investigated the effect of nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) on H and ROS release.

Mast cells were harvested from male Wistar rats (200-400g) by peritoneal lavage with HEPES-buffered Locke's (HBL) solution and purified by centrifugation (450g, 4°C, 5 min), over a 38% (w/v) bovine serum albumin gradient. Purified mast cells (85 - 90% purity; 10^6 cells ml^{-1}) were used in all experiments. Where H release was measured mast cells were incubated with L-NAME (100 μM), or vehicle, in HBL at 37°C for 30 min prior to activation (15 min, 37°C) with Substance P (SP), Nerve Growth Factor (NGF), or Compound 48/80 (48/80). Release of H was determined fluorometrically (Anton & Sayre, 1969) and expressed as net percentage of total cellular H. In experiments where intracellular ROS were measured, mast cells were resuspended in phosphate buffered saline (0.01 M) containing Ca^{2+} (1 mM) and glucose (5 mM), and incubated with L-NAME, or vehicle, for a total of 30 min at 37°C. After 15 min, dichlorofluoroscein diacetate (DCF-DA; 1.25μM final concentration) in 0.1 % methanol was added to the mast cell suspensions. Following incubation, mast cells were washed (200g, 4°C, 5 mins) and resuspended in phosphate buffered saline, prior to the addition of test compound. ROS were measured as the change in DCF fluorescence from basal levels following activation, and expressed as the percentage change in fluorescence. In each experiment determinations were performed in duplicate. Data are expressed as the mean ± s.e. mean of the values from the number of experiments shown.

48/80 (0.1-100 μg ml^{-1}; n=5), SP (0.05-50 μM; n=6) and NGF (1pg-10 μg ml^{-1}; n=5) caused a significant ($p<0.05$, Student's t-test) concentration-related release of H with $EC_{(1/2max)}$ values of 251 ± 3 ng ml^{-1}, 25 ± 1 μM and 708 ± 7 ng ml^{-1} respectively. The release of H at the basal level, and in response to 48/80, NGF, and SP was increased in the presence of L-NAME. 48/80 (n=4), SP (n=6), and NGF (n=4) also caused concentration-related generation of ROS with maxima of 404 ± 170 % at 100 μg ml^{-1}, 150.6 ± 31% at 50 μM, and 115 ± 14.5% at 0.1 μg ml^{-1} respectively, with $EC_{(1/2\ max)}$ values of 3.93±0.5 μg ml^{-1}, 731 ± 2nM and 0.185 ± 0.005 ng ml^{-1}. Threshold concentrations of NGF (1pg ml^{-1}) and SP (50nM) for ROS generation were less than those for H release (0.1μg ml^{-1} and 5μM). In the presence of L-NAME (n=4), 48/80, SP, and NGF induced ROS generation was markedly increased (577 ± 38% at 100 μg ml^{-1}, 335 ± 49% at 50 μM, and 425 ± 50% at 0.1 μg ml^{-1} respectively).

Our data show that RPMC release H and intracellular ROS in response to non-immunological stimuli in a concentration dependent manner. These effects are enhanced by L-NAME suggesting that endogenous NO inhibits mediator release. Furthermore, NGF and SP promote ROS generation from mast cells at concentrations which do not cause significant H release. Since these concentrations of substance P have been shown to exert other physiological effects (Maggi *et al.*, 1994), we believe that NGF and SP induced ROS generation by mast cells may be relevant to neurogenic inflammation.

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5P LERISETRON Pk-Pd *IN VIVO*: EXPERIMENTAL RESULTS AND COMPARISON WITH A PHARMACOKINETIC (UNBOUND CONCENTRATION) PHARMACODYNAMIC THEORY

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The pharmacologic effect is related to unbound (Cu) rather than to total concentrations (C). Therefore, the pharmacodynamic (PD) parameters (i.e. EC_{50}) related to the total C cannot predict directly effect-time *in vivo*. Especially so when the unbound fraction is altered. The effect- C parameters of lerisetron (L) (a novel 5-HT_3 antagonist) from two experimental situations, with the common factor of an increase in protein binding, are compared to those from a pharmacokinetic (PK) theoretical model of the Cu (Proost *et al.*, 1996), and is linked to a semi-empirical effect equation based on the mechanism of action (Kenakin, 1993).

The two laboratory setups are: a) Inflammation to Sprague- Dawley female rats (280-300g) caused by turpentine oil (0.5 ml, sc., 48 hours before): TI group (n=10). Control of same species: C_{TI} (n=10). b) Fischer-344 female rats (140-170g) with mammary adenocarcinoma: MA (n=14); Tumor pieces (2x3 mm) of adenocarcinoma line 13762, were implanted sc. by surgical incision three weeks earlier. Control: C_{MA} (n=19). All rats were anaesthetized with urethane (1.2 g Kg^{-1}, i.p.) *PK experiments*: An i.v. bolus (50µg Kg^{-1}) of L was administered and samples withdrawn (0-180 min). Unbound fraction in plasma (fu) was obtained by ultrafiltration and levels of drug by HPLC. Compartmental models were used to describe C vs time profiles. The PK parameters obtained, initialized the model which predicted the drug effect evolution and magnitudes. Receptor binding data were provided by FAES. *PD experiments*: An i.v. bolus (5µg Kg^{-1}) of L was administered. The effect measured (0-180 min) was the inhibition of the Bezold- Jarisch reflex (bradycardia), after i.v. injection of serotonin (16µg Kg^{-1}). The inhibitory Emax relation was used to describe E (%) with measured C levels. Experimental results are summarized in Tables 1 and 2. In both the TI and MA groups the fu was diminished compared to the controls. Distribution volume (Vdss) was reduced, but total clearance (CL) was very diminished only in the MA group. EC_{50} was increased but compensated with the unbound fraction. The values of ECu_{50} in the effect compartment estimated by the model are close to the obtained empirically and so is the prediction of the evolution of the effect (Figure 1). Conclusions: A model based on Cu permits good predictions of the effect, although the drug shows distinct PK behavior under different pathological situations.

Table 1: PK parameters of L (50 µg Kg^{-1})

Animals	CTI	TI	CMA	MA
Number	5	5	13	5
Vdss (l)	0.73±0.12	0.46±0.10**	0.76±0.12	0.21±0.04*
Cl (l min^{-1})	22±4.4x10^{-3}	24±5x10^{-3}	21±4x10^{-3}	63±2x10^{-4}*
fu (%)	14.40±1.40	6.60±1.23*	16.0±0.9	12.0±0.5*

Table 2: PD parameters of L (5 µg Kg^{-1} i.v.)

Animals	CTI	TI	CMA	MA
Number	5	5	6	9
EC_{50}(ng ml^{-1})a	0.38±0.03	1.28±0.07*	0.37±0.05	1.08±0.02*
ECu_{50} [b]	55±6x10^{-3}	84 ±7x10^{-3}	59±8x10^{-3}	130±2.4x10^{-3}*
nc	1.96±0.45	2.4±0.41	9.9±0.64	7.7±0.63

a: Serum concentration producing half of Emax (100%) b: fu x EC_{50}; c: Slope. Values are mean ± s.e.m. The Student's-test was used for comparison of the means. * $p<0.05$; ** $p<0.02$

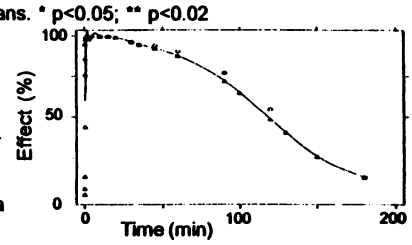

Figure. 1. Example in a rat: Effect (bradycardia reduction) vs time of L in cancer. Model simulation (line-Δ) and experimental data (o).

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6P EVIDENCE THAT POTENTIATION BY CORTICOSTERONE OF THE RESPONSE OF DOPAMINE-SENSITIVE NEURONES IN THE VENTRAL TEGMENTAL AREA TO NMDA IS MEDIATED BY GLUCOCORTICOID (TYPE II) RECEPTORS

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Glucocorticoid hormones and the ventral tegmental area (VTA) are both involved in drug dependence. We have reported (Cho and Little, 1997) that corticosterone (the major glucocorticoid hormone in rats), at 100 nM and above, potentiated the responses of VTA neurones to NMDA (N-methyl-D-aspartate). We now investigate the receptors involved in this effect using aldosterone (an agonist at mineralocorticoid, Type I, receptors) and RU38486 (an antagonist at glucocorticoid, Type II, receptors; Gagne et al., 1985).

Male hooded Lister rats (200-225g) were killed by cervical dislocation and midbrain slices containing the VTA were prepared. Recordings were made from slices perfused with aCSF at 2.3 ml/min, using the interface method. Single unit recordings were made from dopamine sensitive-cells with firing frequencies less than 5 Hz and action potential duration over 2 ms. NMDA (5 µM) was added to the perfusion medium for 5 min, at 15 min intervals; recordings were obtained during the last 1 min of NMDA application. After baseline measurements, corticosterone (500 nM), or corticosterone (500 nM) plus RU38486 (500 nM), or aldosterone (1 nM to 1 µM), or normal aCSF were added and addition of NMDA (5 µM) repeated after 15 min. Higher concentrations of aldosterone were studied on separate slices from lower concentrations. The hormones were dissolved in DMSO, final concentration 0.001%. N values were 4-6 per treatment group (one slice per rat per treatment, one neurone per slice). Student's t-test was used to compare firing rates in parallel recordings.

The mean firing frequency (Table 1) was significantly higher, after addition of NMDA, in slices perfused with corticosterone 500 nM, than in control slices, as reported previously. No significant change in NMDA responses was seen when corticosterone was added with RU39486. Addition of RU39486 alone had no effect on the firing rate. Aldosterone significantly decreased NMDA responses at concentrations of 100 nM and above. None of the treatments altered the firing rates in the absence of NMDA, or affected the inhibitory action of dopamine on the cells.

The results suggest that the increased response to NMDA produced by corticosterone was due to effects of this hormone at glucocorticoid, Type II, receptors. Overton et al (1996), demonstrated potentiation by corticosterone of the effects of glutamate on burst firing in dopamine-sensitive cells in the VTA. On the basis of the effective concentrations, these authors concluded that their effect was mediated by mineralocorticoid, Type I, receptors. It is not clear as yet if this effect is the same as that seen in our studies, but if so, the different conclusion drawn concerning the receptor type involved may have been due to the fact that Overton et al. (1996) were recording in vivo, rather than in vitro, and used adrenalectomised animals.

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Table 1. Firing rates (Hz) in presence of NMDA, 5 µM, mean ± s.e.m., cort = corticosterone, RU = RU38486, aldo = aldosterone
*P<0.05 compared with concurrent recordings in nomal aCSF (controls)

Controls	Cort 500 nM	Cort 500 nM+RU 500 nM	RU 500 nM	Controls	Aldo 100 nM
2.39 ± 0.09	3.45 ± 0.22*	2.13± 0.3	2.65 ± 0.38	2.55 ±0.26	1.71 ± 0.13*

7P MPP⁺-EVOKED HYDROXYL RADICAL FORMATION IN THE RAT STRIATUM *IN VIVO* IS ALTERED BY THE MONOAMINE OXIDASE INHIBITORS SELEGILINE AND CLORGYLINE, BUT NOT PARGYLINE

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Infusion of 1-methyl-4-phenylpyridinium (MPP^+) into the striatum increases hydroxyl radical ($OH^·$) production by autoxidation of dopamine and/or inhibition of mitochondrial complex I (Chiueh et al, 1992; Smith & Bennett, 1997)). The monoamine oxidase (MAO) inhibitor, selegiline, is used in the treatment of Parkinson's disease to increase dopamine availability. Additionally, selegiline may possess neuroprotective activity, however, whether the mechanism involves inhibition of MAO is not clear. For this reason we have compared the effect of the selective MAOB inhibitor, selegiline, the selective MAOA inhibitor, clorgyline, and the non-selective MAO inhibitor, pargyline, on MPP^+-evoked OH formation and dopamine efflux in the rat striatum *in vivo*.

Microdialysis probes were inserted into the left striatum of male Wistar rats (280-320g) using standard stereotaxic techniques. Probes were perfused at a flow rate of 1µl/min with artificial extracellular fluid (aECF) containing sodium salicylate (5mM) to trap $OH^·$, forming 2,3-dihydroxybenzoic acid (2,3-DHBA). One hour after implantation, dialysate samples were collected every 10 min throughout the experimental period and analysed for levels of 2,3-DHBA and DA by HPLC with electrochemical detection. After a period of 30 min, probes were perfused with the MAO inhibitors, selegiline (10 and 40µM, n=10 and 5), clorgyline (10 and 40µM, n=5 and 6) or pargyline (1mM, n=6) for 60 min. Control rats received vehicle (aECF, n=4). Nomifensine (10µM, n=6) was also used for comparison. MPP^+ (10mM, in the presence of the drug/vehicle) was then perfused for a period of 10 min followed by a further 90 min perfusion with drug or vehicle. Data (expressed mean ± s.e. mean MPP^+-evoked 2,3-DHBA formation or DA efflux) was analysed by one-way ANOVA followed by Dunnett's test.

MPP^+ increased 2,3-DHBA formation (13.3±3.0 pmol/90min). Selegiline (40µM), clorgyline (40µM) but not pargyline (1mM) reduced the MPP^+-evoked increase in 2,3-DHBA formation (Figure) without altering MPP^+-evoked DA efflux (total DA efflux (pmol/100 min): control 133.9±19.8; selegiline (40µM) 108.4±12.4; clorgyline (40µM) 109.5±7.6; pargyline (1mM), 138.2±22.6). Nomifensine decreased both 2,3-DHBA formation and dopamine efflux (2,3-DHBA, 7.9±1.9 pmol/90 min; dopamine, 75.2±15.3 pmol/100 min).

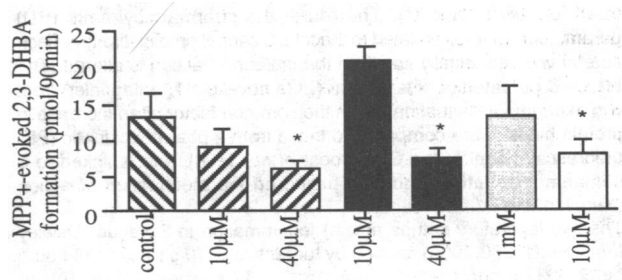

Figure The effect of selegiline, ▨; clorgyline, ■; pargyline, ▭, and nomifensine, □, on MPP^+-evoked 2,3-DHBA formation. * $p<0.05$ compared to control (▨).

Both selegiline and clorgyline concentration-dependently prevented MPP^+-evoked $OH^·$ formation at concentrations that did not alter extracellular levels of dopamine. However, pargyline had no effect on MPP^+-evoked $OH^·$ formation or dopamine efflux. These data suggest that selegiline and clorgyline can protect against MPP^+-induced increase in extracellular fluid $OH^·$ concentrations by mechanisms not involving MAO activity. This may be due to a direct antioxidant action of both selegiline and clorgyline (Wu et al, 1993).

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8P EFFECT OF CAPSAICIN AND RESINIFERATOXIN ON THE RELEASE OF SENSORY NEUROPEPTIDES IN THE RAT ISOLATED TRACHEA

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Capsaicin and resiniferatoxin (RTX) release then deplete substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin (SOM) from the sensory nerve terminals. It has been shown that the release process is Ca^{++} dependent but resistant to tetrodotoxin or omega-conotoxin, suggesting that it takes place without the intervention of axon reflexes or N-type Ca^{++} channels (see Maggi, 1995, Szolcsányi, 1996). Opposite results were reported, however, by using capsaicin in low (10 nM) concentration (see Lundberg, 1996). The aim of this study was to analyse the release of SP, CGRP and SOM from the rat's trachea in response to capsaicin or electrical nerve stimulation and to determine the IC_{50} values of capsaicin and RTX pretreatments on the electrically evoked neuropeptide release.

Tracheae of two adult Wistar rats were placed in oxygenated Krebs solution in an organ bath (1.8 ml) at 37°C. After equilibration the solution was changed 3 times for 8 min (prestimulated, capsaicin exposed or field stimulated with 40V, 0.1 ms and poststimulated). SP, CGRP and SOM content of the fractions was determined by radioimmunoassay and means of 4-6 experiments were taken (Helyes et al., 1997).

Capsaicin (10 nM-10µM) elicited a concentration dependent release of SP, CGRP and SOM with similar EC_{50} values of 190 nM, 230 nM and 77 nM, respectively, taking the effect of 10 µM as maximum. The released SP, CGRP and SOM at the lowest concentration of 10 nM capsaicin was 1.43 ± 0.10, 0.30 ± 0.02 and 0.25 ± 0.01 fmol/mg, respectively. The frequency optimum of field stimulation was low for all three peptides since identical number of 100 or 300 pulses at 0.5, 2 or 10 Hz produced similar effects. The release of SP, CGRP and SOM evoked by capsaicin (10 nM) remained unchanged in the presence of lidocaine (25 mM), tetrodotoxin (1 µM) omega-conotoxin GVIA (300 nM) and agatoxin TK (50 nM) but abolished by Cd^{++} (200 µM). 30 min exposure of capsaicin or RTX followed by a 30 min washout period inhibited the release of SP, CGRP and SOM to electrical field stimulation (1200 pulses at 10 Hz) at different concentrations. The respective IC_{50} values were 20 µM, 0.25 µM and 5.9 µM for capsaicin and 512 nM, 4 nM and 66 nM for RTX.

These data suggest that in the rat's isolated trachea capsaicin releases SP, CGRP and SOM from the sensory nerve endings in similar concentrations but both in the case of capsaicin and RTX the subsequent blocking effect is more pronounced for CGRP followed by SOM and SP. Na^+ channels, N- or P-type of Ca^{++} channels, axon reflexes are not involved in the release process, suggesting our concept (Szolcsányi, 1996) that the release sites of sensory neuropeptides serve also as sensory receptors.

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9P EFFECT OF PRIOR CHRONIC ETHANOL INTAKE ON THE LOCOMOTOR RESPONSE TO AN ENVIRONMENT ASSOCIATED WITH REPEATED NICOTINE ADMINISTRATION

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Strong links are thought to exist between the effects of ethanol and of nicotine; about 95% of alcoholics smoke cigarettes. It is also known that exposure to the environment associated with drug taking can increase the propensity to self-administer these drugs. We previously showed that chronic alcohol intake in mice can increase the sensitising effect of repeated nicotine administration on locomotor activity (Watson et al., 1996). We now examine the response of mice to the environment associated with repeated nicotine administration, and the effects of chronic ethanol intake on this response.

Male TO mice (35-40g), 10-15 per treatment group, were given ethanol by a liquid diet: schedule: control diet for 3 days, then 2 days 3.5% v/v ethanol, 9 days 5% ethanol and 9 days 8% ethanol (ethanol intake 24-28 g/kg/24h). Control groups were pair-fed an equicalorific control diet without ethanol. Locomotor activity was measured by breaking of infra-red beams across the test cages.

Six days after cessation of the ethanol treatment, nicotine (0.4 mg/kg s.c.), or saline vehicle, was injected once daily for 28 days. These injections were given in the animals home cages, with the following exception: Once a week, the animals were placed singly in either the locomotor meter cages, or in grid-floored cages, and acclimatised for 30 min before the injections. On the final day, all of the mice were placed singly in the locomotor meters and their locomotor activity was measured during the 30 min acclimatisation period prior to the final injections.

On the final test day, the mice which previously received chronic ethanol treatment followed by nicotine injections in the familiar environment of the locomotor meters, had significantly higher locomotor activity during the acclimatisation period, ($P < 0.05$, Student's t-test) than the mice which received saline injections after the ethanol diet (Table 1). This was in contrast to the animals which had been given the control diet, where no significant effect of the prior nicotine injections was seen. There were no significant differences between any of the treatment groups in the mice which were not made familiar with the locomotor cages during the nicotine/saline treatment.

These results indicate that after chronic ethanol intake, a "conditioning" effect occurs to the environment associated with nicotine administration This effect could have important implications in the abuse of these two drugs.

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Table 1. Locomotor activity counts prior to injections of nicotine or saline in mice previously given ethanol-containing diet or control diet, and after 27 days of nicotine or saline injections. "Familiar" indicates mice previously placed in the locomotor cages prior to the injections "Novel" indicates mice which were not placed in the locomotor cages during the nicotine/saline treatment. * $P < 0.05$ compared with the ethanol group that received saline injections.

Environment	Control + saline	Control + nicotine	Ethanol + saline	Ethanol + nicotine
Novel	3869 ± 389	4431 ± 431	3983 ± 417	3647 ± 458
Familiar	2624 ± 339	2922 ± 402	2165 ± 236	3297 ± 401*

10P DETAILED BEHAVIOURAL PHENOTYPE CHARACTERISATION OF ApoE KNOCKOUT MICE

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Apolipoprotein E (ApoE) may play a role in neuronal degeneration or regeneration in the CNS and is associated with filamentous amyloid deposits of Alzheimer brain tissue. Homozygous ApoE knockout mice (Apoe^{m1Unc}, Piedrahita *et al.*, 1992) were produced which had been backcrossed through 8 generations onto a C57BL/6 background. We have used SHIRPA (Rogers *et al.* 1997), a comprehensive testing protocol designed to identify and characterise phenotypic variation, to determine the effects of disruption of the ApoE gene on the behavioural phenotype.

The genotype of 20 wildtype (+/+, 10 male, 10 female) and 13 knockout (-/-, 6 male, 7 female) mice was confirmed by pcr on DNA prepared from tail tissue in which three primers were used to amplify a 155bp wild type band and a 245bp band from the targeted allele. Each mouse was tested in all of the following procedures; elevated X-maze, open field test, primary observation of behavioural and neurological measures, accelerating rotarod, spontaneous locomotor activity, holeboard exploration, hot plate test of analgesia and the water maze spatial learning test.

Statistical analyses revealed no significant main effects of gene knockout in the majority of the behavioural measurements in this study (all data were collapsed across gender). There was no significant difference between wildtype and knockout mice on anxiety as measured by proportion of time in the open arms of the elevated X-maze (+/+ 14.0±3.2%, -/- 11.6±4.5%) or in the centre of the open-field (+/+ 12.5±1.5%, -/- 11.9±2.7%). However, in both cases, distance travelled was reduced (X-maze; +/+ 27.4±2.1m, -/- 21.7±3.3m, P=0.066; open field; +/+ 20.4±1.2m, -/- 16.2±1.8m, P<0.05). There were no significant differences in the SHIRPA behavioural observation measurements, although there was a non-significant reduction in rotarod performance (+/+ 62.1±6.5s, -/- 44.8±5.9s, P=0.07) and holeboard exploration (+/+ 49.5±7.3 visits, -/- 31.2±6.6 hole visits, P=0.08). There were no differences in either spontaneous motor activity or analgesia. There was also no significant difference between groups in acquisition of the water maze, as measured by both latency to find the platform over four consecutive training days and time spent in the platform quadrant during the probe trial (+/+ 33.8±4.3%, -/- 32.5±5.6%). There was a trend towards reduced body weight in the knockout mice (+/+ 21.6±0.7g, -/- 19.4±1.3g, P=0.07).

The lack of effect on learning of a spatial task by ApoE knockout mice in this study contrasts with a marked impairment reported by Oitzl *et al.* (1997). However, our findings from the water maze experiment agree with those of Andersen and Higgins (1997). In summary, ApoE knockout appeared to have subtle effects on the behavioural phenotype of mice which were detected by the systematic and comprehensive assessment provided by SHIRPA. The knockout mice tended to be smaller, less able to remain on the accelerating rotarod and had reduced levels of exploratory activity.

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11P BEHAVIOURAL RESPONSIVITY TO THE SELECTIVE D_2-LIKE AGONIST RU 24213 IN TRANSGENIC MICE WITH D_{1A} DOPAMINE RECEPTOR 'KNOCKOUT'

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Dopamine receptors of the D_1-like family [$D_{1A/1}$, $D_{1B/5}$] are known to play an important role in the regulation of psychomotor behaviour, often through important functional interactions with their D_2-like [$D_{2L/S}$, D_3, D_4] counterparts; for example, D_1-like antagonists readily attenuate typical behavioural responses to D_2-like agonists via cooperative/synergistic D_1-like:D_2-like interactions, and can release atypical responses to such agonists via oppositional interactions. However, in the absence of selective agents, the relative roles of D_{1A} and D_{1B} receptors in such phenomena are unclear, though prepotence of the D_{1A} receptor is presumed on anatomical grounds. Furthermore, there may exist an additional D_1-like receptor that does not share the coupling to adenylyl cyclase that defines D_{1A} and D_{1B} receptors (Waddington et al., 1995). Described here is the profile of responsivity to the D_2-like agonist RU 24213 (Deveney & Waddington, 1996) in transgenic mice with targeted gene deletion of the D_{1A} receptor.

D_{1A}'knockout' mice were constructed, bred and genotyped as described previously (Drago et al., 1994; Clifford et al., 1997, 1998). At approximately 10 weeks, black females [n=8 per group] were challenged with 0.1-12.5 mg/kg s.c. RU 24213 and assessed by direct observation over successive 1h periods using an ethologically-based rapid time-sampling behavioural checklist procedure (Clifford et al., 1997, 1998) and stereotypy rating scale (Deveney & Waddington, 1996). Statistical analysis was by analysis of variance (ANOVA), followed by Student's t-test or Mann-Whitney U-test. In wildtypes [$D_{1A}^{+/+}$], RU 24213 dose-dependently induced sniffing [+235%, P<0.001] and a characteristically 'ponderous' rather than normal or rapid locomotion [P<0.001], with a dose-dependent increase in stereotypy score [mean ± s.e.mean: vehicle 0.1 ± 0.1, RU 24213 12.5mg/kg 3.3 ± 0.2; P<0.001]; none of these responses differed significantly in 'knockouts' [$D_{1A}^{-/-}$]. RU 24213 dose-dependently abolished baseline levels of grooming [P<0.001] similarly in each genotype; there were no significant effects of RU 24213 on overall rearing or sifting, and no other atypical behaviours were evident.

Mice with targeted gene deletion of the D_{1A} receptor evidenced unaltered induction of stereotyped sniffing and ponderous locomotion by the selective D_2-like agonist RU 24213. Thus, normal D_1-like:D_2-like interactions appeared operative in $D_{1A}^{-/-}$ mice at the level of behavioural topography. These findings suggest either that a D_1-like receptor other than D_{1A} is the critical element in D_1-like:D_2-like interactions or that compensatory processes consequent to developmental absence of D_{1A} receptors can sustain such D_2-like agonist responses.

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12P INVESTIGATION OF NMDA RECEPTOR LIGAND OCCUPANCY USING *IN VIVO* [^{3}H]-MK-801 BINDING

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The N-methyl-D-aspartate (NMDA) receptor has been extensively characterised *in vitro* using radioligands for the glutamate, glycine, MK-801 and polyamine modulatory sites . [^{3}H]-MK-801 binding has been used as a functional assay to investigate NMDA receptors *in vitro* (Foster *et al.*, 1992). These studies provide information regarding intrinsic activity and affinity of ligands but provide no information concerning occupation of the NMDA receptor by these ligands *in vivo*. Price *et al.* (1988) developed an *in vivo* [^{3}H]-MK-801 binding assay and determined *in vivo* occupancy for compounds acting at the ion channel of the NMDA receptor complex. Here we have used this methodology to determine *in vivo* occupancy for compounds acting at various modulatory sites on the NMDA receptor complex.

Male BKTO mice (20-25 g) were injected with either vehicle or NMDA receptor antagonist. Test compounds or MK-801 (3 mg/kg, i.p.) to determine non-specific binding were injected followed 15 minutes later by [^{3}H]-MK-801 (200 µCi/kg i.v.) . Animals were humanely killed 10 minutes after injection of [^{3}H]MK 801. The forebrain was rapidly removed, and homogenised in 40 volumes 5 mM Tris-acetate buffer (pH 7.4 @ 4°C) and 500 µl aliquots were filtered through Whatman GF/B filters (2 x 5ml wash). Radioactivity in filter and homogenate samples were determined using liquid scintillation counting.

Data obtained for ion channel blockers, glutamate site antagonists and ifenprodil site ligands are shown in Table 1.

Consistent with the results obtained by Price *et al (1988)* the non competetive ion channel blockers MK-801, PCP and ketamine virtually completely displaced MK-801 binding as did the glutamate receptor antagonist CPP. In contrast displacement of [^{3}H]-MK-801 binding by the NR2B-selective compounds (±)CP 101,606, (±)RO 25-6981 and ifenprodil was only partial, as might be expected with compounds which only occupy a subpopulation of NMDA receptors.

Table 1. Inhibition of [^{3}H]-MK-801 binding *in vivo* by NMDA receptor ligands.

Compound	ED_{50}	Max Response
Ion channel site(i.p.)		
MK801	0.17 ± 0.05	89 ± 19
Phencyclidine	0.97 ± 0.29	91 ± 8
Ketamine	5.33 ± 4.39	100 ± 25
Glutamate site(i.p.)		
(±) CPP	2.49 ± 0.50	82 ± 4
Ifenprodil site(s.c.)		
(±)RO 25-6981	2.15 ± 0.40	75 ± 4
(±)CP 101,606	1.43 ± 0.70	56 ± 8
Ifenprodil	5.83 ± 1.15	54 ± 4

ED_{50} = dose (mg/kg) giving 50% of the maximal inhibition. Data are means from two separate experiments with 4 mice per dose ± standard error for goodness of fit. Max Response is % inhibition.

Taken together these results show that *in vivo* [^{3}H]-MK-801 binding can be used to determine NMDA receptor occupancy for ligands acting at various modulatory sites on the NMDA receptor complex

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13P PHARMACOLOGICAL CHARACTERISATION OF THE NOVEL SYNTHETIC HEXAPEPTIDE, Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 AT THE RAT BRAIN ORL_1 RECEPTOR USING [^{3}H]NOCICEPTIN AND [^{35}S]GTPγS BINDING ASSAYS

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Recent radioligand binding and isolated tissue studies have shown that nociceptin (orphanin FQ), the endogenous ligand the ORL_1 receptor is rivalled in affinity and potency but not in efficacy for this site by the synthetic hexapeptide, Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 (Ac-RYYRWK-NH_2) (Nicholson *et al*, 1997). This study has used [^{3}H]nociceptin binding and the agonist-induced increase in guanylyl 5'-[γ-[^{35}S]thio]-triphoshate ([^{35}S]GTPγS) binding to confirm these effects in in areas of native rat brain (frontal cortex and hypothalamus) where ORL_1 receptors are heavily invested (Foddi & Mennini, 1997).

For the [^{3}H]nociceptin assay, membranes prepared from rat (wistar) frontal cortex were incubated with appropriate concentrations of radioligand, displacing drugs and Tris HCl buffer (pH 7.4 at 25°C) for 1 hour at 25°C. Non-specific binding was defined in the presence of 1μM nociceptin/Ac-RYYRWK-NH_2 and inhibition curves were performed at a [^{3}H]nociceptin concentration of 40pM using 14 concentrations of competing drug. For the [^{35}S]GTPγS binding assay, membranes from rat frontal cortex and hypothalamus were incubated for 90mins at 30°C with an appropriate range of agonist concentrations in 50mM Tris HCl buffer (pH 7.4, 25°C) containing 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl, 50μM GDP, 50pM [^{35}S]GTPγS and 10μM peptidase inhibitors (amastatin, phosphoramidon, bestatin & captopril). Basal binding was assessed in the absence of agonist and non-specific binding in the presence of 10μM unlabelled GTPγS. For both assays 0.01% BSA was present and final incubations (volume 0.5ml) were terminated by rapid filtration through Whatman GF/B filters (pre-soaked in 1% polyethylenimine for the ^{3}H but not the ^{35}S assay)

Saturation studies with [^{3}H]Nociceptin defined a uniform population of binding sites of high capacity (B_{max}=325 ± 32 fmol/mg protein) and very high affinity (pK_d=10.70 ± 0.11, n=4). Competition studies showed that this binding was displaced in a monophasic manner by both nociceptin (pK_i = 10.38) and the synthetic hexapeptide, Ac-RYYRWK-NH_2 (pK_i = 10.44)(table 1).

Functional studies in rat cortex showed the endogenous agonist nociceptin to produce a potent (pEC_{50}=8.26) concentration-dependent increase in [^{35}S]GTPγS binding with a maximum stimulation (E_{max}) of 174.3% over basal. The response to Ac-RYYRWK-NH_2, however, was less efficacious (table 1) but of comparable high potency (pEC_{50}=9.07). These results are entirely consistent with those observed in the isolated rat vas deferens assay (Nicholson *et al*, 1997). Similarly in rat hypothalamus a potent apparently full agonist response was elicited by nociceptin (E_{max}=213%; pEC_{50}=8.21) but the potent (pEC_{50}=8.73) partial agonist effect observed with Ac-RYYRWK-NH_2 was of an even lower efficacy (E_{max}=49.5%) than in the cortex (table 1). The reason for this latter effect is unclear but potentially of interest.

This study confirms the existence of ORL_1 receptors in rat brain and that the synthetic hexapeptide, Ac-RYYRWK-NH_2 displays equivalent high affinity and potency but substantially lower efficacy at this site than the endogenous peptide, nociceptin.

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Table 1. Data for nociceptin and Ac-RYYRWK-NH_2 displacement and stimulation of [^{3}H]Nociceptin and [^{35}S]GTPγS binding respectively. Affinity (pK_i), potency (pEC_{50}) and efficacy (E_{max}) values represent means (± sem) taken from 3-5 individual experiments. (For Hill coefficients (nH), sem<0.1).

	[^{3}H]Nociceptin binding	[^{35}S]GTPγS binding			
	cortex	cortex		hypothalamus	
	pK_i (nH)	pEC_{50}	E_{max} (% over basal)	pEC_{50}	E_{max} (% over basal)
Nociceptin	10.38 ± 0.07 (1.01)	8.26 ± 0.17	174.3 ± 9.4	8.21 ± 0.09	212.7 ± 13.5
Ac-RYYRWK-NH_2	10.44 ± 0.03 (0.97)	9.07 ± 0.11	93 ± 9.8	8.73 ± 0.17	49.5 ± 13.3

14P EFFECT OF CHRONIC ANTIPSYCHOTIC DRUGS ON NEUROTENSIN BINDING IN RAT BRAIN REGIONS

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The neuropeptide neurotensin (NT) colocalises with dopamine in brain mesolimbocortical structures. Central levels of NT are reduced in schizophrenia, but increase with the onset of antipsychotic drug action (at 2-3 weeks; Garver *et al*, 1991). This study compared high affinity NT binding in rat brain following acute or chronic treatment with the typical antipsychotic, haloperidol, and the atypical drugs clozapine and zotepine (Needham *et al*, 1996).

Male CD rats (150-250g, Charles River) received acute or chronic (21 days) oral treatment with (mg/kg) clozapine (CZ, 7.72 or 38.6), haloperidol (HAL, 0.246 or 1.23), zotepine (ZOT, 1.02 or 5.1) or vehicle (1% [w/v] tartaric acid; CZ and ZOT, or 5% [w/v] lactic acid in 5% [w/v] glucose; HAL). Control animals received no treatment. 4 h after the last dose the striatum, nucleus accumbens and frontal cortex were removed and frozen. Tissue was homogenised in 40 vols ice-cold Tris buffer (50 mM, pH7.4), then centrifuged (12500 rpm, 4°C, 3 x 10 min). The pellet was suspended (to a concentration of 12.5 mg/ml) in buffer (50 mM Tris, 0.05% BSA, 0.03% bacitracin, 0.1mM EDTA, pH7.4) and a 12-point saturation analysis (final volume 500 μl) was performed using [^{3}H]NT (0.04-20 nM) with 1 μM levocabastine present to define the high affinity receptor. Non-specific binding was defined by 10 μM cold NT. A 40 min incubation was followed by filtration and liquid scintillation analysis of bound [^{3}H]NT.

Acute drug administration produced no significant changes in NT binding (data not shown). Chronic dosing, however, resulted in an increase in both the Kd and Bmax of high affinity NT binding in the striatum following HAL but not CZ or ZOT (Table 1). In contrast, the atypical antipsychotics CZ and ZOT decreased the Kd in mesolimbocortical structures (Table 1).

In agreement with previously reported drug effects on central NT levels in patients (Garver *et al*, 1991), these data demonstrate that antipsychotics alter NT binding after chronic but not acute administration. Furthermore, the limbic specificity of the effects of CZ and ZOT, but not HAL, are consistent with a role for NT in atypical antipsychotic action.

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Table 1: Effect of chronic antipsychotic treatment on NT binding.

	STRIATUM				NUCLEUS ACCUMBENS				FRONTAL CORTEX			
	Lower dose		Higher dose		Lower dose		Higher dose		Lower dose		Higher dose	
	Kd	Bmax	Kd	Bmax	Kd	Bmax	Kd	Bmax	Kd	Bmax	Kd	Bmax
C	1.7±0.1	6.6±0.6	1.1±0.1	2.2±0.3	1.5±0.2	5.9±0.6	2.5±0.2	4.3±0.8	1.6±0.3	3.4±0.8	1.6±0.1	1.9±0.2
V1	1.7±0.2	5.7±0.7	1.1±0.2	2.3±0.2	1.4±0.2	5.6±0.5	2.3±0.5	3.8±0.3	1.7±0.1	3.4±0.4	2.1±0.4	2.6±0.8
V2	1.6±0.1	7.5±0.5	1.4±0.2	3.1±0.1	1.6±0.2	5.9±0.8	2.6±0.3	4.3±0.2	1.4±0.1	3.3±0.8	1.8±0.2	2.0±0.4
HAL	2.6±0.2*	8.5±0.9*	3.0±0.3**	5.0±0.6**	1.9±0.1	5.1±0.4	2.5±0.3	3.6±0.5	1.7±0.1	3.3±0.3	1.5±0.1	1.6±0.1
CZ	1.5±0.2	6.0±0.4	1.0±0.1	2.3±0.2	0.9±0.1$^{\phi}$	3.9±0.8	0.9±0.1$^{\phi\phi}$	1.6±0.1$^{\phi\phi}$	1.3±0.1	2.5±0.2	1.6±0.1	2.1±0.1
ZOT	1.5±0.2	6.1±0.4	1.1±0.2	3.8±0.4	0.7±0.1$^{\phi\phi}$	4.2±0.3	0.5±0.1$^{\phi\phi}$	2.2±0.3$^{\phi}$	0.6±0.1$^{\phi\phi}$	2.2±0.1	0.4±0.1$^{\phi\phi}$	1.6±0.2

Values are mean ± se mean; Kd - nM, Bmax - fmol/mg protein, n = 6-7. *p<0.05, **p<0.01 treatment vs vehicle 1; $^{\phi}$p<0.05, $^{\phi\phi}$p<0.01 treatment vs vehicle 2, following one-way ANOVA and Tukey's post hoc test. C = control (no treatment); V1 = vehicle 1 (5% [w/v] lactic acid in 5% [w/v] glucose); V2 = vehicle 2 (1% [w/v] tartaric acid); HAL = haloperidol (0.246 or 1.23 mg/kg); CZ = clozapine (7.72 or 38.6 mg/kg); ZOT = zotepine (1.02 or 5.1 mg/kg).

15P THE NO DONOR SPM 5186 REDUCES INFARCT SIZE IN A RABBIT MODEL OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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There is good evidence that organic nitrates and NO-donors reduce infarct size in animal models of myocardial ischaemia and reperfusion. The mechanism of this cardioprotective effect is thought to involve (i) reductions in afterload (and hence oxygen demand) and (ii) reduction of neutrophil infiltration (Lefer, 1995). There is some evidence that NO can activate ATP-sensitive potassium channels (K_{ATP} channels) (Murphy & Brayden, 1995). Activation of K_{ATP} channels protects against ischaemic injury and contributes to the cardioprotective effects of 'ischaemic preconditioning' (Hide & Thiemermann, 1996). Here we investigate the effect of the NO donor SPM 5186 on infarct size resulting from regional myocardial ischaemia and reperfusion in the anaesthetised rabbit. We have also investigated whether any observed cardioprotective effect is blocked by the K_{ATP} channel blocker 5-hydroxydecanoate (5-HD).

Male New Zealand white rabbits (2.5-3.0 kg) were premedicated with Hypnorm (0.1 $ml \cdot kg^{-1}$, i.m.). General anaesthesia was induced (20 $mg \cdot kg^{-1}$, i.v.) and maintained with sodium pentobarbitone. The animals were ventilated with room air. A left intercostal thoracotomy was performed, and a ligature was placed around the first antero-lateral branch of the left coronary artery (LAL). Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded. Vehicle (saline) or SPM 5186 (100 μg/kg/min) were infused for 60 min and 15 min after termination of this infusion, the LAL was occluded for 45 min followed by 2h reperfusion. To elucidate whether the activation of K_{ATP} channels contributes to any of the observed effects of SPM 5186, 5-HD (5 mg/kg) was administered as a 2 ml left ventricular bolus 5 min before the saline or SPM 5186 infusion. At the end of the experiment, the LAL was reoccluded and Evans blue dye (2% w/v) was injected into the left ventricle for determination of the area at risk (AAR). Infarct size was determined by incubation of the AAR with nitro-blue tetrazolium (0.5 $mg \cdot ml^{-1}$ for 20 min.). Values are expressed as mean ± s.e.mean (*$p < 0.05$ compared to control by one-way ANOVA followed by Dunnett's test).

Infusion of SPM 5186 (100 μg/kg/min) for 60 min caused a fall in MAP which remained significantly reduced throughout the experiment (control; baseline MAP 67±3, 120 min reperfusion 64±4 mmHg, SPM 5186; baseline MAP 66±3, 120 min reperfusion 55±4 mmHg). SPM 5186 caused a significant reduction in infarct size (expressed as a % of the AAR) from 59±2% (control n=7) to 41±5% (n=7). Pretreatment of the animals with 5-HD did not affect the fall in MAP nor the reduction in infarct size afforded by SPM 5186 (infarct size was 41±5%, n=6). It should be noted that 5-HD alone did not affect haemodynamics or infarct size in saline treated animals. AAR (approx 38%) was not different between groups.

Thus, the NO donor SPM 5186 reduces infarct size caused by myocardial ischaemia and reperfusion in the anaesthetised rabbit. The cardioprotective effect of SPM 5186 is not due to activation of K_{ATP} channels, as the protection was not affected by pretreatment of rabbits with 5-HD. The mechanisms underlying the cardioprotective effect of SPM 5186 are not clear but may involve a reduction of afterload and hence myocardial oxygen demand.

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16P S-NITROSOTHIOLS CAUSE SUSTAINED NITRIC OXIDE-MEDIATED VASODILATATION IN HUMAN SAPHENOUS VEIN: POTENTIAL AS LONG-TERM NITRIC OXIDE DONOR DRUGS IN CORONARY ARTERY BYPASS GRAFTS

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Endothelium-derived nitric oxide (NO) causes vascular relaxation and inhibits platelet aggregation, monocyte adhesion and mitogenesis (Moncada *et al.*, 1991). Reduced NO generation caused by endothelial dysfunction in graft arteries and veins used in coronary artery bypass graft surgery (CABG) is thought to be an important factor in the development of 'vein atherosclerosis'. Organic nitrates (e.g. GTN) and sodium nitroprusside (SNP) are NO donor drugs that are often used to deliver supplementary NO to vessels post-CABG, but GTN engenders tolerance and prolonged administration of SNP can be toxic. Neither GTN nor SNP can be targeted specifically at graft veins. S-nitrosothiols are emerging as possible alternatives to established NO donor drugs. They decompose spontaneously in solution to release NO and do not engender tolerance. Recently, we have shown that a novel S-nitrosated glyco-amino acid, RIG200, causes sustained, NO-mediated vasodilatation in isolated rat femoral arteries (Megson *et al.*, 1997).

Here, we compared the vasodilator effects of RIG200 to those of GTN, SNP and S-nitrosoglutathione (GSNO) in rings of saphenous vein (SV) mounted in organ baths containing oxygenated Krebs buffer solution. A tension of 4 g was applied prior to pre-contraction with phenylephrine (PE; EC_{80} concentration). Endothelial integrity was assessed using ACh (10^{-5} M) and rings were then exposed to increasing concentrations (10^{-7} - 10^{-5} M; 7 min) of NO donor in the presence or absence of the NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM). NO donor drug exposures were interspersed with 10-30 min washout periods to determine whether responses were sustained, and the NO scavenger, ferrohaemoglobin (Hb; 10 μM) was used to assess the role of NO in sustained responses. In separate experiments, rings of SV were treated for 30 min with either GTN, GSNO, RIG200 (all 10^{-5} M) or Krebs solution (control), and responses to PE (EC_{80} concentration) were monitored at timed intervals for 240 min after washout.

	EC_{50} (-L-NAME)	EC_{50} (+L-NAME)	R_{240}	R_{240} (+Hb)
RIG200	0.9±0.20	0.6±0.3	76.0±.5.1	98.7±7.5
GSNO	1.2±0.25	0.4±0.11	93.2±3.5*	107.6±6.4
SNP	0.4±0.12*	0.5±0.22	–	–
GTN	0.1±0.05*	0.03±0.01*	100.2±2.5*	107.7±5.3

Table 1. EC_{50}s (μM) in the absence (n=7) and presence (n=7) of L-NAME (100 μM), and tension existing 240 min after washout of 10^{-5} M NO donor drugs (% pre-existing tension; R_{240}; n=8). The effect of Hb (10 μM) on R_{240} is also included.* - $P<0.05$ vs RIG200 (unpaired two-tailed student's t-test).

Venoselective GTN was the most effective vasodilator in SVs but responses to GTN, like SNP, recovered rapidly. The S-nitrosothiols both caused sustained vasodilatation in SVs. The effect was more pronounced with RIG200. Addition of Hb, either 30 or 240 min after NO donor washout, reversed vasodilatation to RIG200 and GSNO but did not significantly affect tension in GTN or control rings. L-NAME sensitised vessels to RIG200, GSNO and GTN but did not inhibit sustained S-nitrosothiol-induced vasodilatation. Responses to ACh were small in SVs (14.9±3.1% relaxation; n=15), suggesting endothelial dysfunction. These observations are consistent with the hypothesis that RIG200 is retained by SVs and that *in situ* decomposition results in release of sufficient NO to maintain a vasodilator tone. We suggest that pre-treatment with RIG200 might improve patency of vein grafts.

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17P IMPAIRMENT OF NITRIC OXIDE-MEDIATED RESPONSES BY HIGHLY GLYCOSYLATED HUMAN OXYHAEMOGLOBIN IN HUMAN MESENTERIC MICROVESSELS

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Early glycosylation products such as glycohaemoglobin have recently arised as possible mediators of diabetic endothelial impairment. We have previously reported the inhibition of nitric oxide (NO)-mediated responses in rat aortic segments induced by nanomolar concentrations of glycohaemoglobin (physiological plasmatic content of free haemoglobin) only when glycated at pathologic range (Rodríguez-Mañas *et al.*, 1993). Furthermore, this effect was produced by generation of superoxide anions (Angulo *et al.*, 1996). The aim of this work is to confirm the ability of highly glycosylated haemoglobin to interfere on endothelial function in the human vascular bed.

Branches of the human mesenteric artery were obtained from abdominal fat of patients (n=19; aged 47.5±2.8 years; 46% females) suffering surgical intervention unrelated to diabetes, hypertension or vascular disease. Arterial segments were mounted in a myograph for analysis of reactivity of microvessels as previously described (Rodríguez-Mañas *et al.*, 1998). Cumulative vasodilatatory responses to bradykinin (BK; 0.01 to 3 μM) were tested in vessels precontracted with 35 mM K^+.

Addition of 10 μM N^G-nitro-L-arginine metyl ester (L-NAME), an inhibitor of NO-synthase, markedly reduced relaxations evoked by BK (27.8±8.4% vs 92.0±1.2% of control maximal relaxation; n=4 and 5). In the same way, BK-induced responses were inhibited by 1 μM oxyhaemoglobin (43.7±6.2% vs 86.12% of control maximal relaxation; n=3 and 5). However, when physiological concentrations were employed (10 nM) non glycosylated human oxyhaemoglobin (HHb) did not modify relaxations to BK when compared to respective control curves. By contrast, 10 nM glycosylated human oxyhaemoglobin (GHHb) inhibited relaxations induced by BK when the percentage of glycosylation was 10% or higher (14%). (Table 1) Preincubation of vessels with 100 U/ml superoxide dismutase (SOD) prevented the effect induced by 10 nM 14% GHHb (Table 1).

Table 1. Effect of 10 nM non glycosylated (HHb) and glycosylated human oxyhaemoglobin (GHHb) at increasing percentages of glycosylation on pD_2 values for bradykinin in human mesenteric microvessels. Effects of 100 U/ml superoxide dismutase (SOD).

	10 nM		
	Control	Oxyhaemoglobin	
HHb	7.43±0.12 (6)	7.31±0.04 (7)	
GHHb 8%	7.40±0.09 (10)	7.33±0.09 (8)	
GHHb 10%	7.66±0.10 (9)	7.28±0.04* (8)	14% GHHb +SOD
GHHb 14%	7.48±0.07 (19)	7.02±0.08* (9)	7,39±0,16 (9)

pD_2 indicates the -log M of the required concentration of bradykinin to reach the half-maximal relaxation obtained in control conditions. Number of segments is in parenthesis. * P< 0.01 vs respective control curve.

Results show, for the first time in the human vascular bed, that only highly glycosylated human oxyhaemoglobin impairs NO-mediated responses when tested at physiological plasmatic concentrations. This fact support the hypothesis that glycohaemoglobin contributes to the development of vascular dysfunction in diabetic patient.

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18P INFLUENCE OF N^G-NITRO-L-ARGININE METHYLESTER (L-NAME) ON VASOCONSTRICTION INDUCED BY EXOGENOUS AND ENDOGENOUS NORADRENALINE IN RAT TAIL ARTERY

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A lack of effect of L-NAME on α_1-adrenoceptor response was observed in rat tail artery (TA) rings and ascribed to the high efficiency of coupling of these receptors (Tabernero *et al.*, 1996). In the perfused TA, vasoconstrictor responses to noradrenaline (NA;Thorin & Atkinsson, 1994) or to electrical field stimulation (EFS; Vo *et al.*, 1991) where enhanced by inhibitors of the NO-synthase. In TA α_1- and α_2-adrenoceptors participate in the NA induced vasoconstriction (Medgett, 1985). Thus, we aimed to study :i) if responses to exogenous and endogenous NA were influenced to the same extent by L-NAME and ii) if the α_2-adrenoceptor component of the NA response could play a role on the potentiation by L-NAME.

Sprague-Dawley rats were killed by decapitation. TA rings were set up in an organ bath under 7.35 mN resting tension. After a 30 min equilibration period, rings were contrated 3 times with 75 mM KCl. After 30 min each ring was contracted with 0.3 μM NA and relaxed with 1-3 μM acetylcholine to verify the functional state of the endothelium. In a few rings the endothelium was removed. Only rings that relaxed by more than 60% were considered to have intact endothelium. Concentration response curves to phenylephrine (PHE) or frequency response curves to EFS (0.3 msec, 40 V, 0.1-5Hz) control or after 30 μM L-NAME were carried out in absence or presence of 0.1 μM yohimbine. EFS was also applied to a 1 μM PHE contracted vessel, in presence of 1 μM TTX. The influence of L-NAME or endothelial removal was also analyzed. Results obtained with NA are displayed in Table 1.Vasoconstriction induced by EFS ($p<0.01$) but not by NA was enhanced by L-NAME. Yohimbine did not modify the potentiation by L-NAME. EFS (5-20 Hz) relaxed the PHE contracted vessel. L-NAME and the lack of endothelium abolished the relaxation-induced by EFS. These results suggest that α_2-adrenoceptor do not participate in the potentiation of EFS responses by L-NAME. The observed potentiation could be ascribed,at least in part, to the release of NO from endothelium. However, the possibility that exogenous and endogenous NA could interact with different populations of α_1-adrenoceptors and so influenced in a different way by L-NAME should not be excluded.

Table 1 Parameter estimates for noradrenaline induced vasoconstriction . (**$p<0.005$ control responses in absence *vs* presence of yohimbine)

	E_{max} (mN)	pEC_{50}	n
Without yohimine			
Control	11.20±1.40	6.20±0.08**	6
L-NAME	12.00±1.39	6.18±0.08	6
With yohimbine			
Control	12.35±1.60	5.96±0.09	6
L-NAME	13.90±1.60	5.92±0.09	

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19P PACING-INDUCED HEART FAILURE UPREGULATES ENDOTHELIAL NITRIC OXIDE SYNTHASE IN LEFT ATRIUM BUT NOT VENTRICLES

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In humans with congestive heart failure, the alterations in expression of cardiac nitric oxide synthase (NOS) isoforms is unclear (DeBelder et al., 1993, 1995; Haywood *et al.*, 1996; Thoenes *et al.*, 1996). To date, no study has determined in unison the activity and expression of both endothelial NOS (eNOS) and inducible NOS (iNOS) in the failing heart and how this may be reflected by changes in plasma nitrate/nitrite (NO_x^-) levels, a measure of total NO production. Moreover, the cardiac chamber specificity of changes in NOS is unknown. We determined this in an experimental model of pacing-induced heart failure in dogs.

Adult male mongrel dogs (23±1 kg) were paced via the right ventricle at 250 beats·min^{-1} for 21 days (n=7) and compared to dogs paced for 2 days (n=5) and non-paced control animals (n=8). Hemodynamic and echocardiographic assessments were done before the initiation and within 30 min after the cessation of pacing. Thereafter, dogs were anaesthetized with pentobarbital (50 mg·kg^{-1} i.v.) and left atrial and ventricular tissues were used for measurement of NOS activities by the conversion of [^{14}C]-L-arginine to [^{14}C]-citrulline (Khadour *et al.*, 1997). Cardiac eNOS expression was determined by Western blot analysis and quantified by densitometry. Plasma NO_x^- and creatinine levels were also measured. Data are expressed as mean ± s.e.mean. One-way ANOVA with Tukey's post hoc test were used to assess statistical significance ($P<0.05$).

Heart failure was evidenced by both hemodynamic changes and clinical signs after 21 days but not 2 days of pacing (45% reduction in left ventricular dP/dt and 55% decrease in ejection fraction; 35% increase in heart rate with clinical evidence of ascites and edema). Plasma NO_x^- did not change after pacing for 2 days (21±4 μmol·l^{-1}) from a pre-pacing level of 19±1, but rose significantly after both 7 (43±6) and 21 (31±2) days of pacing. Plasma creatinine levels were not significantly altered. In controls, left atrial Ca^{2+}-dependent NOS activity was significantly greater than that of right or left ventricular activities (0.45±0.03, 0.29±0.04 and 0.24±0.03 pmol·min^{-1}·mg^{-1} protein, respectively). Left atrial Ca^{2+}-dependent NOS activity significantly increased by 50% after 21 days, but not after 2 days of pacing (0.68 ± 0.01 and 0.41±0.11 pmol·min^{-1}·mg^{-1} protein, respectively). The expression of eNOS protein in left atrial tissues was also enhanced at 21 days and was due to greater levels of the 150-kDa, but not the 135-kDa, protein. These findings were accompanied by signs of atrial but not ventricular hypertrophy after 21 (but not 2) days of pacing. Pacing for 2 or 21 days was not associated with detectable changes in ventricular Ca^{2+}-dependent NOS activity or eNOS expression. Ca^{2+}-independent NOS activity (iNOS) was not detected in any cardiac tissue.

In dogs with pacing-induced heart failure there is enhanced production of NO in the left atrium which is associated with upregulation of the 150-kDa eNOS. This may play a role in the structural and/or functional changes seen in this chamber.

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20P LONG-TERM L-ARGININE ADMINISTRATION REDUCES NEPHROSCLEROSIS IN UNINEPHRECTOMIZED SPONTANEOUSLY HYPERTENSIVE RATS

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Progressive renal dysfunction is characterized by the development of glomerulosclerosis and secondary renal interstitial fibrosis (Diamond & Karnovsky, 1988). We have investigated if chronic changes in nitric oxide (NO) synthesis are able to reduce or exacerbate renal damage in a rat model with renal dysfunction.

Female uninephrectomized spontaneously hypertensive rats (UNX-SHRs) were treated during six months with D-or L-arginine (D-or L-ARG, 1mg/kg/day in drinking water) alone or in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 1mg/kg/day the first month and 0.5mg/kg/day the next five months in the drinking water). Mean blood pressure (MBP) was measured in conscious UNX-SHRs by the tail-cuff procedure. Plasma nitrite (NO_2^-) and proteinuria (Prot.) were analyzed respectively by the Griess reaction (1879) and the Bradford method (1976). At the end of the study, 5μm sections of the remnant kidney were stained with syrium red for morphometric evaluation of the glomerular and tubulo-interstitial fibrosis. Digital image analysis was performed with a specific software (Fibrosis HR®, Master Diagnóstica, Granada, Spain).

Table1 Effects of different treatments on MBP (mm Hg), NO_2^-(μM) and Prot. (mg/24h) expressed as mean ± SEM (ANOVA plus Scheffe's test).

	n	MBP	NO_2^-	Prot.
D-ARG	12	203±7	13.5±2.5	3.9±0.6
L-ARG	13	214±7	22.6±2.0^a	4.0±0.5
L-NAME+D-ARG	14	261±5^{ab}	12.0±1.7^b	5.6±0.8
L-NAME+L-ARG	14	261±6^{ab}	12.2±2.3^b	6.4±1.1

a P<0.05 versus D-ARG; b P<0.05 versus L-ARG.

In the L-ARG group glomerular size and interstitial fibrosis area were significant reduced when compared with other three treated-groups (Table 2).

Table 2 Effects of different treatments on glomerular size (GS; mean ± SEM; Newman-Keuls multiple-comparison test) and fibrosis area (F.A.) (median; Kruskal-Wallis multiple-comparison z-value test)(n=9).

		F.A.	
	GS (mm^2 x 10^{-3})	glomerulus (mm^2 x 10^{-3})	interstitium (mm^2 x 10^{-3})
D-ARG	12.87±0.18	3.51	8.84
L-ARG	11.94±0.22^a	3.49	8.45^a
L-NAME+D-ARG	12.73±0.24^b	3.70^{ab}	9.48^{ab}
L-NAME+L-ARG	12.27±0.24	3.64^b	9.30^b

aP<0.05 versus D-ARG; b P<0.05 versus L-ARG.

These results show that chronic treatment with a low dose of L-ARG is able to reduce histological renal damage, a response which is not necessary linked with MBP and proteinuria.

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21P MECHANISMS CONTRIBUTING TO THE INTERACTION OF DIAZEPAM WITH THE CASCADE OF EVENTS FOLLOWING β-ADRENOCEPTOR STIMULATION IN ISOLATED ATRIA

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In previous *in vivo* studies on the reversal of chloroquine poisoning, an enhanced positive inotropic effect was observed with combined administration of diazepam and adrenaline (Hughes & Coker, 1997). The aim of the present study was to investigate the mechanisms by which diazepam may potentiate the positive inotropic response to β-adrenoceptor stimulation in isolated cardiac tissue.

The left atria of male Wistar rats (330±3g) which had been given heparin (1000 units kg^{-1} i.p.) then killed by a blow to the head followed by exsanguination, were isolated, suspended in oxygenated Krebs solution at 37°C and electrically stimulated at 1Hz. Developed tension (mN) was measured with isometric force transducers. All tissues (n=6 for each drug and control group) were incubated with 3μM phenoxybenzamine, 3μM indomethacin and 30μM 8-(*p* sulfophenyl) theophylline, to eliminate extraneuronal uptake, inhibit prostaglandin synthesis, and block adenosine receptors respectively.

(a) Cumulative concentration-response curves for isoprenaline were obtained in the presence (after 15 min incubation) of 10μM diazepam or its vehicle, propylene glycol (1% v/v).
(b) In separate tissues, propranolol (1μM) was added to the Krebs solution, and concentration-response curves for forskolin were obtained in the presence of 10 μM diazepam or vehicle.
(c) In further studies, 2-deoxyadenosine (100μM), an adenylyl cyclase inhibitor, was included and concentration-response curves for dibutyryl-cAMP were constructed in the presence of 10μM diazepam or vehicle.

Curves were fitted to individual concentration-response curves by a 4 parameter logistic equation, and EC_{50} values determined.

Table 1. EC_{50} values for isoprenaline, forskolin and dibutyryl-cAMP in the presence of either diazepam (10μM) or propylene glycol (1% v/v).

	Vehicle	Diazepam
(a) Isoprenaline(nM)	1.6 (0-4.8)	0.4 (0.2-8.4)*
(b) Forskolin (nM)	348 (0.05-1000)	64 (19-130)*
(c) dibutyryl-cAMP(μM)	26 (10-47)	31 (10-64)

Values are geometric mean (95% Confidence Intervals), n=6. *P<0.05; two-tailed Mann-Whitney U test versus vehicle control.

Diazepam (10μM) produced a leftward shift of the isoprenaline concentration-response curve, indicated by a reduction in EC_{50} (Table 1), a response which was not mediated via effects upon extraneuronal uptake, prostaglandin synthesis or stimulation of adenosine receptors. Propranolol did not abolish the leftward shift, by diazepam, of the forskolin concentration-response curve. However, diazepam failed to potentiate the effects of dibutyryl-cAMP in the presence 2-deoxyadenosine. This suggests that diazepam-induced potentiation of the inotropic response to β-adrenoceptor agonism is not due to direct or indirect stimulation of β-adrenoceptors, but is dependent upon functional adenylyl cyclase.

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22P INVOLVEMENT OF DELTA OPIOID RECEPTOR SUBTYPES IN ISCHAEMIC PRECONDITIONING AGAINST INFARCTION IN THE RAT ISOLATED HEART

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Recent studies have suggested that opioid receptors could play a pivotal role in ischaemic preconditioning (PC) against infarction in the rat heart in situ (Schultz et al., 1995). Activation of the delta (δ) opioid receptor subtype was shown to elicit increased survival time during hypoxia in mice (Mayfield & D'Alecy, 1994). The present study sought to examine whether stimulation of δ-receptors is involved in PC in isolated rat hearts, by attempting to inhibit protection with the selective δ-receptor antagonist naltrindole (NTL), and to mimic it with the δ-receptor agonist [D-ala[2], D-leu[5]] enkephalin (DADLE).

Male Long-Evans rats (260-340g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and heparinized (200 units i.p.). Hearts were rapidly excised and perfused in the Langendorff mode with modified Krebs buffer (37 C; 95% O_2/ 5% CO_2) at constant pressure (100 cm H_2O). Left ventricular developed pressure was measured using a water-filled balloon coupled to a pressure transducer. This was inserted into the left ventricle and diastolic pressure set at 4 mmHg. A ligature was placed around the left coronary artery for the induction of regional ischaemia. After 15 min stabilization, hearts were assigned randomly to one of six treatment groups of n=6: Control,PC (2 x 5 min of global ischaemia interspersed with 5 min reperfusion), NTL (0.1 μM), PC + NTL, DADLE (0.01 μM; 2 x 5 min of drug perfusion interspersed with 5 min drug-free periods) or DADLE 1.0 μM. NTL was administered 5 min prior to the PC protocol until the start of the test ischaemia. After treatment, the coronary artery was occluded for 35 min followed by 120 min reperfusion. Infarct size , as a percentage of the ischaemic risk zone, was then assessed by tetrazolium staining as described previously (Bugge & Ytrehus, 1995).

The results are presented in Table 1. PC significantly reduced infarct size compared to control. The δ-receptor antagonist NTL reversed this protective effect of ischaemic PC but had no influence on infarct size in controls. Pharmacological PC with DADLE 0.01μM mimicked the cardioprotective effects of ischaemic PC. However, protection against infarction was less marked with the higher concentration of DADLE (1 μM). There were no statistically significant differences in haemodynamic variables with respect to treatment.

Table 1. Infarct size (% of risk zone)

Group	Infarct size
Control	50.6 ± 1.9
PC	18.5 ± 1.9*
NTL 0.1 μM	48.6 ± 3.5
PC + NTL 0.1 μM	47.0 ± 4.3
DADLE 0.01 μM	20.1 ± 2.4*
DADLE 1.0 μM	35.4 ± 3.0*

mean ± s.e.mean. *P< 0.05 compared with control, 1-way ANOVA with Bonferroni correction.

These data indicate that ischaemic PC is mediated through δ-opioid receptors in the isolated rat heart, since the protection afforded was abrogated by NTL and mimicked by a low concentration of DADLE. The mechanism behind the attenuated protection observed with the higher concentration of DADLE warrants further investigation.

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23P EFFECTS OF THE NOVEL CARDIOSELECTIVE K_{ATP} CHANNEL BLOCKER HMR 1883 ON K_{ATP} CHANNELS IN GUINEA PIG HEARTS AND ON RAT PANCREATIC β-CELLS

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Hypoxia induces opening of ATP-sensitive (K_{ATP}) channels in heart muscle cells, facilitating the generation of reentrant arrhythmias. It was demonstrated that the sulfonylurea glibenclamide significantly reduced the incidence of ventricular fibrillation in a canine model of sudden cardiac death (Billman *et al.* 1993). However, glibenclamide is not selective for cardiac cells. In order to obtain a cardioselective K_{ATP} channel blocker we developed the sulfonylthiourea HMR 1883 (1-[[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methyl -thiourea) and compared its effects in cardiac and pancreatic tissues with those of glibenclamide.

Isolated ventricular myocytes were prepared from guinea pig hearts and investigated in the whole-cell mode of the patch-clamp technique. K_{ATP} channels were opened by 1-10 µM rilmakalim. Action potentials were induced by field-stimulation and were recorded under current-clamp conditions. The action potential duration at 90% repolarization (APD_{90}) was calculated. The extracellular pH was 6.5.

HMR 1883 and glibenclamide had no effects in the absence of rilmakalim, but inhibited the rilmakalim-activated current in a dose-dependent manner, yielding half-maximal inhibition with 0.8 µM and 8 nM, respectively. When action potentials were recorded, 0.25 µM rilmakalim shortened the APD_{90}, which was antagonized half-maximally with 0.4 µM HMR 1883 and 10 nM glibenclamide, respectively.

Isolated guinea pig hearts were perfused in the Langendorff mode. The effect of hypoxia was investigated by gassing the perfusion solution with 20% O_2, 75% N_2 and 5% CO_2. Under this condition the coronary flow (CF) increased from 12.2±0.9 ml/min to 16.4±0.6 ml/min (mean±s.e.m., n=5). Subsequent addition of 1 µM glibenclamide caused a reversible decrease of CF to 11.4±0.8 ml/min. In contrast, 1 µM HMR 1883 had no significant effect (hypoxia:15.7±0.6 ml/min, +drug: 15.9±0.6 ml/min, $p>0.05$, n=4) and 10 µM inhibited the hypoxia-induced increase in CF slightly (hypoxia:18.7±0.4, +drug: 17.0±0.7 ml/min, $p<0.05$, n=7).

Effects on pancreatic β-cells were investigated in the rat insulinoma cell RINm5F by means of the whole-cell configuration of the patch-clamp technique. The cell potential (PD) was recorded in the current-clamp mode. In order to obtain stable PDs, all experiments were performed in the presence of 100 µM diazoxide, yielding a mean PD of -80.2±1.4 mV (n=11) in the absence of drugs. Application of glibenclamide depolarized the PD dose-dependently with a half-maximal concentration of 9 nM. In contrast, more than 10 µM HMR 1883 was needed to cause half-maximal depolarization.

In conclusion, HMR 1883 inhibits K_{ATP} channels in cardiac cells at concentrations below 1 µM. HMR 1883 (1 µM) has no effect on the CF under hypoxic conditions, nor does it depolarize rat pancreatic β-cells. Therefore, this cardioselective K_{ATP} channel blocker is considered to be a promising substance for the prevention of ischaemia-induced ventricular fibrillation in hearts.

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24P THE K_{ATP} CHANNEL BLOCKER HMR 1883 ATTENUATES THE EFFECTS OF ISCHAEMIA ON MAP DURATION AND IMPROVES SURVIVAL DURING LAD OCCLUSION IN THE ANAESTHETISED PIG

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K_{ATP} channels are activated when myocardial tissue becomes ischemic. Activation of K_{ATP} channels is thought to be involved in the shortening of action potential duration (APD) during myocardial ischemia (Wilde&Janse, 1994). Regional ischemia results in heterogeneity in $[K^+]_o$ and in APD dispersion both considered to be proarrhythmic mechanisms affecting impuls generation and propagation (Billman, 1994). Consequently, blockade of K_{ATP} channels may be antiarrhythmic by selectively prolonging APD in the ischemic region and, thus, ameliorating the differences in the APD. Therefore, we investigated the in vivo-effects of HMR 1883, 1-[[5-[2-[5-chloro-o-anisamido) ethyl]-2-methoxyphenyl]sulfonyl]-3-methyl-thiourea, recently identified as a selective blocker of cardiac K_{ATP} channels, on ischemic APD and on the rate of survival in a model of sudden cardiac arrhythmic death induced by regional ischemia.

The effect on APD was assessed by recording epicardial monophasic action potentials (MAP) from the ischemic region in pentobarbitone-anesthetized pigs (n=14, male, 22-35 kg) subjected to 3 occlusion (5')-reperfusion (15') cycles of a small branch of the circumflex coronary artery. Ischemia shortened MAP_{50} duration in the control group (n=7) by 51±4 ms. HMR 1883 (3 mg/kg i.v.) given 5 min before the 3rd occlusion attenuated the shortening to 21±5* ms ($P<0.05$,Student's t test).

The effects of HMR 1883 on ischemia-induced fatal arrhythmias were determined in pentobarbitone-anesthetized pigs (n=12) with LAD occlusion-reperfusion (20 and 30 min). HMR 1883 (3 mg/kg i.v.) reduced the incidence of ventricular fibrillation during ischemia as compared to the control group (Figure 1), however, reperfusion arrhythmias were not affected. In this model of sudden cardiac death, flecainide (2 mg/kg i.v.) showed a strong proarrhythmic effect. No animal survived the first 5 min of the LAD occlusion.

HMR 1883 had no effect on the ECG before the occlusion, however, during ischemia the ST segment depression was attenuated as compared to the control group (0.11 ± 0.05* vs. 0.35 ± 0.06 mV at 5 min). Moreover, the ischemia induced prolongation of the QJ-time was also significantly attenuated (15 ± 3* ms vs. 43 ± 5 ms).

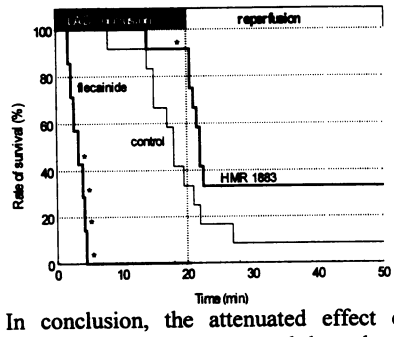

Figure 1. Survival rate during occlusion and reperfusion. *P<0.05, compared to control, Fisher's exact probability test. N=12 for control and HMR 1883, and N=7 for flecainide.

In conclusion, the attenuated effect of ischemia on MAP duration and ST-segment, and the reduced mortality with HMR 1883 suggest that blockers of cardiac K_{ATP} channels could have beneficial effects in the treatment of ischemia-related arrhythmias and sudden cardiac death.

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Billman, G.E. (1994) *Cardiovasc. Res.* 28:762-769

25P EFFECTS OF THE CARDIOSELECTIVE K_{ATP} CHANNEL BLOCKER HMR 1883 ON VENTRICULAR FIBRILLATIONS IN CONSCIOUS POSTINFARCTED DOGS

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The activation of ATP-sensitive potassium channels (K_{ATP}) during myocardial ischemia leads to potassium efflux and reductions in action potential duration. The resulting heterogeneity in refractory period could alter impulse conduction and thereby trigger ventricular fibrillation (Billman, 1994). Drugs that inactivate the K_{ATP} should attenuate these changes in electrical conduction and thereby prevent malignant arrhythmias. However, most K_{ATP} antagonists also alter pancreatic and vascular channels. Recently, a novel cardioselective K_{ATP} antagonist, HMR 1883, 1-[[5-[2-[5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methyl-thiourea has been developed which may offer cardioprotection without the untoward effects of existing compounds. Therefore, HMR 1883 was evaluated for its effects on the susceptibility to ventricular fibrillation (VF) using an unanesthetized canine model of sudden death in comparison with the non-selective antagonist glibenclamide.

Thirty-five mongrel dogs (15.4-19.1 kg) were anesthetized and instrumented to measure left circumflex coronary blood flow, left ventricular pressure and ventricular electrogram as previously described (Billman & Hamlin, 1996). A hydraulic occluder was also placed around the circumflex artery and an anterior wall infarction was made by ligating the left anterior decending artery. After a 3 week recovery period, the dogs were classified as to the susceptibility to VF by a 2 min coronary occlusion during the last min. of exercise (Billman & Hamlin, 1996). Eighteen animals had VF (susceptible) while 17 did not (resistant). On subsequent day the exercise plus ischemia test was repeated (susceptible dogs) after pretreatment with either HMR 1883 (3.0 mg/kg i.v. n=13) or glibenclamide (1.0 mg/kg i.v., n=7). Both HMR 1883 and glibenclamide prevented VF (Fisher's Exact test $p<0.01$) in 11 of 13 and 6 of 7 animals, respectively.

Blood glucose and plasma insulin were evaluated in fasted (16 h prior to food) dogs. Glibenclamide elicited significant (ANOVA $p<0.01$) decreases in blood glucose (4.20 ± 0.08 mmol/l to 2.75 ± 0.14 mmol/l after 240 min, n=4) whereas after HMR 1883 blood glucose remained unchanged (4.44 ± 0.18 mmol/l to 4.57 ± 0.11 mmol/l over 240 min, n=6). Plasma insulin in the HMR 1883 group (n=6) increased slightly but non-significantly from 13.4 ± 1.1 to 18.0 ± 2.9 µEq/ml after 10 min whereas glibenclamide (n=4) increased plasma insulin significantly from 8.7 ± 1.45 to 38.7 ± 5.64 µEq/ml after 20 min.. Glibenclamide also significantly reduced the reactive hyperemia induced by a 15 s coronary occlusion (control 427 ± 102 %, glibenclamide 311 ±73 % payback, a 30 % reduction) while HMR 1883 did not affect payback (415 ± 133 %).

In summary, the blockade of myocardial K_{ATP} channels can prevent ventricular fibrillation. Furthermore, HMR 1883, in contrast to glibenclamide, protects against these malignant arrhythmias without altering either coronary vascular (no effects on reactive hyperemia) or pancreatic (no significant effects on insulin or glucose) K_{ATP} channels.

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26P THE BENEFIT OF ISCHAEMIC PRECONDITIONING IS NOT ABOLISHED BY THE CARDIOSELECTIVE K_{ATP} CHANNEL BLOCKER HMR 1883 IN RABBITS

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The benefit of ischaemic preconditioning (PC) is abolished by nonselective K_{ATP}-blockade with glibenclamide (GLIB) (Toombs *et al.*, 1993). Newly developed cardioselective K_{ATP}-blockers such as the sulfonylthiourea HMR 1883 (1-[[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methylthiourea) reveal a marked antifibrillating activity in different experimental models. In infarct studies in rabbits we investigated the effects of the novel cardioselective K_{ATP}-channel blocker, HMR 1883, on PC and compared them with the nonselective K_{ATP}-blocker GLIB.

New Zealand White rabbits of either sex (2.2 to 3.0 kg) were anaesthetized with an intramuscular injection of ketamine (50 mg/ml) and xylazine (10 mg/ml) solution at a volume of 0.6 ml/kg body weight. Anaesthesia and fluid volume was maintained by constant infusion of xylazine, 2 mg/ml in heparinized saline, 0.15-0.25 ml/min via the jugular vein. After tracheotomy and artificial ventilation with room air and supplementation with carbogen (tidal volume: 10 ml/kg frequency: 40 inflations/min), thoracotomy was performed and the animals subjected to a 30 min occlusion period of a branch of the left descending coronary artery (LAD) followed by 2 hours reperfusion. For PC experiments LAD was additionally twice occluded for 5 min followed by 10 min reperfusion before the long-lasting ischemia. At the end of the experiment the left ventricle was cut in slices and infarct mass was evaluated by TTC staining and expressed as a percentage of area at risk. The rabbits were randomly selected (n=7/group) to receive (i.v.) saline vehicle, GLIB (0.3 mg/kg) or HMR 1883 (3 mg/kg) 5 min before occlusion or 5 min before the first PC. The doses used were found to be antifibrillating in different experimental models in rats, pigs and dogs. Statistics: Multiple sample groups were compared by means of the distribution-independent Kruskal-Wallis H test followed by the nonparametric Mann-Whitney *U* test, where $P<0.05$ was considered statistically significant. Data are expressed as mean ± s.e.mean.

Myocardial risk mass as a percentage of left ventricular mass did not differ between groups and ranged from 56±4% to 59±5%. The same was true for the ratio of left ventricular mass to 100 g body weight (79±2% and 93±4%, respectively). Myocardial infarct mass as a percentage of area at risk in the saline vehicle group without PC was 41±3% and in the PC vehicle group 21±4% ($P<0.05$). GLIB given prior to PC or prior to the long ischemia totally reversed the PC effect (42±2% and 55±4%, respectively). In contrast, HMR 1883 under the same conditions did not interfere with PC (21±3% and 26±2%, respectively).

The novel antifibrillatory compound, HMR 1883, a K_{ATP}-channel blocker with a predominant effect on potassium channels in cardiomyocytes, did not abolish PC in rabbits measured as myocardial infarct mass, whereas GLIB totally reversed this powerful endogenous cardioprotective mechanism. This suggests that the sarcolemmal ATP-sensitive potassium channels are not primarily involved in the mechanism of PC. From these results we suggest that GLIB possibly acts intracellularly on mitochondrial K_{ATP}-channels, which could be responsible for PC.

Toombs, C.T., Moore, T.L, Shebuski, R.J., (1993) Cardiovasc. Res. 27:617-622.

27P POTASSIUM CHANNEL MODULATION BY CIS-5,8,11,14,17-EICOSAPENTAENOIC ACID IN RAT PORTAL VEIN CELLS

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Using whole-cell voltage clamp conditions, Asano *et al.* (1997) demonstrated that the ω-3 polyunsaturated fatty acid cis-5,8,11,14,17-eicosapentaenoic acid (EPA) activated a slowly-developing K^+ current in a rat smooth muscle (A7r5) cell line. Although these workers showed that the current was TEA- and Cs^+-sensitive, no detailed examination of its properties was carried out. The aim of the present study was to clarify the effects of EPA on vascular smooth muscle K^+ channels.

Portal vein cells from male Sprague-Dawley rats (200-250g) were isolated by enzymatic treatment. The effects of 10 μM EPA (10 min exposure) on K^+ currents were recorded using whole-cell voltage-clamp conditions under a quasi-physiological K^+ gradient at room temperature. Voltage-sensitive K^+ currents were elicited by stepping from -90 mV to a series of test potentials (-80 to +50 mV, 10mV increments). Cells were held at -10 mV (to inactivate voltage-sensitive K^+ currents) and then stepped to the same test potentials to determine the effect of EPA on currents carried by large-conductance, Ca^{2+}-sensitive K^+ channels ($I_{BK(Ca)}$) or those ($I_{K(ATP)}$) induced by 10 min exposure to the ATP-sensitive K^+ channel opener, levcromakalim (LK, 3.3 μM). All values are mean ± s.e.mean.

As shown in Table 1, EPA produced a large increase in an outward current with little effect on the zero-current level. Under Ca^{2+}-free conditions, in the one cell in which it was tested, a current of similar magnitude at +50 mV was induced by EPA (control, 355.4 pA; EPA, 2181 pA). The EPA-induced current was fully reversed by 200 nM iberiotoxin, consistent with induction of $I_{BK(Ca)}$ by EPA. Inhibition of levcromakalim-induced $I_{K(ATP)}$ by EPA was evident at -40 mV (negative to the activation threshold potential of BK_{Ca}) but was masked at +50 mV by the induced $I_{BK(Ca)}$. Difference currents suggested that EPA had also inhibited Ca^{2+} channels.

	Zero-current potential (mV)	Current (pA) at test potential	
		-40 mV	+ 50 mV
control (n=7)	-31 ± 3	-4 ± 2	228 ± 60
EPA (n=7)	-36 ± 3	-7 ± 8	1967 ± 276
control (n=3)	-43 ± 3	1 ± 1	128 ± 14
LK (n=3)	-60 ± 5	41 ± 19	292 ± 85
LK+EPA(n=3)	-48 ± 5	8 ± 5	1384 ± 284

Table 1: Effect of EPA (10 μM) and LK (3.3 μM) on currents elicited by stepping to potentials from -10 mV.

Stepping from -90 mV to -20 mV (a potential negative to the activation threshold for BK_{Ca}) induced a voltage-sensitive (delayed-rectifier) K^+ current, $I_{K(V)}$, which was inhibited by EPA (control, 78 ± 18 pA; EPA, 14 ± 13 pA; n=4).

EPA shows a complex profile of activity similar to that of NS1619 in rat portal vein cells:- calcium-independent activation of BK_{Ca} and inhibition of $I_{K(V)}$, $I_{K(ATP)}$ and I_{Ca} (Edwards et al., 1994). Further experiments are in progress to elucidate the underlying mechanism.

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Edwards, G., Niederste-Hollenberg, A., Schneider, J. *et al.* (1994) *Br. J. Pharmacol.* 113, 1538-1547.

28P EFFECTS OF THE α_2 ADRENOCEPTOR AGONIST UK 14304 ON LIPOGENESIS IN MICE FOLLOWING CHRONIC ETHANOL TREATMENT

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UK 14304, a highly selective α_2 agonist, has been shown to inhibit adipose tissue hormone sensitive lipase (HSL), a key enzyme of lipolysis, via a reduction in cAMP levels. Chronic ethanol treatment increases the sensitivity of mice to the effects of UK 14304 (Shih & Taberner, 1995). In addition, chronic ethanol treatments have been shown to produce dose dependent changes in lipid metabolism: brown adipose tissue (BAT) HSL was reduced, white adipose tissue (WAT) HSL increased, (Shih & Taberner, 1997).

Two groups of 12 adult, male CBA/Ca mice were given pelleted rodent diet *ad lib* with either water (control) or 24% (v/v) ethanol (CED) for 4 weeks; a further two groups received either a control liquid diet (CLD) or ethanol (7% v/v) liquid diet (ELD), *ad lib* for 5 days (Jelic *et al.*, 1998). UK 14304 or saline, as control, was administered i.p., one hour prior to measurement of lipogenesis, at 2mg/kg to control and CLD fed animals and 1mg/kg to ethanol treated animals. *In vivo* lipogenesis was assessed by measuring the incorporation of $[^3H]$ into total lipid extracted from BAT and WAT samples removed one hour after administration of $[^3H]$-H_2O and expressed as μg atoms H h^{-1} mg $tissue^{-1}$ (Mercer & Trayhurn, 1983). Differences between groups (means ± s.e.mean) were analysed by Student's t test.

UK 14304 reduced lipogenesis in BAT and WAT in ethanol treated and control mice. BAT lipogenesis was significantly reduced by UK 14304 in non-ethanol-drinking control mice: 90.0 ± 19.7 vs 8.32 ± 1.32 ($P < 0.01$) and in CED mice: 40.3 ± 2.8 vs 15.2 ± 4.7 ($P < 0.05$). UK 14304 significantly depressed WAT lipogenesis in control animals from 8.85 ± 1.19 to 3.13 ± 0.40 ($p<0.01$).

Ethanol drinking (CED) reduced BAT lipogenesis but had little effect on WAT lipogenesis, whereas the ELD decreased lipogenesis in both BAT and WAT. WAT lipogenesis was significantly greater ($P < 0.05$) in the CLD group 46.1± 8.8, compared to the pellet fed controls 13.2 ± 4.4, possibly due to the higher fat intake of the CLD group. Both ethanol regimes significantly reduced BAT tissue weight (mg): 93.9± 6.1 vs 51.5 ±2.6 in CED, 93.8 ±8.5 vs 51.1± 3.9 in ELD ($P < 0.001$). The ELD also reduced the BAT % lipid: 54.9 ± 4.8 vs 27.7 ± 2.7 ($P < 0.001$). BAT % lipid content was significantly higher in CLD mice, 54.9 ± 4.8, compared to pellet fed controls, 32.2 ± 3.0, reflecting the difference in lipogenesis, but there was no difference in BAT weight.

We conclude that UK 14304 and low doses of ethanol have an additive effect to suppress lipogenesis and lipolysis in BAT and that this tissue is more sensitive to ethanol than WAT.

CA Williams is a Daphne Jackson Research Fellow.

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Shih, M.F. & Taberner, P.V. (1997) *Br. J. Pharmacol.* 120, 721-727.

29P CALCIUM-DEPENDENT INHIBITION OF AGONIST-STIMULATED CYCLIC AMP ACCUMULATION IN HUMAN U373 MG ASTROCYTOMA CELLS

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Ca^{2+}-sensitive isoforms of adenylyl cyclase may play an important role in the interaction between Ca^{2+} and cAMP signalling pathways (Cooper *et al.*, 1995). Such 'cross-talk' could be of particular interest in glial cells, in which cyclic AMP can regulate the secretion of cytokines and trophic factors. We present evidence here that the human U373 MG astrocytoma cell line, which has several of the properties of Type 2 astrocytes, possesses a Ca^{2+}-inhibitable isoform of adenylyl cyclase.

U373 MG cells were cultured as described previously (Young *et al.* 1998) and seeded onto multiwell plates. Sub-confluent monolayers were incubated at 37°C for 3 h in DMEM F12 medium containing 3 μCi (0.12 nM) [^{3}H]-adenine and then equilibrated for 10 min in Ca^{2+}-free HEPES medium containing 0.5 mM isobutylmethylxanthine (IBMX). Stimulation with agonists for 30 sec to 10 min in HEPES medium (in mM: NaCl 152.5, KCl 5.4, $CaCl_2$ 1.8, NaH_2PO_4 1, $MgSO_4$ 0.8, glutamine 0.6, glucose 5.5, HEPES 5) was terminated by addition of 5% trichloroacetic acid (final concentration) and [^{3}H]-cAMP separated by the method of Salomon *et al.*, (1974). Correction for variations in cell density between wells was made by measuring the amount of [^{3}H]-ATP formed and corrections were normally also made for the recovery of ATP and cyclic AMP.

Basal accumulations of cyclic AMP in U373 MG cells did not differ consistently in the presence or absence of 1.8 mM Ca^{2+} with or without 5 μM thapsigargin. Forskolin, 10 μM, acting alone produced a 10 ± 1 fold stimulation of cyclic AMP accumulation over a 4 min incubation period (n=3). However, the response to 10 μM forskolin + 5 μM thapsigargin in normal Ca^{2+}-containing medium was only 34 ± 2% (n=3) of the response to forskolin + thapsigargin when Ca^{2+} was omitted from the medium. Since phosphodiesterase activity is blocked by IBMX, the implication of this observation is that store-refilling-induced Ca^{2+} entry results in inhibition of the cyclase. Thapsigargin (5 μM) also inhibited cyclic AMP accumulation induced by 1 μM isoprenaline in U373 MG cells (EC_{50} for isoprenaline 0.10 ± 0.01 μM, maximum response after 4 min incubation 12 ± 2 fold of basal). The extent of the inhibition (68 ± 2% inhibition, n=3) was similar to that of the response to forskolin and was apparent at all times measured between 30 s and 10 min.

Histamine stimulates an increase in intracellular Ca^{2+} in U373 MG cells via activation of H_1-receptors, although the initial peak response can return to basal levels within 2-3 min (Young *et al.*, 1998). Histamine (100 μM) on its own had no significant effect on the level of cyclic AMP, but inhibited the response to 1 μM isoprenaline in normal Ca^{2+}-containing medium, measured over a 1 min incubation period, by 65 ± 3% (3). The IC_{50} for the inhibition by histamine was 1.3 ± 0.3 μM. The inhibition produced by 10 μM histamine was reversed by 1 μM mepyramine, although paradoxically mepyramine itself caused some inhibition of the response to isoprenaline alone.

These data provide evidence for the presence of at least one Ca^{2+}-sensitive isoform of adenylyl cyclase in human U373 MG astrocytoma cells, which can be inhibited by agonists, such as histamine, coupled to phosphoinositide hydrolysis and hence Ca^{2+} mobilisation and store-refilling-induced Ca^{2+} entry.

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Salomon Y. *et al.* (1974) *Anal. Biochem.* **58** 541-548.
Young K.W. *et al.* (1998) *Br. J. Pharmacol.*, **123**, 545-557.

30P CYCLIC AMP AND cGMP DIFFERENTIALLY REGULATE THE PRODUCTION OF THE CALCIUM-MOBILISING METABOLITES, NICOTINIC ACID ADENINE DINUCLEOTIDE PHOSPHATE AND CYCLIC ADP-RIBOSE

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Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are potent intracellular calcium releasing agents (Genazzani and Galione, 1997). In order that cADPR and NAADP may be established as endogenous messengers for calcium release, the existence of intracellular enzymes capable of metabolising these molecules must be demonstrated. In addition intracellular levels of cADPR and NAADP should be under the control of extracellular stimuli. The aim of this study was to determine whether mammalian cells and sea urchin eggs possess the enzymatic machinery to metabolise NAADP and cADPR. Since cGMP stimulates the synthesis of cADPR in the sea urchin egg (Galione et al, 1993), we investigated how these enzymes may be under the control of receptor-coupled second messengers.

Sea urchin egg homogenates were prepared as described previously (Sethi et al, 1996) and rabbit (2.5kg male New Zealand) artery homogenates by glass dounce homogenisation (pestle D) and centrifugation (2,000 *g*, 12 min) in 250mM sucrose and 20mM HEPES. Cyclic ADPR or NAADP synthesis was detected by incubating homogenates with the substrates β-NAD^+ (2.5mM) or β-$NADP^+$ (250μM) plus nicotinic acid (7mM), respectively. Degradation was measured by incubating homogenates with 5μM cADPR or NAADP. Aliquots of the incubation reaction (5μl) were removed and tested for cADPR or NAADP content using the sea urchin egg bioassay as described previously (Sethi et al, 1996). All incubations with added cyclic nucleotides contained IBMX (100μM) to prevent their break-down by phosphodiesterases. Incubations were at 17°C and 37°C for urchin and rabbit samples, respectively.

Both sea urchin egg and rabbit artery homogenates were shown to possess the enzymatic machinery to synthesise and degrade cADPR or NAADP. Degradation activities for cADPR were 0.6±0.03 and 10±2.5 nmol/mg/hr, and for NAADP were 0.169±0.04 and 4.0±0.5 nmol/mg/hr for sea urchin egg and artery homogenates respectively. In the sea urchin egg cGMP selectively enhanced cADPR production (EC_{50} = 3 μM) and cAMP potentiated NAADP production (where 30μM cAMP produced a 2-fold increase in NAADP production and 10μM forskolin enhanced synthesis to the same extent). The effect of cAMP in increasing NAADP production was also observed in the rabbit artery extracts, but cGMP and cAMP did not appear to modulate the synthesis of cADPR. These results are summarised in the table below.

	sea urchin egg homogenate mean ± sem (nmol/mg/hr) (n ≥ 5)			rabbit artery homogenate mean ± sem (nmol/mg/hr) (n ≥ 5)		
	control	+ 30μM cAMP	+ 30μM cGMP	control	+ 30μM cAMP	+ 30μM cGMP
cADPR synthesis	0.14±0.01	0.11±0.03	0.26±0.2	14±1.7	12.9±0.3	12.3±2.0
NAADP synthesis	0.10±0.004	0.21±0.02	0.10±0.007	7.7±1.3	27 ±6.2	7.8±1.5

In summary, we have shown that the synthesis of two calcium-mobilising pyridine nucleotides, NAADP and cADPR are differentially regulated by cAMP or cGMP, respectively. These results show for the first time that NAADP levels are modulated by intracellular signals and may be coupled to cell surface receptors via a cAMP-dependent mechanism.

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31P COORDINATE REGULATION OF RECEPTOR FOR ACTIVATED C-KINASE AND PROTEIN KINASE C IN BRAINS OF MORPHINE-TREATED RATS

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Protein kinase C (PKC) isozymes are a family of proteins that phosphorylate and regulate the activity of G protein-coupled receptors, such as opioid receptors, and other signalling proteins. Upon activation, PKC isozymes translocate from the cytosol to various cellular compartments: plasma membrane, nuclear surface, cytoskeletal and other structures (Mochly-Rosen, 1995). PKC binding to specific intracellular receptors (receptors for activated C-kinase, RACKs) defines the isozyme localisation and facilitates the accessibility of specific substrates. Because opiate drugs have been shown to modulate PKC in rat and human brains (Busquets *et al.*, 1995; Ventayol *et al.*, 1997), this study was designed to assess the effect of morphine on RACK1 levels, and on some of its ligands, α and βPKC.

Male Sprague-Dawley rats (250-300 g) were used. Immunoblotting techniques, using specific monoclonal (anti-RACK1) or polyclonal (anti-PKC) antibodies, followed by chemiluminescence detection and image analysis quantitation, were used to determine the abundance of these proteins in brain (frontal cortex).

Acute morphine (30 mg kg^{-1}, i.p., 2 h) induced similar increases in the levels of RACK1 (33±6%, n=8, P<0.001), and of αPKC (35±7%, n=4, P<0.001) and βPKC (24±7%, n=8, P<0.05). In contrast, chronic morphine (30 mg kg^{-1}, i.p., 3 times daily for 5 days) was associated with down-regulation in the abundance of RACK1 (22±3%, n=8, P<0.001) and α and βPKC (16±6%, n=7, P<0.05 and 16±5%, n=5, P<0.001, respectively). Naloxone-precipitated withdrawal (2 mg kg^{-1}, i.p., 2 h) induced a marked up-regulation of these proteins, when compared with chronic morphine-treated rats (RACK1, 41±4%, n=6; αPKC, 51±3%, n=4; βPKC, 62±7%, n=3; P<0.005). Spontaneous opiate withdrawal (48 h) also induced increases in the levels of RACK1 (37±4%, n=3; P<0.01), αPKC (52±7%, n=3; P<0.01) and βPKC (52±7%, n=3; P<0.01). For all treatments together, there was a positive and significant correlation between the levels of RACK1 and those of αPKC or βPKC measured in the frontal cortex of the same animals (Figure1).

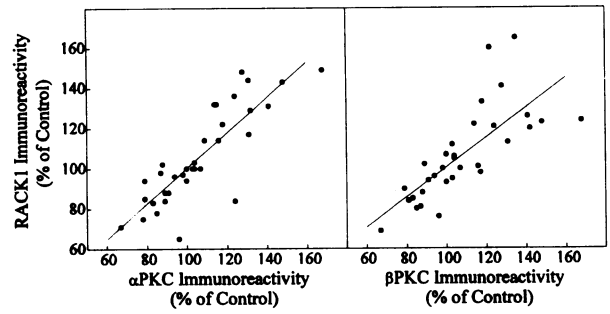

Figure 1. Scatterlots and linear regression showing the relation between levels of RACK1 and αPKC or βPKC in the frontal cortex of the same rats after various treatments (saline vehicle, acute and chronic morphine, and spontaneous and naloxone-precipitated withdrawal). Data are expressed as percentage of naive control rats. Data were best described by the expressions: y = 12 + 0.88 x (r = 0.85, n=35, P<0.0001, αPKC) and y = 26 + 0.77 x (r = 0.75, n=32, P<0.0001, βPKC).

These results clearly indicate that RACK1 is modulated during early and late stages of morphine addiction, as well as during morphine withdrawal. Recently, changes in the expression of RACK1 have been associated with parallel increases or decreases in PKC activity (Padanilam & Hammerman, 1997). The modulation of RACK1 by morphine may result in more pronounced changes in PKC-mediated phosphorylation. In this context, the regulation of the PKC/RACK system may be of relevance in the cellular and molecular processes of opioid addiction.

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32P ADP CAN INDUCE AGGREGATION OF HUMAN PLATELETS VIA BOTH $P2Y_1$ AND P_{2T} RECEPTORS

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While studies with AR-C67085 (formerly FPL 67085), a potent (pK_B 8.9) and selective P_{2T} receptor antagonist (Humphries *et al.*, 1995) indicate that this receptor mediates ADP-induced platelet aggregation, the role of $P2Y_1$ receptors in aggregation has not been previously investigated. We have shown that not only can both receptors mediate aggregation, but that the nature of the response is dependent upon the relative contribution from each of these receptors. In this study, AR-C67085 and the $P2Y_1$ antagonist, A3P5P (adenosine-3'-phosphate-5'-phosphate: pK_B 6.05, Boyer *et al.*, 1996) were used to investigate the roles of the $P2Y_1$ and P_{2T} receptors in ADP-induced aggregation. Blood was collected from healthy human donors into syringes containing heparin (final concentration 10 U ml^{-1}) and centrifuged (240 *g*, 15 min) to produce platelet rich plasma. ADP-induced aggregation was measured optically, using PAP-4 aggregometers, in the absence and presence of AR-C67085 (3 - 100 nM) or A3P5P (3 - 100 μM). The effects of ADP, AR-C67085 and A3P5P were quantified (Table 1) using three indices of aggregation: maximum extent (the maximum degree of aggregation); final extent (the degree of aggregation after ≥ 8 min); rate (initial rate of aggregation). Control aggregation responses to ADP were transient at low concentrations and sustained at higher concentrations. When aggregation was transient to some degree, maximum and final extents differed, whereas when aggregation was sustained, maximum and final extents were the same. AR-C67085 and A3P5P both inhibited aggregation in a concentration-dependent manner. A3P5P produced parallel rightward displacement of all three indices, although only in the case of rate was this consistent with competitive antagonism (Table 1). In contrast, AR-C67085 caused competitive rightward displacement of final extent and had minimal effect on rate. Its effect on maximum extent was more complex: selective competitive displacement of only the upper part of the ADP concentration-response curve resulted in a concentration-dependent reduction in slope.

Table 1: Quantification of effects of AR-C67085 and A3P5P on ADP-induced platelet aggregation in heparinised human platelet rich plasma

	Final extent	Maximum extent	Rate
	pA_{50}: mean ± s.e. (n = 4 - 6)		
ADP	5.95 ± 0.08	6.23 ± 0.07	6.47 ± 0.13
	pK_B or pA_2 & (Schild slope): mean ± s.e. (n = 4 - 6)		
AR-C67085	**8.54 ± 0.07** (0.95 ± 0.02)	**8.59 ± 0.09** (1.02 ± 0.05)	see text
A3P5P	*5.49 ± 0.39* (0.67 ± 0.10)	*5.47 ± 0.25* (0.73 ± 0.03)	**5.46 ± 0.15** (1.04 ± 0.10)

It is concluded that ADP induces aggregation of human platelets via two pharmacologically distinct receptors. The initial, rapid, transient response appears to be mediated predominantly by the $P2Y_1$ subtype, while P_{2T} receptor stimulation appears of particular importance in determining the final extent of sustained aggregation. The differential effects of AR-C67085 and A3P5P on the components of the response suggest a complex interaction between the P_{2T} and $P2Y_1$ pathways, the nature and significance of which remain to be defined.

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33P FUNCTIONAL EVIDENCE FOR ALLOSTERIC EFFECTS OF CIBACRON BLUE AT THE P2X$_4$ RECEPTOR

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Functional studies have provided evidence that the interactions of the P2 antagonists with the P2X$_4$ receptor are complex with reports that compounds may inhibit, potentiate or have no effect on receptor function (Buell *et al.*, 1995, Bo *et al.*, 1995, Séguéla *et al.*, 1996, Soto *et al.*, 1996). We have previously demonstrated that d-tubocurarine and cibacron blue can allosterically affect the binding of [^{35}S]ATPγS to the rat P2X$_4$ receptor (Michel *et al.*, 1997). In this study we have examined the effects of d-tubocurarine and cibacron blue on the rat recombinant P2X$_4$ receptor, stably expressed in HEK293 cells, to determine if these compounds also exhibited allosteric effects in functional studies.

Whole cell patch clamp recordings were made at 22°C using Cs-aspartate containing electrodes (2.5-5MΩ) and drugs were rapidly applied via a U-tube (see Chessell *et al.*, 1997). In a HEPES buffered extracellular medium, application of ATP produced concentration-dependent inward currents (holding potential -90mV). However, these currents were subject to application-dependent run down which prevented obtaining reproducible data. The run down was independent of inter-application wash-time and was not prevented by inclusion of Mg-ATP or Ca^{2+} in the electrode. In contrast, when recordings were made from groups of 20-40 cells, reproducible responses could be obtained every 5min with no run down. Hence all further experiments were performed on groups of cells. The data are the mean ± s.e.mean of 5-6 experiments.

In initial studies the ability of compounds to alter the response to an EC_{50} concentration of ATP (6μM) was examined. The agonist was applied every 5min and each concentration of antagonist was superfused over the cells for 5min prior to ATP administration. d-Tubocurarine (1-300μM) did not affect the response to 6μM ATP.

Low concentrations (3-100μM) of cibacron blue caused a potentiation of the response to ATP (EC_{50} = 3.3 ± 1.2μM, maximum potentiation = 158.5 ± 13.5% of response to 6μM ATP). Higher concentrations (300μM) of cibacron blue did not significantly change the response to ATP. To examine these effects further, concentration-effect curves to ATP were obtained in the presence and absence of cibacron blue. Cibacron blue caused a concentration-dependent decrease in the EC_{50} of ATP with no increase in the maximum response (see table).

Cibacron blue (μM)	ATP EC_{50} (μM)	Max. Response†
0	6.08 ± 0.82	151.6 ± 3.9
3	3.94 ± 0.69*	158.7 ± 18.1
10	2.20 ± 0.19*	158.5 ± 14.0
30	1.52 ± 0.01*	169.5 ± 9.3

* Significantly different from control, P<0.05
† As a % of initial response to 10μM ATP

Co-administration of cibacron blue with ATP, without pre-incubation, did not potentiate the responses, but instead produced inhibition at high concentrations (25.7 ± 7.5% inhibition at 300μM).

The finding that cibacron blue could either potentiate or inhibit P2X$_4$ receptor-mediated responses depending upon the concentration or the pre-incubation period employed may explain previous conflicting reports on the effects of antagonists on this receptor. The demonstration that cibacron blue could increase the potency of ATP would be consistent with an allosteric regulation of the P2X$_4$ receptor.

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34P DIFFERENTIAL PHARMACOLOGICAL CHARACTERISATION OF HUMAN RECOMBINANT MELATONIN mt$_1$ AND MT$_2$ RECEPTORS

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Melatonin is the principle hormone involved in the regulation of mammalian circadian systems. Two human melatonin receptors, mt$_1$ and MT$_2$ (current IUPHAR melatonin receptor nomenclature), have been identified. They are seven transmembrane G-protein-linked receptors which couple negatively to adenylyl cyclase (Reppert *et al.*, 1996). Here we compare the pharmacology of the inhibitory effects of melatonin and melatonin analogues on cyclic AMP (cAMP) levels in cells stably transfected with these receptors.

Human mt$_1$ and MT$_2$ receptors were stably expressed in CHO cells (Browning *et al.*, 1998). Following a 60 min incubation in DMEM containing 300μM isobutylmethylxanthine, confluent cells were incubated with agonist in the presence or absence of antagonist. 60 min later cells were stimulated with 30 μM forskolin for 15 min. cAMP was extracted by incubating with ice cold ethanol for 30 min and measured using an SPA cAMP assay (Biotrak, Amersham Life Sciences). Data are means ± s.e.mean of 3-11 replicates.

Melatonin produced a concentration-dependent inhibition of forskolin-stimulated cAMP in CHO mt$_1$ and MT$_2$ cells with similar potencies (Table 1) and maximum responses of 83±4 and 64±3% respectively. This effect was mimicked by a range of indolic and non-indolic analogues of melatonin, all of which were full agonists with respect to melatonin, with the exception of 6-OH-melatonin, which had an intrinsic activity of 0.82±0.05 (P<0.05) at the MT$_2$ receptor. The potency of these agonists, relative to melatonin (PR), was generally similar at both receptors. However, 2-I-melatonin was 33 fold more potent than melatonin at the mt1 receptor, whilst 6-Cl-melatonin was 25 fold less potent. Both of these agonists were equipotent with melatonin at the MT$_2$ receptor. The melatonin antagonist luzindole (0.1-100μM) competitively antagonised responses to melatonin at both receptor subtypes, with no effect on Hill slope or maximum response (ANOVA). Competition analyses yielded Schild slope estimates of 1.00±0.10 and 0.94±0.07 at mt$_1$ and MT$_2$ respectively, and revealed a 54-fold higher affinity for the MT$_2$ receptor (Table 1). In CHO mt$_1$, but not MT$_2$ cells, luzindole augmented forskolin-stimulated cAMP levels in a concentration dependent manner (118±15% at 100μM), suggesting that mt$_1$ receptors are constitutively active and that luzindole is an inverse agonist in this system. Incubation of mt$_1$ and MT$_2$ cells in 100ng/ml pertussis toxin abolished the inhibitory effects of melatonin, confirming that these responses may be mediated by G_i.

Table 1: Agonist and antagonist potencies at mt$_1$ and MT$_2$ receptors.

	mt$_1$		MT$_2$	
Drug	$pEC_{50}(pA_2)$	PR	$pEC_{50}(pA_2)$	PR
2-I-melatonin	11.02±0.13	0.03	10.14±0.16	0.50
melatonin	9.53±0.16	1.00	9.74±0.05	1.00
S20098*	9.38±0.24	1.42	10.34±0.09	0.30
6-Cl-melatonin	8.14±0.12	24.6	9.77±0.21	1.02
GR196429*	7.98±0.14	36	9.06±0.08	4.61
6-OH-melatonin	7.22±0.09	205	7.36±0.12	231
N-acetyl-5-HT	5.68±0.12	7150	6.61±0.09	1170
Luzindole*	(6.08±0.10)		(7.81±0.17)	

*Browning *et al.*, 1997.

These data suggest that melatonin, 2-I-melatonin, 6-Cl-melatonin and luzindole could be used as tools to discriminate pharmacologically between native mt$_1$ and MT$_2$ receptors. However, differences in the abilities of melatonin receptor subtypes to display constitutive activity may complicate the interpretation of functional data obtained from recombinant receptor systems.

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35P THE USE OF [^{35}S]GTPγS BINDING TO ASSESS G PROTEIN COUPLING OF HUMAN TYPE 2 METABOTROPIC GLUTAMATE RECEPTOR

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Metabotropic glutamate receptor type 2 when expressed in cultured cell lines appears to be negatively coupled to the effector enzyme adenylate cyclase via G-proteins of the G_i family (Tanabe *et al.*, 1992). However, to date no report in which the functional interaction between this receptor sub-type and individual $G_i\alpha$-like G-protein subclasses has been described. In the present study we have investigated the functional interaction of recombinant human mGluR2 with both endogenous and over-expressed pertussis toxin sensitive G-proteins (G_i1-3α) utilising [^{35}S]GTPγS binding assay.

Human embryonic kidney (HEK293T) cells were transfected with either pcDNA3 (vector control), human mGluR2 cDNA alone or human mGluR2 together with each of the wildtype G_i1-3α G-protein classes. Cultured cells were maintained in DMEM, 10% (v:v) foetal calf serum and 2mM L-glutamine. Assays were performed in 96 well plates at room temperature using 10μg/well of fresh cell membranes reconstituted in saponin supplemented (10mg/l) assay buffer (20mM HEPES pH7.4, 10mM $MgCl_2$, 100mM NaCl) with 0.3nM [^{35}S] GTPγS and 40μM GDP in the presence or absence of L-glutamate agonist applied for 30min. Wheatgerm agglutinin SPA beads (0.5mg/well) were added whilst mixing for a further 30min. The plates were then spun at 1500g for 5min and bound radioactivity measured.

In membranes from control cells transfected with pcDNA3 alone, no L-glutamate stimulation on the binding of [^{35}S]GTPγS was observed (basal, 854.3±32.8; +Glu, 845±37.6 cpm/10μg protein). However, a clear elevation of [^{35}S]GTPγS binding was detected at a concentration of 300μM L-glutamate in cells expressing human mGluR2 alone (basal, 824.3±35.9; +Glu, 1632.3±56.4 cpm/10μg protein). Moreover, in membranes derived from cells expressing human mGluR2 together with $G_i1\alpha$, $G_i2\alpha$ or $G_i3\alpha$ a more pronounced increase in SPA binding above basal levels was observed (Table 1).

To pharmacologically characterise in more detail the agonist stimulated G-protein activation, dose response curves to L-glutamate were generated by application of the agonist at the concentrations $1x10^{-7}$M-$3x10^{-4}$M. The L-glutamate potentiation of [^{35}S]GTPγS binding was concentration-dependent with an EC_{50} of 13.7±1.1μM. In all cases examined, agonist mediated stimulation of [^{35}S]GTPγS binding to membranes from mGluR2 expressing cells was sensitive to pertussis toxin (50ng/ml) following pre-incubation of the cultured cells for 20hour prior to preparation of cell membrane protein, reducing binding to sub-basal values (basal, 824.3±35.9; +PTX 558±23.8 cpm/10μg protein). Such a dramatic response was also observed using membranes with co-expression of receptor along with G_i1-3α as shown in Table 1.

Taken together these results provide evidence that human mGluR2 has the capacity to interact with and stimulate pertussis toxin sensitive $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ in the particular mammalian expression system adopted. Furthermore, over-expression of G-proteins increases the signal:noise ratio in the assay.

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Table 1. [^{35}S]GTPγS Binding Data

Transfection	Basal (cpm)	L-glu (300μM, cpm)	PTX (50ng/ml, cpm)	% stimulation over basal
mGluR2	824.3±35.9	1632.3±56.4	558.0±23.8	98.0±56.4
mGluR2+$G_i1\alpha$	336.7±16.2	1187.0±15.9	279.3±3.9	252.5±1.3
mGluR2+$G_i2\alpha$	489.0±10.6	1639.3±31.8	418.4±2.7	235.2±1.9
mGluR2+$G_i3\alpha$	206.7±26.9	974.3±26.4	165.3±5.5	371.4±2.7

36P THE EFFECT OF IRON CHELATORS ON THE NEUROTOXICITY OF ARTEMISININ DERIVATIVES *IN VITRO*

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The antimalarial drug artemisinin and its derivatives display neurotoxicity in animal studies *in vivo* (Brewer *et al.*, 1994) and in neuronal cells *in vitro* (Fishwick *et al.*, 1995). Their antimalarial activity and toxicity may be due to an interaction of iron with the endoperoxide bridge of the derivative to produce toxic free radicals (Meshnick *et al.*, 1994). To support this hypothesis we have previously shown that iron in the form of haemin enhances the *in vitro* neurotoxicity of artemisinin derivatives which contain an endoperoxide bridge (Smith *et al.*, 1997). In this study, we have examined the effects of novel iron chelators on the neurotoxicity of dihydroartemisinin in the neuroblastoma NB2a cell line.

NB2a cells were plated on to 48-well plates and after 24 h, the cells were preincubated for 2 h with the following iron chelators (150 μM, a concentration at which they had no significant effect on their own): 1,2-dimethyl-hydroxypyrin-4-one (CP20), 1,methyl-2,ethan-1-ol-hydroxypyridin-4-one (CP40), desferrioxamine and desferrioxamine conjugated to hydroxyethyl starch (DFO-HES). The cells were induced to differentiate by addition of serum-free medium plus 0.5 mM dibutyryl cyclic AMP in the presence or absence of 1 μM dihydroartemisinin with or without iron chelator. After a further 24 h, the cells were fixed with 4% (w/v) formaldehyde, stained with Coomassie Blue and neurite length measured by light microscopy with automated image analysis.

Dihydroartemisinin significantly inhibited neurite outgrowth to 22 ± 9.2 (SD) % of control values ($p<0.001$, n=3, two way ANOVA & Bonferroni-modified t-test). The cell-permeant iron chelators CP20 and CP40 completely protected against the inhibition of neurite outgrowth: 99 ± 13.5 and 100 ± 4.2 % of controls respectively (both $p<0.001$, n=3). The semi-permeant iron chelator desferrioxamine partially protected against the inhibition: 75 ± 9.5 % of controls ($p<0.001$, n=3, two way ANOVA & Newman-Keuls test) and the non-cell-permeant DFO-HES did not protect against the inhibition (10 ± 9.3 % of controls).

These results suggest dihydroartemisinin produces its toxicity by an iron-dependent mechanism that is consistent with the intracellular generation of toxic free radicals.

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37P TRANSDERMAL NITROGLYCERIN PREVENTS LEUKOCYTE ACTIVATION AND GASTRIC BLOOD FLOW REDUCTION INDUCED BY NSAIDs

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Clinically used doses of transdermal nitroglycerin protects the rat gastric mucosa against damage induced by indomethacin (Barrachina et al., 1995). Gastrolesivity by NSAIDs implies a reduction in blood flow and the activation of inflammatory responses in the gastric mucosa (Wallace et al., 1993). We have now analyzed the effects of this mode of administration of an NO-donor on these mechanisms of gastric injury. Conscious male rats received indomethacin (20 mg kg^{-1}, s.c.) 30 min after application of transdermal patches releasing nitroglycerin (NGC, 166 ng kg^{-1} min^{-1}) or placebo (PP). Three hours later, animals were killed and gastric lesions measured. Two further groups of experiments were performed in anaesthetized (pentobarbital, 50 mg kg^{-1}, i.p.) rats. In the first one, the effect of indomethacin (20 mg kg^{-1}, s.c.) on gastric mucosal blood flow (GMBF) was evaluated by laser-Doppler flowmetry using an ex vivo gastric chamber preparation. Results were expressed as % of basal value. In the second one, intravital microscopy was used to analyze the effect of transdermal nitroglycerin on leukocyte-endothelial cell interactions in the mesenteric microcirculation. Indomethacin was administered by superfusion (50 µg ml^{-1}) and the number of rolling (NR), rolling velocity (RV), adherence (A) and emigration (E) of leukocytes quantified. In both cases, indomethacin was administered thirty minutes after patch application and GMBF, inflammatory events and blood pressure registered for 60 min.

Animals treated with transdermal NGC exhibited a 70±13% (n=7, $p<0.05$) diminution in the level of gastric damage induced by indomethacin (30±5 mm, n=7). This dose or NGC did not modify blood pressure.

Table 1: Effect of indomethacin in control (PP) and NGC pretreated rats.

min	0		30		60	
	PP	NGC	PP	NGC	PP	NGC
GMBF	100	100	89±2^{+}	96±7	84±6^{+}	96±8
NR	16±3	11±1	48±13^{+}	10±2*	34±6	11±5*
A	1.5±0.2	2.7±0.7	8.8±1.6^{+}	3.6±0.8*	9.8±2.3	2.5±1.5*
RV	75±17	52±13	44±6	39±4	44±8	47±15
E	2.5±0.5	1.0±0.5	3.8±0.6	2.6±0.3	4.5±0.9	3.0±1.0

($n \geq 5$; mean ± s.e.m). $^{+}p<0.05$ vs respective basal values and $^{*}p<0.05$ vs respective PP values.

In conclusion, gastroprotective doses of transdermal nitroglycerin prevent the reduction of mucosal blood flow and the leukocyte-endothelial cells interaction induced by indomethacin.

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38P THE SYNTHESIS OF NITRIC OXIDE IN THE BRAINSTEM AND IN THE HYPOTHALAMUS MODULATES THE INHIBITION BY BOMBESIN OF GASTRIC ACID SECRETION

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Nitric oxide (NO) has been implicated in the central regulation of gastric acid secretion (GAS) (Esplugues *et. al.*, 1996). The neuropeptide bombesin inhibits GAS when administered in the central nervous system (Tache *et. al.*, 1981) and bombesinergic neurons have been located in nuclei implicated in the regulation of gastric responses such as the paraventricular nucleus of the hypothalamus (PvN) and the dorsal motor nucleus of the vagus (DMN). Both nuclei also exhibit substantial NO-synthase activity. The aim of the present study was to evaluate the role of NO in the inhibition by bombesin of GAS. Sprague Dawley rats under urethane anaesthesia (1.6 g kg^{-1}) were perfused intragastrically with saline (0.9 ml min^{-1}). GAS was stimulated either by centrally mediates stimuli (distesion of the stomach 15 cm H_2O; insulin 0.75 u.i. kg^{-1}, i.p.) or by the peripherally acting secretagogue pentagastrin (100 µg kg^{-1}, i.p.). Prior to acid stimulation, rats were placed in the stereotaxic apparatus and bombesin administered either intracisternally (40 ng kg^{-1}, 10 µl) or in the PvN (12 ng kg^{-1}, 100 nl). In some cases L-NAME (80 µg kg^{-1}, 100 nl) was injected in the PvN or in the DMN. Controls groups received only vehicle (PBS). The effect of exogenously administered NO was evaluated in animals with a dialysis tube (i.d. 0.22 mm, o.d. 0.31 mm) implanted into de dorsal vagal complex (DVC) and receiving an i.v. infusion of pentagastrin (8 µg kg^{-1} h^{-1}). Once GAS reached a stable plateau, a solution of SNAP (25 mM, 150 µl h^{-1}) was perfused through the dialysis tube. Results are shown as $\Delta\mu Eq\ H^{+}$ 100 g^{-1} during the appropiate experimental period for each stimulus: distension 120 min; insulin 60 min; pentagastrin 30 min. (mean ± S.E.M.; n≥6).

Administration of L-NAME in the DMN or in the PvN reverses the acid inhibition induced by central bombesin.

STIMULUS	CONTROL	BOMBESIN	
			+L-NAME
DISTENSION	47.3 ± 10	4.1 ± 2.2 *i.c.	38 ± 6.6# DMN
INSULIN	43 ± 9.7	0.9 ± 0.3 *i.c.	33.1 ± 7# DMN
PENTAGAST.	9.7 ± 1.7	2 ± 0.6 *i.c.	8.5 ± 1# DMN
	9.4 ± 2.4	2.7 ± 0.3* i.c.	9.8 ± 2.3# PvN
	13.7 ± 2.8	6.2 ± 2.6 *PvN	17 ± 6.8# DMN

$p < 0.05$: * vs control; # vs bombesin. (ANOVA + Tukey test)

The administration of SNAP by microdialysis induced an immediate diminution of GAS stimulated by the infusion of pentagastrin, which reached its maximal inhibitory effect (40 ± 7 %; n=4) 20 minutes after the beginning of the SNAP perfusion.

These results suggest that bombesin inhibits GAS, induced either by central or peripherally acting stimuli, by a central mechanism which requires the synthesis of nitric oxide in the PvN and in the DMN. The fact that exogenous NO also inhibits GAS suggests that NO plays a major role in the CNS inhibition of gastric acid responses.

Esplugues J.V., Barrachina, M.D., Beltran B. *et al* (1996) *Proc. Nat Acad Sci* 93, 148393-14844.

Tache Y., Marki, W., Rivier, J.(81) *Gastroenterology* 81, 298-302.