209P STUDIES OF THE EFFECTS OF SCA40 IN RELAXING BOVINE TRACHEALIS MUSCLE AND IN INHIBITING ISOENZYMES OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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SCA40 relaxes guinea-pig isolated trachea and Laurent *et al.* (1993) proposed that this action results from the opening of large conductance Ca^{2+}-sensitive K^+-channels (BK_{Ca}). This proposal was based on the findings that K^+-channel inhibitors such as charybdotoxin (ChTx) and a K^+-rich medium antagonised SCA40. However, Cook *et al.* (1995) showed that the antagonism caused by these agents was offset by nifedipine (1μM), suggesting that this antagonism was functional and attributable to the promotion of Ca^{2+} influx. The aims of the present study were to examine the relaxant action of SCA40 in bovine trachealis and its activity against isoenzymes of PDE isolated (method of Elliott *et al.*, 1991) from bovine and guinea-pig tissue.

Cumulatively-applied SCA40 (10nM-10μM; 6min tissue contact), isoprenaline (Iso; 0.1nM-1mM; 4min tissue contact) and levcromakalim (Lev; 1nM-1μM; 8min tissue contact) each relaxed histamine (460μM)-precontracted bovine isolated trachealis. A K^+-rich (80mM), but isosmolar, Krebs solution antagonised SCA40 (2-fold), antagonised Iso (5.5-fold) and profoundly depressed the log concentration/effect curve for Lev. Nifedipine (1μM) did not modify the relaxant actions of SCA40, Iso or Lev. Nifedipine (1μM) offset the antagonism of SCA40 and Iso provided by the K^+-rich medium but failed to prevent the equivalent antagonism of Lev. ChTx (100nM) antagonised (5-fold) Iso but did not antagonise SCA40. Iberiotoxin (100nM) did not antagonise SCA40. Microelectrode recording showed that SCA40 (1μM and 10μM) only weakly hyperpolarised bovine trachealis cells (3.0 ± 0.6mV and 2.3 ± 0.7mV; mean ± s.e.mean; n=6 respectively), whereas Lev (10μM) induced hyperpolarisation of 13.3 ± 1.5mV (n=10). SCA40 (1μM and 10μM) failed to stimulate significant $^{86}Rb^+$ efflux from bovine trachealis preloaded with the radiotracer (method of Chiu *et al.*, 1993) whereas Lev (10μM) evoked a 200% increase in the efflux rate coefficient.

SCA40 (10nM-100μM) and theophylline (1μM-1mM) each inhibited cyclic nucleotide phosphodiesterase (PDE) isoenzyme types I, III and IV. SCA40 was most potent against the type III isoenzyme (-log IC_{50} 7.16 ± 0.23; n=4) derived from guinea-pig cardiac ventricle, less potent against the type IV isoenzyme (5.39 ± 0.22; n=4) from bovine trachea and was a relatively weak inhibitor of the type I isoenzyme (<4.00; n=4) from guinea-pig cardiac ventricle. Theophylline was less selective and less potent than SCA40 in inhibiting the three isoenzymes (-log IC_{50} values 3.59 ± 0.10, 3.34 ± 0.08 and 3.50 ± 0.07 for isoenzyme types III, IV and I respectively).

The present findings lend support to the suggestion (Cook *et al.*, 1995; Macmillan *et al.*, 1995) that the opening of BK_{Ca} channels does not play an important role in the tracheal relaxant activity of SCA40. Our studies of isoenzymes derived from animal tissue confirm the human data of Cook *et al.* (1995) that SCA40 is a selective inhibitor amongst the isoenzymes of PDE, being of greatest potency against the type III isoenzyme and having second greatest potency against the type IV isoenzyme. Inhibition of PDE isoenzymes III and IV could account for the tracheal relaxant activity of SCA40.

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210P EFFECTS OF IBERIOTOXIN ON THE MECHANICAL AND ELECTRICAL ACTIVITY OF GUINEA-PIG TRACHEALIS MUSCLE

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Iberiotoxin (IbTx) is a highly selective inhibitor of the large conductance, Ca^{2+}-sensitive K^+-channel (BK_{Ca}) (Galvez *et al.*, 1990; Suarez-Kurtz *et al.* 1991). In the present study of guinea-pig isolated trachealis we have examined the effects of this agent *per se* on the mechanical and electrical activity of the muscle cells and whether blockade of BK_{Ca} by IbTx alters the sensitivity of the tissue to spasmogenic agents.

In guinea-pig trachealis muscle treated with indomethacin (2.8μM), IbTx (100nM, 60 min tissue contact) evoked spasm equivalent to 74.2 ± 3.7% (mean ± s.e.mean; n=25) of the maximal response to carbachol (CCh). IbTx-induced spasm was abolished by nifedipine (1μM), reduced (by 28%) by atropine (1μM) but not modified by either tetrodotoxin (TTx; 3.1μM) or tissue pretreatment with capsaicin (1μM for 30 min). Cumulatively-applied CCh (10nM - 10μM), histamine (Hist; 100nM - 1mM) and KCl (5 -120mM) each caused concentration-dependent tension development in indomethacin (2.8μM)-treated trachealis. IbTx (100nM, 20 min preincubation) caused a leftward shift in the log concentration-effect curve for Hist and elevated the lower part of the log concentration-effect curves for both CCh and KCl. The pD_2 value for each of the three spasmogens was significantly increased in the presence of IbTx (CCh 6.48 ± 0.03 and 6.87 ± 0.09; Hist 4.73 ± 0.09 and 5.40 ± 0.18; KCl 1.52 ± 0.02 and >2.30; mean ± s.e.mean from at least six tissues in the absence and presence of toxin respectively). Neither atropine (1μM) nor a mixture of atropine (1μM) plus TTx (3.1μM) modified the shape or position of the cumulative log concentration-effect curve for Hist. Tested in the presence of the atropine and TTx combination, IbTx again caused a significant increase in the pD_2 value for Hist (4.84 ± 0.12 and 5.42 ± 0.09 in the absence and presence of IbTx respectively; n=6). Intracellular microelectrode recording (Dixon & Small, 1983) revealed that the resting membrane potential of the trachealis cells was -44.2 ± 0.5mV (mean ± s.e.mean, n=57 cells) and most cells exhibited slow wave activity. IbTx (100nM) did not alter resting membrane potential (-44.9 ± 1.0mV, n=21) but converted the spontaneous slow waves into regenerative action potentials. Atropine (1μM) did not alter resting membrane potential (-44.3 ± 1.0mV, n=16) and did not modify slow wave activity. In the presence of a mixture of atropine (1μM) and IbTx (100nM) there was a small increase in resting membrane potential (to -49.7 ± 1.0mV, n= 20) and slow waves were converted into regenerative action potentials.

We conclude that the spasmogenic action of IbTx is partly due to the (possibly indirect) activation of muscarinic cholinoceptors. The discharge of neuronal action potentials or the release of excitatory neuropeptides does not significantly contribute to the spasmogenic action of IbTx. The failure of IbTx to reduce the resting membrane potential of the trachealis cells suggests that BK_{Ca} channels do not help to determine the resting membrane potential. However, the ability of the toxin to convert spontaneous electrical slow waves into regenerative action potentials suggests that BK_{Ca} channel opening may be promoted by the Ca^{2+} influx associated with the depolarising phase of the slow waves and BK_{Ca} channel opening thereby prevents the development of the slow wave into a regenerative action potential. The ability of IbTx to potentiate carbachol, histamine and KCl suggests that BK_{Ca} channel opening may modulate the sensitivity of the trachealis muscle to spasmogens.

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211P CHROMATE-INDUCED FIBROGENESIS ASSOCIATED WITH THE DEVELOPMENT OF CONTRACTILE MYOFIBROBLASTS

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Inhalation of hexavalent chromium-containing materials gives rise to irritant and other toxic damage with respiratory impairment as a result. Although not substantially documented, it is to be assured that pulmonary fibrosis would be a consequence of th inhalation of hexavalent chromium-containing materials; along with nasal septal perforation and asthma. Fibrogenic effects of chromium compounds have been shown experimentally in the rat. It is also known that fibrotic lung lesions provoked by a variety of agents, e.g. bleomycin, paraquat and silica, may also be associated with myofibroblast cell developments sufficient to impart contractile properties to affected tissues (Hicks & Hemati, 1994). Contractile activity, attributable to such cells, has been demonstrated to result from influence of some distinctive substances, e.g. oxidising agents, and the possibility has been suggested that inhalation of air pollutant by such materials could thus provoke respiratory distress. The possibility that this might be the case with hexavalent chromium fibrogenesis, has been investigated to assess enhancement of contractile activity associated with fibrogenesis and myofiboblast development in the rat lung, taking particular account of the time course after chromate treatment. Female Sprague-Dawley rats (200-250g) were treated with single doses (50mg/kg) of sodium chromate, intratracheally, after 1 to 6 weeks lung strip preparations were isolated from normal (n=6) or treated animals. Weighed, parenchymal strips (2.5 x 2 x 20mm) were suspended in 95% O_2/5% CO_2 gassed Krebs solution at 37°C and resting tension of 1g. Tissue tension was measured isometrically and standardised in terms of length (in situ) and weight. Administration of ascending doses of mepyramine (1 x 10^{-5} to 5 x 10^{-3}M) or sodium tungstate (1 x 10^{-5} to 5 x 10^{-3}M) caused dose related, reversible and repeatable contractions, in both control and chromate treated tissues. Chromate treated tissues showed significant increase ($P<0.05$ or 0.01) in contractility with a time-related progress (as shown in fig. 1 for the effect of mepyramine). Such contractions were associated with development of myofibroblasts and fibrosis which were also observed histologically. There was no corresponding enhancement of smooth muscle contractions evoked by agents such as barium ions.

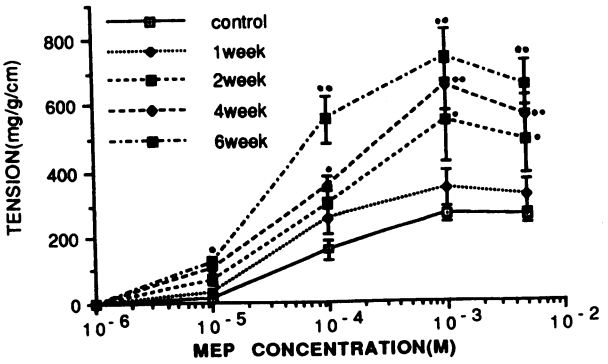

*Fig. 1: Effect of mepyramine on control or chromate treated lung tissues. Data significantly different from control value are indicated *P<0.05 and **P<0.01*

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212P EFFECT OF CYCLOPIAZONIC ACID ON CONTRACTIONS PRODUCED BY TACHYKININ NK_1 AND NK_2 RECEPTOR AGONISTS IN THE CIRCULAR MUSCLE OF GUINEA-PIG COLON

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Previous studies have shown a striking difference in contraction produced by selective stimulation of tachykinin NK_1 vs NK_2 receptor in the circular muscle of guinea-pig colon: the response mediated by the NK_1 receptor is strongly inhibited by nifedipine, while that mediated by NK_2 receptor is largely nifedipine-resistant (Zagorodnyuk et al., 1994). In the present study we aimed to clarify the mechanism(s) underlying contraction mediated by tachykinin NK_1 vs NK_2 receptor by testing cyclopiazonic acid (CPA, 3 μM for 60 min), a selective inhibitor of sarcoplasmic reticulum calcium (Ca^{2+}) pump (e.g. Uyama et al., 1992), against contractile responses produced by NK_1 ([Sar^9]substance P sulfone) and NK_2 ([βAla^8]NKA (4-10)) receptor-selective agonists. All the experiments were performed on isolated circular muscle strips from guinea-pig colon, in the presence of indomethacin (10 μM) and atropine (1 μM).

[Sar^9]substance P sulfone (10 nM) produced a contraction characterised by a peak phasic and a sustained tonic component (measured at 1 and 15 min from bath application of the agonist) averaging 75 ± 2 and 43 ± 3 % (n=7) of the maximal response to KCl (80 mM). CPA slightly reduced the phasic response to [Sar^9]substance P sulfone (16 ± 4 % inhibition, n=7, $P<0.05$) and markedly suppressed the tonic component (89 ± 3 % inhibition, n = 7, $P<0.01$).

[βAla^8]NKA (4-10) (10 nM) produced a sustained contraction of the colonic muscle, averaging 69 ± 5 % and 73 ± 4 % (n=7) of the response to KCl, at 1 and 15 min from application of the agonist; the corresponding components of contraction obtained in the presence of CPA were slightly (18 ± 7 and 21 ± 5 % inhibition, $P < 0.05$, n=7, respectively) depressed as compared to controls. In the presence of 1 μM nifedipine (60 min before) the response to [βAla^8]NKA (4-10) was depressed in its early component (32 ± 6 % inhibition at 1 min, n=8) only. CPA produced a slight inhibition (15 ± 9 and 33 ± 10 % at 1 and 15 min, respectively) of the nifedipine-resistant response to [βAla^8]NKA (4-10), similar to that observed in the absence of nifedipine. We conclude that a CPA-sensitive Ca^{2+} store plays a crucial role in producing the tonic increase in tension following stimulation of the NK_1 receptor: since this response is abolished by nifedipine (Zagorodnyuk et al., 1994), a Ca^{2+}-induced Ca^{2+}-release is postulated to occur. By contrast, Ca^{2+} mobilization from a CPA-sensitive store seems to be of minor importance for NK_2 receptor -mediated responses.

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213P COMPARISON OF THE EFFECTS OF VARIOUS METHODS OF CELL CULTURE ON THE ACTIVATION OF THE TRANSFECTED HUMAN NK_2 RECEPTOR

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Recombinant receptor preparations offer convenient systems in which to determine both affinity and efficacy of novel pharmacological agents. The level of receptor expression, the G-protein complement of the host cell line and the ability of the receptor to couple correctly are crucial to the success of any functional assay. This study aimed to determine whether the functional response and ligand binding parameters of the cloned human NK_2 receptor expressed in CHO cells were altered by different methods of culturing and harvesting.

CHO-H-NK_2 cells were cultured as monolayers, in suspension or attached to microcarrier beads. Monolayers were harvested by scraping, or by brief exposure to either trypsin (0.25% v/v) or a non-enzymatic cell dissociation solution (CDS). K_D and B_{max} were determined using the NK_2 receptor antagonist [^{3}H]-SR-48968 (Edmonds-Alt *et al.*, 1992) against the NK_2 receptors expressed in the different cell preparations. Non-specific binding was defined by 1 μM SR-48968. Intracellular Ca^{2+} concentration changes following stimulation by the NK_2 receptor agonist [β-Ala^8]-NKA(4-10) were measured using a Perkin-Elmer LS-50B fluorimeter. Cells (1.5 x 10^6/ml) were loaded with 5 μM fura 2-AM (60 min, 37 °C) in Krebs-HEPES Buffer pH 7.4 containing 2% foetal calf serum.

Measurable K_D values and the B_{max} values for monolayer cells were similar. Cells cultured in suspension gave a higher B_{max} value than cells cultured as monolayers. The presence of microcarrier beads prevented the determination of K_D and B_{max} values by interfering with ligand binding methods. [β-Ala^8]-NKA(4-10) caused a dose-dependent elevation of intracellular Ca^{2+}, except in cells harvested by scraping or trypsin. The low viability (<50%) obtained with scraped cells may explain why a functional response was not observed. Although the viability of trypsinised cells was higher than that of scraped cells (>85%), a steady base line was not maintained in the functional assay. This may be due to damage to the cell membrane by trypsin. Cells cultured as monolayers (harvested with CDS), in suspension, or attached to microcarrier beads had high viability (>85%) and did not show a significant difference in their response to [β-Ala^8]-NKA(4-10). The EC_{50} values compared favourably with previously reported data (Subramanian *et al.*, 1994).

Table 1: Analysis of different methods of cell culture on the activation of the transfected human NK_2 receptor in CHO cells.

Method of culture/harvest	K_D (nM)	B_{max} (pmol/mg protein)	EC_{50} (nM)
CDS	0.22 ± 0.026	8.19 ± 0.47	2.28 ± 0.46
Trypsin	0.42 ± 0.067	12.86 ± 1.66	MU
Scraped	0.29 ± 0.032	8.48 ± 0.28	MU
Suspension	0.37 ± 0.019	24.60 ± 4.93	1.93 ± 0.24
Microcarrier beads	MU	MU	1.94 ± 0.53

MU - measurements unobtainable. Values are means ± s.e.mean. N = 4.

In conclusion, the affinity of the NK_2 receptor for [^{3}H]-SR-48968 is unaffected when CHO-H-NK_2 cells are cultured in suspension or as monolayers, harvested with different agents. Culturing CHO-H-NK_2 cells as monolayers, in suspension or attached to microcarrier beads does not significantly affect the functional response of the NK_2 receptor. The results indicate that functional responses can be measured on cells whilst still remaining attached to microcarrier beads. However harvesting monolayer cells by scraping or by trypsinization is unsuitable for functional assays. This study suggests that suspension or monolayer cells harvested using CDS are the preferred methods of cell culture for CHO-H-NK_2 cells as both binding and functional results can be obtained.

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214P THE CALCIUM CHANNEL ANTAGONIST, NIMODIPINE, GIVEN BEFORE PRACTICE IN A TEST OF ATAXIA, INCREASES THE DEVELOPMENT OF TOLERANCE TO ETHANOL

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Dihydropyridine calcium channel antagonists have previously been shown to decrease both the development of tolerance to ethanol (Dolin and Little, 1989) and withdrawal signs (Whittington et al., 1991), when given chronically with ethanol. In the former study, the effects of a calcium channel antagonist, given at the same time as the ethanol, were studied on tolerance to the ataxic actions of ethanol, and the animals were tested on a rotorod every other day during the chronic treatment. We have now investigated the effects of the dihydropyridine calcium channel antagonist, nimodipine, given 2h prior to the ethanol, with testing on the rotorod carried out during the 2h interval.

Male Wistar rats (200g) were given a 7 day treatment with ethanol, 2 g/kg i.p., once daily. Nimodipine, 50 mg/kg, suspended in tween 80, 0.05%, was given i.p., 2h before each ethanol injection. Separate groups of rats (n = 10) were given either tween then saline, tween then ethanol, nimodipine then ethanol, or nimodipine then saline. Ataxia testing was carried out using a rotorod (6 r.p.m.) and the animals were tested on the rod on days 1, 3 and 5 of the ethanol treatment (day 1 = first drug treatment).The times were measured which the animals were able to stay on the rotating rod after the ethanol injections and the results are shown in Table 1 as mean ± s.e.m. (s). Statistical comparisons were by the Mann-Whitney U test.

Rats which received ethanol plus tween vehicle showed significant tolerance to ethanol by the 7th day of drug treatment. Animals which were given nimodipine prior to the ataxia testing and ethanol administration, however, developed much greater tolerance to ethanol, compared with those given ethanol plus vehicle. Administration of nimodipine alone did not affect the actions of ethanol given on the 7th day.

The results are in contrast to the previous demonstration of decreased tolerance to ethanol, and indicate that the effect of a calcium channel antagonist on tolerance development is crucially dependent on the time of drug administration. The increase in the ability of the animals to stay on the rotating rod after repeated administration of nimodipine prior to testing may be related to the improvement in memory and learning produced by this drug in other paradigms (eg. Deyo et al., 1989).

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Table 1. Times (s, mean ± s.e.m) for which rats were able to stay on the rotating rod after acute i.p. ethanol, 2 g/kg, on day 7. Times in minutes are the times after the acute injection of ethanol. * $P < 0.05$ vs saline + tween, † $P < 0.05$ vs ethanol + tween

Chronic + Acute	15 min	30 min	45 min	60 min	75 min	90 min	105 min
Saline + tween	3.8±0.8	4.6±0.7	6.5±1.0	10.2±1.8	16.0±2.0	38.4±9.2	67.8±15
Ethanol + tween	4.8±1.3	6.6±1.9	12.3±3.2	22.7±2.6*	40.1±14.3*	100±24*	143±17.3*
Ethanol + nimodipine	12.4±6.2	21.3±9.1	52.5±23.1	94.6±24.2*†	127.4±21.2*†	159±13.3*†	178±2.1*
Saline + nimodipine	5.3±1.5	5.5±0.7	6.8±3	14.6±2.8	24±5.2	47.7±16.2	93±17.8

215P PROTECTION OF NITRIC OXIDE FROM HYDROQUINONE BY SOME PHYSIOLOGICAL ANTIOXIDANTS

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It has recently been demonstrated that the presence of superoxide dismutase (SOD) within nitrergically-innervated tissues protects the neurotransmission process from inhibition by superoxide anion-generating quinones (Martin et al., 1994; Lilley & Gibson, 1995) However, under certain experimental conditions, simple quinones such as hydroquinone (HQ) or 1,4-benzoquinone do not generate superoxide anions nor do they inhibit nitrergic transmission, although they do block responses to exogenous nitric oxide (NO; Lilley & Gibson, 1995). This raises the possibility that other antioxidant mechanisms, in addition to SOD, may act to protect nitrergic transmission. Here, we have assessed the ability of several physiological antioxidants to protect NO from inhibition by HQ, using the mouse anococcygeus muscle.

Mouse anococcygeus muscles were set up for the isometric recording of relaxations to NO as described previously (Hobbs et al., 1991); muscle tone was raised with 50μM carbachol in all cases. Authentic NO added to Krebs solution was detected by a chemical microsensor (Lilley & Gibson, 1995). α-tocopherol (α-TOC) was dissolved in dimethylsulphoxide and all other drugs in distilled water. Results were expressed as mean±s.e. (n of at least 5), and statistical analysis was by Student's t test.

Authentic NO (3-60μM) produced concentration-related relaxations of carbachol-induced tone; 15μM NO produced a relaxation of 48±5% and was used as a standard concentration in subsequent experiments. Relaxations to NO were inhibited in a concentration-related manner by HQ (10-400μM); 100μM HQ inhibited responses to NO by 52±10% and was used in further studies. This inhibitory effect of HQ on NO was decreased in the presence of reduced glutathione (GSH; 5-100μM); 100μM GSH reversed the inhibitory effect of HQ by 67±9%. Ascorbate also protected NO from HQ, although to a lesser extent, 400μM ascorbate producing a reversal of 41±10%. α-TOC (100-400μM) and SOD (250U ml^{-1}) had no effect on the inibition of NO by HQ. The protective effect of GSH was related to the -SH group since oxidised glutathione (GSSG; 100μM) produced no protection of NO; L-cysteine on the other hand was as effective as GSH (51±15% reversal of the effect of HQ by 100μM L-cysteine).

NO produced a concentration-related signal when added to an organ bath containing the microsensor. 15μM NO produced a signal of 123±14mV; the signal was significantly ($P<0.05$) reduced by 100μM HQ (to 26±12mV). This inhibition was unaffected by 250U ml^{-1} SOD (25±3mV) but was reversed by 100μM GSH (83±13mV).

These results confirm that, in our hands, HQ acts as a scavenger of NO and not as a superoxide anion generator (Hobbs et al., 1991; Lilley & Gibson, 1995). Further, they show that sulphydryl-containing antioxidants (Brave et al., 1993), and ascorbate, can protect NO from inhibition by HQ, suggesting that several physiological antioxidant mechanisms might act in concert to protect nitrergic transmission, and perhaps to prevent formation of toxic metabolites. It will be of interest to determine whether, in tissues with reduced antioxidant function, nitrergic relaxations become vulnerable to HQ and other scavengers.

E.L. is an MRC student.

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216P NITRIC OXIDE MEDIATES RELAXATIONS OF THE FROG OESOPHAGUS TO TRANSMURAL ELECTRICAL STIMULATION

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A role for nitric oxide (NO) as a non-adrenergic, non-cholinergic (NANC) relaxant transmitter has been established in the lower oesophageal sphincter (Tøttrup *et al.*, 1991) but few studies have examined the oesophageal body. In most species including man the oesophageal body contains both striated and smooth muscle but it is the latter which has intrinsic as well as extrinsic innervation. We have examined the role of NO in oesophageal function using tissue from the frog which contains only smooth muscle. (Inglefinger, 1958).

Circular strips (c.2mm wide) from the oesophageal body of the frog (*Rana temporaria)* of either sex, weighing 14-25g, were mounted longitudinally in a 25ml organ bath, within a pair of platinum ring electrodes, under a tension of 1g. Responses were measured using an isometric transducer. The tissues were bathed in Tyrodes containing indomethacin and guanethidine (10^{-6}M) and aerated with 95% O_2 and 5% CO_2 at room temperature.

In the presence of a maximal carbamylcholine chloride induced contraction (10^{-5}M), electrical field stimulation (1-10Hz, 2ms, 30V, 10s every 5min) produced frequency dependant relaxations. These relaxations were not inhibited by TTX (10^{-6}M) (n=4) which indicates a possible non-neuronal source of relaxant transmitter. Incubation with the NO synthase inhibitor L-NOARG (10^{-4}M) for 25min between random frequency response curves (n=8) resulted in the complete abolition of the relaxant responses at all frequencies tested. The inactive isomer D-NOARG (10^{-4}M) had no effect (n=4). The effect of L-NOARG was completely reversed (n=4) by a 25min incubation with L-arginine ($5x10^{-3}$M), the biological precursor of nitric oxide, but not D-arginine (n=4). Preincubation with the phosphodiesterase type V inhibitor, SK&F 96231 (2-(2-propoxyphenyl)-6-purinone), for 25min (10^{-5}M) between random frequency response curves caused an increase in both the size (see Table 1) and duration of the response (n=5).

Table 1. The effect of SK&F 96231 on EFS induced relaxations (g). Values are means ± s.e.mean, n=5.

	1Hz	2Hz	4Hz	8Hz	10Hz
CONTROL	0.17 ± 0.05	0.23 ± 0.05	0.26 ± 0.05	0.29 ± 0.05	0.34 ± 0.05
SK&F 96231	0.25 ± 0.04	0.32 ± 0.04	0.37 ± 0.03	0.41 ± 0.04	0.46 ± 0.04

This study suggests that NO may be the sole NANC relaxant transmitter in the frog oesophageal body evoked by electrical field stimulation but the source of NO is possibly non-neuronal.

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217P ET RECEPTORS INVOLVED IN THE BLOOD PRESSURE RESPONSE TO ENDOTHELINS IN THE RAT

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We have investigated the role of ET_A and ET_B receptors in mediating the blood pressure (BP) response to endothelin (ET) receptor agonists in rats. We have used 'untreated rats' and rats which have been repeatedly challenged with ET-1or BQ3020, 'desensitised rats'. Male Alderley Park rats (280 - 320g) were anaesthetised with halothane and artificially respired through a tracheal cannula with 40% O_2, 60% N_2. Rats were pithed and the right carotid artery and jugular vein cannulated for measurement of mean arterial pressure (MAP) and administration of drugs respectively. In 'untreated rats', cumulative dose response curves (DRCs) to big ET-1, ET-1 or the selective ET_B agonist BQ3020, (Ihara et al., 1992) were constructed. In 'desensitised rats', repeated doses of either ET-1 (0.1 nmolkg^{-1}) or BQ3020 (0.7 nmolkg^{-1}) were administered at 10-15 min intervals until both the depressor component of the response had been abolished and the pressor component of the response had stabilised. A cumulative DRC to one of the agonists was then constructed. The dose to induce an increase in MAP of 30 mmHg (ED_{30mmHg}) was estimated for each agonist.

Repeated dosing with ET-1 resulted in desensitisation of the depressor component of the BP response. Following depressor desensitisation, an increase in the pressor sensitivity of the rats to both ET-1 (approx. 10 fold) and BQ3020 (approx. 20 fold) was observed (see Table 1) indicating the involvement of a depressor component in the BP responses to these agonists. The remaining pressor response to BQ3020 was not susceptible to inhibition by the selective ET_A antagonist BMS182874 but was sensitive to inhibition by the ET_B selective antagonist BQ788 (Ishikawa et al. 1994; see Table 2), indicating that the ET_B constrictor response was not desensitised to the same extent as the ET_B dilator component. Pressor responses to big ET-1 were not affected by previous desensitisation with ET-1 (see Table 1), nor by pretreatment with BQ788 (0.3 mgkg^{-1} iv induced mean dose ratio shift of 0.88 ± 0.13 (n=4)), indicating that ET_B receptors are probably not involved in the blood pressure responses to big ET-1.

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Table 1. Effect of ET receptor agonists in untreated rats and rats desensitised to the depressor effects of ET-1.
Results are expressed as means with confidence limits. * p<0.001 significant difference from untreated group, Students t-test.

	Untreated rats			Desensitised rats		
Agonist	Resting MAP (mmHg)	ED_{30mmHg} (nmolkg^{-1})	n	Resting MAP (mmHg)	ED_{30mmHg} (nmolkg^{-1})	n
ET-1	59.2 ± 1.5	0.21 [0.15 - 0.30]	6	60.5 ± 3.8	0.021 [0.010 - 0.042] *	6
BQ3020	58.2 ± 1.3	2.46 [1.83 - 3.32]	6	59.7 ± 2.9	0.12 [0.03 - 0.46] *	3
Big ET-1	62.7 ± 2.2	0.25 [0.18 - 0.35]	6	64.2 ± 1.9	0.30 [0.18 - 0.49] NS	6

Table 2. Effect of ET receptor antagonists on DRC to BQ3020 in rats desensitised to the depressor effects of BQ3020.

Antagonist	Dose (mgkg^{-1})	Dose ratio shift	n
BMS 182874	30	0.96 [0.47 - 1.86]	4
BQ788	0.3	3.90 [2.05 - 7.41]	4

218P DIRECT POSITIVE CHRONOTROPIC EFFECT OF ANGIOTENSIN III IN PITHED RATS AND ISOLATED RAT ATRIA

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It is well known (Knape & van Zwieten, 1988) that angiotensin II (Ang II) induces a tachycardiac response in the pithed rat, primarily by stimulating the sympathetic ganglia thus leading to the release of noradrenaline. *In vitro* studies have also shown that Ang II increases the spontaneous contractile frequency in cultured neonatal rat heart myocytes (Allen *et al.*, 1987) and in guinea-pig isolated atria (Nakashima *et al.*, 1982). In the present study, we investigated the direct positive chronotropic effect of angiotensin III (Ang III), a major degradation product of Ang II, in the pithed rat and in isolated rat right atria preparations.

Pithed rat preparations were set up as described previously (Knape & van Zwieten, 1988). When the circulation had stabilized, saline, prazosin (0.1 mg kg^{-1}) or propranolol (1 mg kg^{-1}) was injected intravenously. Twenty minutes later, the increase in heart rate (HR) provoked by Ang III i.v. was measured 15 min after saline or one of the AT-antagonists had been administered: the AT_1-selective antagonist losartan (3 and 10 mg kg^{-1} i.v.) and the AT_2-selective antagonist PD123177 (25 mg kg^{-1} i.v.). Isolated rat right atria preparations were set up in a gassed Tyrode solution at 37°C, at a tension of 0.5 g, and the spontaneous contractile frequency (beats min^{-1}) was recorded. The effects of cumulative additions of Ang III in the absence and presence of one of the following compounds were established: prazosin (1 μM), propranolol (1 μM), amastatin (10 μM), losartan (10, 100 and 300 nM), PD123177 (1 μM) and staurosporine (5 and 50 nM). The data are presented as means ± s.e.mean for n observations. Significance ($p < 0.05$) was tested by one way analysis of variance or Student's t test.

In pithed rat preparations Ang III (1 - 100 nmol kg^{-1}) i.v. caused a dose-dependent increase in HR by maximally 108.3 ± 6.1 beats min^{-1} (pD_2: 8.42 ± 0.05; n = 26). Propranolol but not prazosin reduced the maximal response to Ang III in pithed rats by 71.4 ± 4.2% (n = 8; $p < 0.01$). In isolated rat right atria preparations, cumulative additions of Ang III (0.3 nM-1 μM) caused a concentration-dependent increase in frequency (pD_2: 7.96 ± 0.03; E_{max}: 34.7 ± 0.4 beats min^{-1}; slope: 0.73 ± 0.03; n = 10). Prazosin and propranolol did not influence the concentration-response curves of Ang III. Amastatin caused a 6 fold increase in the potency of Ang III and significantly steepened the slope of the Ang III curves (pD_2: 8.74 ± 0.01; E_{max}: 34.0 ± 0.2 beats min^{-1}; slope: 1.19 ± 0.04; $p < 0.05$; n = 7). Staurosporine (5 and 50 nM) dose-dependently shifted the concentration-response curves of Ang III to the right and significantly decreased the maximal response by 46.1 ± 5.6% and 65.6 ± 4.8% ($p < 0.01$; n = 6 - 8), respectively. Both in pithed rats (propranolol-pretreated and -nonpretreated) and in isolated preparations, losartan but not PD123177 caused parallel rightward shifts of the curves for Ang III without changing the maximal responses.

In conclusion, both in the *in vivo* and *in vitro* experiments, exogenous Ang III induces a direct dose-dependent chronotropic effect which is mediated via AT_1-receptors located in the rat atria. The effect is independent of the adrenergic system. The effect is potentiated by the inhibition of Ang III degradation and decreased by the inhibition of protein kinase formation.

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219P AFFINITY AND BIODISTRIBUTION OF AN IODINATED DERIVATIVE CGP 12177 DEVELOPED FOR THE IMAGING OF CARDIAC β-ADRENOCEPTORS *IN VIVO*

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Cardiac β-adrenoceptors (β-AR) are known to be down-regulated in patients with chronic heart failure, because of a sustained increase in catecholamine levels. The aim of the present study was to develop suitable radioligands to image these particular receptors in vivo using Single Photon Emision Tomography (SPECT).

We compared the in vitro affinity of 4- (3-t-butylamino-2-hydroxypropoxy)-benzimidazole-one ((±)CGP12177), (S)-4-(3-t-butylamino-2-hydroxypropoxy)-5-iodo-benzimidazole-2-one (*S*-CYBL2B), 4-(3-t-butylamino-2-hydroxypropoxy)-7-iodo-benzimidazole-2-one (CYBL2A), respectively, for the β-adrenoceptor, using [125I]-Iodocyanopindolol as the radioligand. The Ki-values (nmol.l^{-1}, mean ± s.e., n=5), were 1.17±0.42, 28786±9255, 11.06±2.08, for CGP12177, CYBL2A, CYBL2BS, nadolol and inadolol, respectively. Subsequently, *S*-CYBL2B was tested in vivo in male New Zealand White rabbits (2.5-3.5 kg). Rabbits received an intravenous injection of 50 μCi of *S*-CYBL2B (specific activity > 5000 Ci/mmol) and were killed at 5,15,30,50 minutes, 1,2,4 and 24 hours after injection, respectively. Organs were removed, weighed and radioactivity was measured. Radioactivity levels in the left ventricle (LV) and in the lungs, expressed as % [injected dose x kg body weight]/g tissue, are listed in the table (mean ± s.e., n=3 per time point).

Table 1. Radioactivity levels of [^{123}I]*S*-CYBL2B in lung and left ventricular tissue of male New Zealand White rabbits at different time points after injection.

	LV	Lung
5 min	0.28 ± 0.07	1.49 ± 0.63
15 min	0.46 ± 0.04	0.89 ± 0.03
30 min	0.20 ± 0.03	0.66 ± 0.11
50 min	0.21 ± 0.01	0.50 ± 0.04
1h	0.20 ± 0.01	0.46 ± 0.03
2h	0.13 ± 0.002	0.33 ± 0.02
4h	0.07 ± 0.009	0.21 ± 0.002
24h	0.02 ± 0.002	0.08 ± 0.01

In a separate set of experiments animals received an pre-injection of 0.1 μmol propranolol i.v. in order to vdetermine specific binding in vivo. Although uptake of *S*-CYBL2B in the lungs could be suppressed significantly (0.63 ± 0.09 in the controls versus 0.33 ± 0.02, n=5, in the pretreated animals, $p<0.05$), uptake in the left ventricle could not be blocked by pre-injection of 0.1 μmol propranolol i.v. (0.24 ± 0.02 in the controls versus 0.20 ± 0.009, n=5, in the pretreated animals, $p>0.05$).

In conclusion, although *S*-CYBL2B shows high affinity in vitro for the receptor, due to high non-specific binding in vivo, this radioligand is probably not suitable for the imaging of cardiac β-adrenoceptors in vivo.

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220P CARDIAC SYMPATHETIC NEURONAL ACTIVITY AND FUNCTION IN THE EARLY PHASE OF LEFT VENTRICULAR VOLUME AND PRESSURE OVERLOAD

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Chronic cardiac overload is associated with increased cardiac sympathetic activity and depressed sympathetic neuronal function. In the present study we evaluated, in a rabbit model, the short term effects of cardiac overload on cardiac sympathetic neuronal activity by measuring β-adrenoceptor density and affinity (Bmax and Kd, respectively) and the myocardial noradrenaline (NA) concentration. We also analyzed cardiac sympathetic neuronal function as reflected by presynaptic re-uptake mechanisms, using [^{123}I]-metaiodobenzylguanidine ([^{123}I]-MIBG). This compound is handled by the neuronal re-uptake mechanisms similar to NA, but it is not subjected to enzymatic degradation via MAO or COMT.

In 9 male New Zealand White rabbits (2.5-3.5 kg) (group 1) volume overload (aortic valve perforation) and pressure overload (banding of the abdominal aorta on a suprarenal level) was induced in a two-stage surgical procedure. 5, age-matched animals were sham operated (group 2). Echocardiography was performed at baseline and 2 weeks after the second operation. Three weeks after the last operation, animals were killed 90 minutes after the intravenous administration of 50 μCi [^{123}I]-MIBG.

All data assessed 3 weeks after the last operation, for both groups of animals, are listed in the table.

Table 1. Left ventricular and biochemical characteristics of rabbits subjected to cardiac overload and sham operated animals.

	Group 1	Group 2
LV/BW ratio (10^{-3})	2.75 ± 0.29*	1.89 ± 0.13
LVEDD (cm)	1.57 ± 0.15*	1.35 ± 0.17
LVFS (%)	38.2 ± 0.57	36.9 ± 8.2
Bmax (fmol.mg^{-1} prot.)	167 ± 36*	224 ± 36
Kd (pmol.l^{-1})	46 ± 8*	71 ± 4
Cardiac NA (ng.g^{-1})	1004 ± 394*	1643 ± 109
[^{123}I]-MIBG	2.2 ± 0.58	1.8 ± 0.44

LV=left ventricle, BW= body weight, EDD= end diastolic diameter, FS= fractional shortening. Concentration of [^{123}I]-MIBG [% injected dose/g x kg]. Data are expressed as mean ± S.D.. *= significantly different ($p<0.05$) from the sham operated animals (group 2) using the Mann-Whitney U test.

The two-stage operation resulted in cardiac overload as is reflected by an increased LV/BW ratio and LVEDD. β-Adrenoceptor density significantly decreased, whereas affinity increased. NA proved to be significantly lower in left ventricular tissue of animals with cardiac overload. Myocardial [^{123}I]-MIBG uptake remained unaltered, thus indicating preservation of the neuronal re-uptake process.

These data suggest that although cardiac sympathetic activity is increased, neuronal function is preserved in early cardiac overload. Therefore, pharmacological reduction of this increased cardiac sympathetic drive may be beneficial in the early phase of cardiac overload, even when heart failure is not overt.

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221P COMPARISON OF THE ETA RECEPTOR POTENCY OF SELECTIVE AND NON-SELECTIVE ET ANTAGONISTS *IN VIVO* AND *IN VITRO*

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Comparison of the activity of selective (ETA) and non-selective (ETA/ETB) ET receptor antagonists on the *in vivo* ET-1 mediated pressor response is complicated by involvement of both ETA and ETB receptors (Flynn et al, 1994). BQ 788 is a selective ETB receptor antagonist which inhibits both the ETB receptor mediated vasodilator and constrictor responses (Ishikawa et al, 1994). In the presence of BQ 788, the ET-1 pressor response is mediated exclusively via ETA receptor activation (Sargent et al, 1995). In this study, we determined the dose-dependent effect of ETA receptor antagonists (BQ 123, BMS 182874 and PD 156707); and mixed ETA/ETB receptor antagonists (SB 209670 and bosentan) (Battistini et al, 1995) on the pressor response to ET-1 in the presence of BQ 788 in the pithed rat. In addition, we determined the antagonist potency of these compounds on the contractile response to ET-1 in rat isolated aorta.

Male Sprague Dawley rats (250 - 350 g) were anaesthetised with isoflurane, pithed and artificially respired. The jugular vein and carotid artery were cannulated for administration of vehicle or test compound and measurement of blood pressure and heart rate. BQ 788 (3 mg/kg) and all test compounds were given i.v. 10 min prior to the ET-1 cumulative dose response curve (0.01 - 10 nmol/kg). In a separate group of animals, the aorta from male Sprague Dawley rats was isolated and connective tissue removed. The aorta was cut into 3 mm rings, endothelium removed and placed in an organ bath containing oxygenated Krebs at 37°C. The tissues were equilibrated for 60 min under a resting tension of 2g. Tissues were then incubated with vehicle or ET antagonists for 30 min (n=6-8 per group). Contractile response to ET-1 in the presence of protease inhibitors and 0.1% bovine serum albumin was measured using standard techniques and compared to a reference 3 μM phenylephrine response and the potency of the antagonists expressed as pKB values. Statistical analysis of *in vivo* and *in vitro* data consisted of one-way analysis of variance followed by Student Newman Keul's t-test with $p< 0.05$ considered significant.

In the pithed rat, each of the antagonists produced rightward, parallel dose-dependent shifts in the ET-1 drc when compared to vehicle control. In the presence of antagonist maximum responses were achieved enabling calculation of an *in vivo* pA2. The dose-ratio for each antagonist was calculated as the shift from vehicle control at a dose of ET-1 which caused a 40 mm Hg change in diastolic blood pressure. The rank order of the apparent pA2 values *in vivo* shows no clear correlation with the pKB values derived from the rat aorta (Table 1). The reasons for this may be numerous, such as varying degrees of protein binding and differing metabolic stability of the compounds. In conclusion, these results illustrate that this model is capable of demonstrating dose-dependent antagonism of a number of standard ET antagonists and that ETA antagonist potency of these compounds can be accurately determined. However, the potency values determined and the relative rank order of a series of compounds may differ from *in vitro* values as a result of physiological influences.

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Sargent C.A., Brazdil R., Flynn D.A. et al., (1995) J. Cardiovasc. Pharmacol. 226 (suppl 2), S216-S218.

Table 1. Apparent *in vivo* pA2 values in the pithed rat and *in vitro* pKB values in isolated rat aorta against ET-1

Compound	SB 209670	PD 156707	BQ123	Bosentan	BMS 182874
In vivo apparent pA2	7.0 ± 0.2	7.2 ± 0.2	6.5 ± 0.1	6.0 ± 0.1	5.1 ± 0.2
In vitro pKB rat aorta	9.2 ± 0.4	8.4 ± 0.3	7.2 ± 0.6	7.1 ± 0.2	5.8 ± 0.4

All values are expressed as geometric mean ± 95% C.I. All comparisons are significantly different ($p< 0.05$) from one another except SB 209670 vs PD 156707 *in vivo* and BQ 123 vs Bosentan *in vitro*.

222P A BIOASSAY FOR MEASUREMENT OF LEVELS OF ET RECEPTOR ANTAGONISTS IN PLASMA AND CORRELATION WITH *IN VIVO* POTENCY

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Measurement of drug levels in plasma by HPLC can be time consuming and necessitates the development of specific assays for individual drugs. In this study, we describe the development of a general and rapid bioassay which quantitates the activity of endothelin receptor antagonists, parent and/or active metabolite, in plasma. In addition, we compared the plasma values with the antagonism of ET-1 induced increases in blood pressure in the pithed rat. The endothelin antagonists investigated were the ETA antagonist, PD 156707 (7.5 $\mu molkg^{-1}$) (Doherty *et al.*, 1995) and two ETA/ETB antagonists; bosentan (75 $\mu molkg^{-1}$) (Clozel *et al.*, 1994) and SB 217242 (75 $\mu molkg^{-1}$) (Battistini *et al.*, 1995).

Male Sprague Dawley rats (250-350g), fasted overnight, were dosed orally with vehicle or test compound either 90 or 180 minutes prior to the cumulative ET-1 dose-response curve (drc ; 0.01-10 $nmolkg^{-1}$; (n=4 to 11)). One hour prior to ET-1 administration rats were anaesthetised with isoflurane, pithed and artificially respired. The carotid artery and jugular vein were cannulated for measurement of diastolic blood pressure (DBP) and administration of the selective ETB-receptor antagonist BQ 788 (3 mkg^{-1}). The addition of BQ 788 enabled examination of the effect of agonist/antagonist on ETA-receptors only (Sargent *et al.*, 1995). Before administration of ET-1, 0.5ml of blood was sampled and plasma prepared. The antagonist shifts in the drc to ET-1 were compared with vehicle controls, by determining the doses of ET-1 necessary to produce a 40mm Hg rise in DBP. Results were expressed as geometric mean ± 95% confidence limits. Levels of parent compound and/or active metabolite in plasma were measured using an ET-receptor competition binding assay. ETA receptors prepared from cultured rat A10 cells were incubated in Millipore 96 well Multiscreen plates with 20pM [^{125}I] ET-1 in CO_2 independent tissue culture medium (Life Technologies) containing BSA (0.02% w/v), bacitracin (0.04% w/v) and EDTA (1.0mM). Reactions were incubated for 2 h at 37°C, terminated by vacuum filtration, the filters washed and counted. Plasma samples from the pithed rats were diluted such that they fell on the linear portion of a standard curve to parent compound prepared with 10% (v/v) control plasma. This plasma volume was maintained throughout the assay by diluting samples with 10% plasma. Total and non-specific binding were determined using 10% plasma and 10% plasma plus 500nM unlabelled ET-1. Results are expressed as plasma equivalents of parent compound as arithmetic mean ± s.e.mean (μM).

In the pithed rat, all three antagonists caused a significant parallel rightward shift from control drc's to ET-1. At 90 and 180 minutes, the shifts of the ET-1 drc produced by SB 217242 were 16 (8, 29) and 15 (9, 26), by bosentan were 13 (6, 28) and 16 (11, 23) and by PD 156707 were 33 (25, 45) and 5.7 (3.7, 8.7), respectively. Plasma levels of parent compound and/or active metabolite at 90 and 180 minutes were 6.1 ± 2.1 μM and 8.8 ± 2.3 μM with SB 217242, 23.4 ± 7.3 μM and 25.4 ± 2.7 μM with bosentan and 2.9 ± 0.6 μM and 0.5 ± 0.1 μM with PD 156707, respectively. Rank order correlation between shifts and plasma concentration was calculated for individual animals for each compound at 90 and 180 minutes using Spearman's rank correlation test. Significance was reached with each individual compound ($p<0.05$). Due to differences in potencies of the three compounds and/or metabolism, no correlation was observed when the plasma levels and shifts for all the compounds were combined. In conclusion, the receptor bioassay constitutes a rapid universal method of determining the concentration at the ETA-receptor of ET antagonists and/or their active metabolites in plasma. Furthermore, there appears to be good correlation between individual plasma values for each compound and their *in vivo* potency. An advantage of measuring the activity of parent and/or active metabolite is that the correlation with function should be better than with parent alone.

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223P THE INFLUENCE OF NITRIC OXIDE IN THE ROSTRAL VENTROLATERAL MEDULLA OF THE ANAESTHETISED RAT DURING TEMPERATURE REGULATION

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Intraventricular injection of nitric oxide synthase (NOS) inhibitors produces hyperthermia in the rat (Gourine 1994). A possible site of action could be the rostral ventrolateral medulla (RVLM), an area which possesses NOS-containing neurons (Vincent & Kimura, 1992) and which regulates vasomotor tone in the skin circulations of the tail and feet, thereby influencing heat loss or heat gain from the body (Key & Wigfield, 1992). The role of NO in the RVLM has been investigated by assessing the effects of injections of the NO donor 3-morpholinosydnonimine-N-ethylcarbide (SIN-1) or the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) into the RVLM during thermoregulatory adjustments.

Experiments were performed on 24, urethane-anaesthetised (1.8g. kg^{-1} i.p.), male Wistar rats (250-300 g) in a temperature controlled room (23±0.5°C). Mean arterial BP, heart rate and respiratory rate were monitored and changes in the degree of dilatation or constriction in the cutaneous circulations of the hind and front paws, dorsal skin surface and tail were assessed non-invasively by monitoring surface skin temperature (SS_T) with thermocouples (type K, Ni/Cr:Ni/Al). SS_T's were compared for linearity against blood flow using a laser Doppler flow probe. Body temperature (B_T) was measured rectally and maintained or adjusted using a small thermostatically-controlled heating blanket. Electrical stimulation (25Hz, 0.1 ms, 50-100μA, 3-5 min) and/or SIN-1 (0.1 or 0.2μl) was delivered through insulated stainless steel cannulae (60μm tip diameter). L-NAME was injected bilaterally through glass micropipettes (20 μm tip, 0.1 or 0.2μl), using D-NAME or artificial CSF (pH 7.3) as controls. The positions of the cannulae were verified histologically.

Raising the B_T to induce hyperthermia produced vasodilatation of the tail and foot circulations as indicated by the sudden and marked rise (6.36±0.2°C) in their SS_T's, as the B_T rose above 38.3-38.6°C. Only small (1.5±0.2°C) increases in SS_T's were noted in other skin areas. Electrical stimulation of the medial region of the RVLM in these hyperthermic rats (n=3) selectively reduced tail and foot SS_T's by 2.9±0.4°C ($P<0.05$). Injection of SIN-1 (400 or 800ng), but not artificial CSF (n=3), 5 mins prior to stimulation, reduced the attenuating effect of electrical stimulation by 76.6±10% ($P<0.05$, Student's t-test).

The increase in tail and foot SS_T's, which occurred during hyperthermia (i.e. BT>38.6°C) was reduced by 50±16% (n=4, $P<0.05$) following the injection of L-NAME (40 μg), but not after D-NAME (40 μg) or artificial CSF (n=4).

In the normothermic or hypothermic rat SIN-1 (400 or 800ng) produced only small, non-significant increases in tail or foot SS_T's (n=6). Similarly, the bilateral injection of L-NAME (20 or 40 μg) into the RVLM produced no significant change in SS_T's (n=4).

The results suggest that NO is involved in the control of sympathoexcitatory outputs from the RVLM which occurs during thermoregulation.

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224P EFFECTS OF SUBCHRONIC ADMINISTRATION OF NIRAVOLINE, A NOVEL AQUARETIC COMPOUND, ON WATER AND ELECTROLYTE RENAL EXCRETION IN CIRRHOTIC RATS

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Niravoline is a new selective kappa opioid agonist which has been reported to possess potent aquaretic properties both in normal animals (Hamon et al., 1995) and in healthy volunteers (Sinnassamy et al., 1994). It has also been shown to be able to improve impaired renal water metabolism in cirrhotic rats after acute administration (Bosch-Marcé et al., 1995). We have previously shown that during a 2-week treatment period in normal rats, the diuretic effects of niravoline showed no sign of desensitization (Hamon et al., 1995). Since the behaviour of the drug could be very different in pathological situations, we decided to analyze its effects after subchronic treatment in cirrhotic rats.
Cirrhosis was induced in 9 to 11-week old male Sprague Dawley rats according to the method of Kountouras et al (1984) : after anaesthesia with isoflurane, the bile duct was ligated with two silk threads and cut between the two ligatures ; the animals were then allowed to recover. A secondary biliary cirrhosis then developed and about 60% of the rats died during the 3 weeks which followed surgery. After having controlled the serum bilirubin and transaminases levels, treatment with niravoline (0.3 mg kg^{-1} bid, s.c.) (n= 13) was started 3 weeks after bile duct ligation. A control group (n =12) received the same volume of saline. Diuresis measurement was performed on the 15th day of treatment, one and two h after the last injection ; urine electrolytes and osmolality were measured on the 0 - 2 h samples. Two groups of normal rats (n = 10 per group) received either saline or niravoline using the same experimental protocol as above.
In normal rats, niravoline induced a significant increase in urine output during the two observation periods, the urine volumes being increased by 2.7 and 4.6 during the first and second h respectively, as compared with controls. Total urine volumes at the end of the 2 h collection period were 3.25 ± 0.72 and 9.98 ± 1.17 ml kg^{-1} in normal control and normal treated rats respectively. Urine output in cirrhotic rats was about 2 times higher than that of normal control rats (79.7 ± 14.8 vs 41.9 ± 9.8 μl kg^{-1} min^{-1} for the 0-1 h period). Nevertheless niravoline injection produced a 2.8 fold increase in urine elimination during the 0-1 h period. This effect was short lasting since it had disappeared during the 1-2 h period. Total urine volumes at the end of the 2 hour collection period were 7.45 ± 1.50 and 16.06 ± 3.56 ml kg^{-1} in cirrhotic control and cirrhotic treated rats, respectively. No significant modification of Na^+ or K^+ excretion was observed in the different treated groups, although a tendency for a decrease in Na^+ excretion appeared in the niravoline-treated cirrhotic rats. It must be noted that basal electrolyte elimination was higher in cirrhotic than in normal rats. A marked and similar decrease in urine osmolality was induced in both normal and cirrhotic rats by niravoline from 598 ± 99 to 280 ± 30 mosm kg^{-1} H_2O ($p < 0.01$) in normal rats and 543 ± 92 to 314 ± 83 mosm kg^{-1} H_2O ($p < 0.05$) in cirrhotic rats. Therefore, a subchronic treatment with niravoline in cirrhotic rats produced the same aquaretic effect as in normal rats, and did not seem to elicit any sign of desensitization.

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225P *IN VIVO* CARDIOVASCULAR EFFECTS OF THE α-ADRENOCEPTOR AGONIST S 18149 IN THE ANAESTHETIZED DOG

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S 18149, (5S)-spiro[(1,3-diazacyclopent-1-ene)-5:2'-(7'-methyl-1',2',3',4'-tetrahydronaphtalene)] fumarate, has been described as a partial agonist at α_1-adrenoceptors (Cordi et al., 1995). S 18149 is a potent but partial agonist at both α_1- and α_2-adrenoceptors in vitro and in the pithed rat (Verbeuren et al., this meeting). The aim of the present study was to investigate the cardiovascular effects of S 18149 in vivo in the dog.

The experiments were performed in pentobarbitone anesthetized dogs of either sex. Respiration was monitored, body temperature maintained at 38 °C and blood gases were controlled. The following recordings were performed: arterial blood pressure, heart rate, renal sympathetic nerve activity by bipolar stainless steel electrodes, subcutaneous temperature by a needle probe under the plantar skin and arterial blood flow by electromagnetic flowmeters. The drug, dissolved in 5 % glucose solution, was administered i.v. through the brachial vein.

S 18149 at 5 µg/kg induced a moderate and short lasting increase in mean arterial blood pressure (+22±2 mmHg mean±sem) associated with bradycardia (-38±7 beats/min, n=10) and sympatho-inhibition (-71±6 %, n=7). The compound produced a marked and sustained decrease in the carotid artery and the cutaneous saphenous artery blood flow (-62±4, n=7 and -67±7 %, n=6). The subcutaneous temperature was also decreased (-0.56±0.06 °C). The decrease in the muscular gracilis artery blood flow was weaker (-35±4, n=5) and of shorter duration. The renal artery blood flow (n=6) and the cardiac output (n=5) were not altered by the drug. In baroreceptor reflex denervated dogs, the bradycardia and the sympatho-inhibition were abolished (n=5).

Previous injection of the α_1-adrenoceptor antagonist prazosin (0.1 mg/kg) or the 5-HT_{1D} antagonist GR127935 (30 µg/kg) did not significantly alter the decrease in carotid artery blood flow or the increase in arterial blood pressure induced by S 18149 (5 µg/kg i.v.). However after treatment with the α_1-α_2-adrenoceptor antagonist phentolamine (1 mg/kg) or the α_2-adrenoceptor antagonists idazoxan (300 µg/kg) or methoxy-idazoxan (30 µg/kg), the decrease in carotid blood flow induced by S 18149 (5 µg/kg i.v.) was significantly lower (-11±5 % n=5, -19±1 % n=5 and -13±2 % n=5 respectively); the increase in arterial blood pressure was also inhibited (+8±1, +7±1 and +5±1 mmHg).

These results indicate that 1) the decreases in heart rate and sympathetic nerve activity induced by S 18149 are due to an activation of the baroreflex, 2) S 18149 selectively decreases the carotid and cutaneous blood flow, which may suggest an action of the drug on arteriovenous shunts, 3) the latter action of the compound is due to an activation of α_2-adrenoceptor. S 18149 is currently studied as a putative venotropic agent.

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226P DIRECT AND LONG-TERM ELECTROPHYSIOLOGICAL EFFECTS OF AMIODARONE IN DOG CARDIAC PURKINJE AND VENTRICULAR MUSCLE FIBRES

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It has been reported that acute and chronic treatments with the antiarrhythmic drug amiodarone causes different ECG changes in patients (1). Therefore, the cellular electrophysiological effects of chronic (6 weeks, 50 mg/kg/day, p.o.) and acute (5µM directly in the tissue bath) amiodarone administration were studied in dog cardiac ventricular muscle and Purkinje fibres by applying the conventional microelectrode technique (2). At 1 Hz stimulation frequency, chronic amiodarone treatment lengthened the repolarization of the ventricular (from 227.8±6.3 ms, n=20 to 262.3±5.2 ms, n=21; $p<0.01$) but shortened that of the Purkinje fibres (from 337.6±9.2 ms, n=21 to 308.3±7.1, n=19; $p<0.05$). Direct superfusion of 5 µM amiodarone on ventricular muscle and Purkinje fibres obtained from chronically treated dogs did not change the action potential duration (APD) in ventricular muscle but further shortened that in Purkinje fibres (from 309.7±13.6 ms to 281.9±11.9 ms, n=8; $p<0.05$). Neither the chronic nor the direct amiodarone application prevented the APD shortening in ventricular muscle evoked by 10 µM pinacidil, suggesting that amiodarone does not interfere with the ATP-dependent potassium channels. The difference of the APD between ventricular and Purkinje fibres in untreated controls (110 ms) decreased to 46 ms in fibres obtained from dogs chronically treated with amiodarone and increased to 185 ms in the presence of 30 µM sotalol (a class III antiarrhythmic drug) used in our experiments for comparison. Amiodarone (5 µM) applied directly abolished the early afterdepolarization (induced by 1 µM dofetilide + 2mM CsCl) in 5 out of 6 experiments. Amiodarone (5 µM) caused strong use-dependent V_{max} block with relatively fast onset (rate constant = 1.23±0.13 action potential, n=5) and offset (time constant = 364±62.5 ms, n=5) kinetics which best resembled those observed earlier with lidocaine and mexiletine (2). These results suggest that amiodarone differs from class III antiarrhythmic drugs, and this unique combination of cellular electrophysiological effects might be best classified as a new type I/D class of antiarrhythmic action (Supported by OTKA T 016651 grant).

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227P REGIONAL HAEMODYNAMIC RESPONSES TO VASODILATORS IN CONSCIOUS, TRANSGENIC [(mRen-2)27], HYPERTENSIVE RATS

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It is generally claimed that vasodilator function is impaired in hypertension, but it may not be (see Gardiner *et al.*, 1994). Since it is feasible that results depend on the experimental model considered, we assessed responses to vasodilators in transgenic (mouse Ren-2 renin gene insertion) hypertensive (TG) rats and their normotensive controls (Hannover Sprague-Dawley (SD) rats). Male, TG and SD rats (350-500 g) were anaesthetised (sodium metho-hexitone, 40-60 mg kg^{-1} i.p., supplemented as required) and had pulsed Doppler probes and and intravascular catheters implanted to allow renal (R) mesenteric (M) and hindquarters (H) haemodynamics to be monitored in the conscious state. Three min, i.v. infusions of acetylcholine (ACh; 56 nmol kg^{-1} min^{-1}), bradykinin (BK; 36 nmol kg^{-1} min^{-1}), salbutamol (SA; 2 nmol kg^{-1} min^{-1}), and sodium nitroprusside (SNP; 64 nmol kg^{-1} min^{-1}) were given in random order separated by at least 15 min. Mean blood pressure (MAP) was higher ($P < 0.05$, Mann-Whitney U test) in TG rats (173 ± 6 mm Hg, mean ± s.e. mean, n = 8) than in SD rats (105 ± 2 mm Hg, n = 10), and the former had lower ($P < 0.05$) vascular conductance (VC) in all 3 beds (TG rats; RVC = 36 ± 4; MVC = 34 ± 3; HVC = 29 ± 4 [kHz mm Hg^{-1}]10^3; SD rats: RVC = 66 ± 6; MVC = 76 ± 6; HVC = 42 ± 2 [kHz mm Hg^{-1}]10^3). Heart rate (HR) was not different (SD rats = 340 ± 7; TG rats = 371 ± 14 beats min^{-1}). TG rats showed a greater hypotensive and lesser mesenteric vasodilator response to SNP than SD rats (Table 1). SA evoked a similar fall in MAP in SD and TG rats, but in the latter the increases in VC were less. In SD rats, BK caused marked tachycardia but no significant fall in MAP, in spite of increases in MVC and HVC. In TG rats, BK evoked a clear hypotension, but a diminished tachycardia, and no increase in HVC. Elsewhere we have shown that the BK-induced increase in HVC is due to adrenal medullary-mediated activation of β_2-adrenoceptors (Gardiner *et al.* 1992). Hence, the changes in the responses to BK, SA and SNP are consistent with impairment of vascular as well as cardiac (Böhm *et al.*, 1994) β-adrenoceptors, and possibly adrenal medullary catecholamine release in TG rats. However, there was no evidence for a reduction in the ACh-mediated increase in RVC in TG rats (Table 1), so they resemble vasopressin-deficient hypertensive rats which appear to have normal nitric oxide-mediated vasodilator responses in the kidney (Gardiner *et al.*, 1994).

Table 1. Integrated (areas under or over curves) cardiovascular changes during infusion of vasodilators in SD and TG rats. Values are mean ± s.e. mean; * $P < 0.05$ for response, † $P < 0.05$ versus SD rats. Units: HR = beats; MAP = mm Hg min; RVC, MVC, HVC = [kHz mm Hg^{-1}]10^3.

	Bradykinin			Salbutamol	
	SD rats	TG rats		SD rats	TG rats
HR	305 ± 30*	199 ± 26*†	HR	135 ± 13*	162 ± 36*
MAP	15 ± 5	-31 ± 9*†	MAP	-23 ± 6*	-20 ± 4*
RVC	21 ± 7	26 ± 3*	RVC	22 ± 5*	9 ± 2*†
MVC	94 ± 10*	81 ± 9*	MVC	15 ± 3*	5 ± 3†
HVC	20 ± 6*	7 ± 3†	HVC	92 ± 12*	46 ± 13*†

	Sodium nitroprusside			Acetylcholine	
	SD rats	TG rats		SD rats	TG rats
HR	211 ± 23*	255 ± 17*	HR	236 ± 18*	258 ± 23*
MAP	-26 ± 2*	-59 ± 10*†	MAP	-19 ± 6*	-41 ± 15*
RVC	13 ± 3	19 ± 3*	RVC	62 ± 11*	46 ± 7*
MVC	83 ± 9*	56 ± 5*†	MVC	-23 ± 7	-14 ± 3*
HVC	-10 ± 2*	-5 ± 3	HVC	33 ± 14	19 ± 12

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228P HAEMODYNAMIC EFFECTS OF LIPOPOLYSACCHARIDE (LPS) INFUSION IN CONSCIOUS, VASSOPRESSIN-DEFICIENT BRATTLEBORO RATS

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Recently, we have described the regional and cardiac haemodynamic changes occurring during infusion of a low dose of LPS which causes only slight hypotension in conscious rats (Gardiner *et al.*, 1995c). Notably, in this model, pretreatment with a non-selective endothelin antagonist unmasks a profound hypotensive and mesenteric vasodilator response to LPS (Gardiner *et al.*, 1995b). In other studies we have shown that after 24 h infusion of LPS the renin-angiotensin system and vasopressin exert some influence on haemodynamic status (Gardiner *et al.*, 1995a). However, it is not known if the congenital absence of vasopressin compromises haemodynamic responses to LPS. To explore this possibility, we have examined the haemodynamic effects of LPS (E coli serotype 0127 B8, 150 μg kg^{-1} h^{-1}) in conscious, vasopressin-deficient (Brattleboro) rats.

Male, homozygous, Brattleboro rats (350-450 g) were instrumented with intravascular catheters and pulsed Doppler flow probes to monitor renal, mesenteric and hindquarters haemodynamics (all surgery carried out under sodium methohexitone anaesthesia, 40-60 mg kg^{-1} i.p.). Experiments began at least 24 h after the last surgical intervention (catheterization).

During a 24 h infusion of saline (n = 8) there were no consistent haemodynamic changes.

Table 1 shows that during LPS infusion there were 2 episodes of moderate hypotension, with persistent renal vasodilatation, transient mesenteric vasoconstriction, and hindquarters vasoconstriction followed by dilatation. These changes are largely similar to those seen in control (i.e., Long Evans) rats during LPS infusion (Gardiner *et al.*, 1995a).

Table 1. Cardiovascular variables before and during LPS infusion in Brattleboro rats (n = 7). RVC, MVC, HVC = renal, mesenteric, and hindquarters vascular conductance respectively. Values are mean ± s.e. mean; *$P < 0.05$ vs baseline (Friedman's test).

Time (h)	HR (beats min^{-1})	MAP (mm Hg)	RVC ([kHz mm Hg^{-1}]10^3)	MVC	HVC
0	310 ± 7	110 ± 3	69 ± 7	98 ± 7	40 ± 5
1	350 ± 8*	100 ± 4*	86 ± 9*	95 ± 12	47 ± 7
5	379 ± 12*	106 ± 6	103 ± 12*	85 ± 5*	36 ± 5*
8	378 ± 12*	97 ± 4*	98 ± 10*	73 ± 9*	45 ± 6
24	340 ± 18	106 ± 6	107 ± 12*	86 ± 12	61 ± 7*

Administration of the angiotensin (AT-1) receptor antagonist, losartan (10 mg kg^{-1} i.v.), after 24 h of LPS infusion caused a marked fall in MAP (-26 ± 5 mm Hg), tachycardia (38 ± 3 beats min^{-1}) and increases in RVC, MVC and HVC (42 ± 7, 55 ± 10, and 14 ± 2 units, respectively). The effects of losartan on MAP and MVC were about double those seen in Long Evans rats under similar conditions (Gardiner *et al.*, 1995a).

These findings indicate that, in contrast to endothelin, vasopressin does not play an indispensable role in maintaining blood pressure during LPS infusion, because, in its absence, activation of the renin-angiotensin system can compensate.

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229P CHARACTERISATION OF ENDOTHELIUM-DEPENDENT RELAXATIONS IN THE ISOLATED PERFUSED RAT SUPERIOR MESENTERIC ARTERIAL BED: THE INVOLVEMENT OF EDHF

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Recent interest has focussed on the possibility that endothelium-dependent relaxations are, in addition to nitric oxide, mediated by a putative endothelium-derived hyperpolarizing factor (EDHF). In the present study the contribution of nitric oxide to endothelium-dependent vasorelaxations to carbachol (Randall & Hiley, 1988) has been assessed by inhibition of nitric oxide synthase using N^G-nitro-L-arginine methyl ester (L-NAME) (Parsons *et al.*, 1994). The possible involvement of potassium channels in mediating the relaxations has been investigated by using glibenclamide, as an ATP-sensitive potassium (K_{ATP}) channel inhibitor, and tetraethylammonium (TEA) as a potassium channel blocker.

Male Wistar rats (250-360g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1} i.p.) and the mesenteric arterial bed was cannulated and perfused with oxygenated Krebs-Henseleit solution at 5 ml min^{-1} (Randall & Hiley, 1988). Perfusion pressure was continuously monitored by a pressure transducer placed close to the inflow cannula. Following 30 min equilibration, perfusion pressure was raised (ca. 100mmHg) by addition of methoxamine (1-40μM) and dose-response curves were constructed for the endothelium-dependent vasodilator carbachol in the absence and presence of either 100μM L-NAME, or 10μM glibenclamide, or 10mM TEA, or L-NAME plus TEA.

Basal perfusion pressure was 24.3±1.4mmHg (mean±s.e.mean, n=15) and was increased by 87.5±4.8mmHg following the addition of methoxamine. Carbachol caused dose-related relaxations of established tone (ED_{50}=180±30pmol, R_{max}= 88.2±3.2%, n=7). In 4 preparations treated with L-NAME carbachol was significantly (P<0.001; Student's t-test) less potent (ED_{50}=660±110pmol), with a maximum relaxation of established tone (R_{max}) of 88.9±2.4%. In preliminary experiments using the isolated rat ileum L-NAME did not influence the contractile responses to carbachol (Amoah & Randall, unpublished observations), indicating that L-NAME does not exhibit any anti-muscarinic properties at 100μM. In the 8 preparations treated with 10μM glibenclamide there were no significant differences in the vasorelaxant properties in the absence (ED_{50}=240±40pmol, R_{max}=89.2±1.3.%) or presence (ED_{50}=260±70pmol, R_{max}=86.6±2.7%) of the inhibitor. However, in the preparations treated with 10mM TEA the dose-response curve was significantly (P<0.001) shifted to the right (ED_{50}=1.97±0.14nmol vs 180±30pmol) with a depression of the maximum (R_{max}=74.6±3.2 vs 88.2±3.2%). In preparations treated with L-NAME plus TEA there was a reduction in the R_{max} (83.9±4.5%) for the relaxant effects of carbachol and the ED_{50} (14.8±6.9nmol) was significantly (P<0.001) greater.

The results demonstrate that in the mesenteric arterial bed a small proportion of the endothelium-dependent relaxation to carbachol is mediated by nitric oxide. Part of the nitric oxide-independent component is mediated via the activation of potassium channels which do not include K_{ATP}, and would therefore show the characteristics of EDHF; the nature of which is currently being investigated.

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230P CHARACTERISTICS OF HUMAN UMBILICAL ARTERY ENDOTHELIAL CELLS MAINTAINED IN A LOW OXYGEN ENVIRONMENT

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We have previously demonstrated the successful culture of human umbilical artery endothelial cells which retain phosphoinositide responses to histamine, ATP and thrombin (Hawley *et al.*, 1995). Most studies using cells from the umbilical vessels are performed at oxygen tensions far in excess of what the vessels would normally be exposed to *in situ* (pO_2 circa 16mmHg: Wulf, 1964). This study demonstrates the establishment and maintenance of these cells in a low (3%) oxygen environment and their viability with respect to their functional phosphoinositide responses.

Human umbilical artery endothelial cells (HUAEC) were isolated as previously described in detail (Hawley *et al.*, 1995). Cells were maintained in medium 199 containing 10% human serum, 10% foetal calf serum, 2mM glutamine, 90μg/ml heparin and 25μg/ml endothelial cell growth supplement. Cells were then grown in either a 21% O_2 (HUAEC21) or a 3% O_2 (HUAEC3) environment maintained at a humidified 37°C with 5% CO_2. All other manipulations of HUAEC3 were performed in a custom built low oxygen microbiological safety cabinet which provides an atmosphere of 3% O_2 by displacement with nitrogen gas. Media for HUAEC3 was pre-gassed in a 3%O_2 environment for several hours before use. Accumulation of ^{3}H-inositol phosphates was measured on confluent cell monolayers as described previously in detail (Hawley *et al.*, 1995). Cellular ATP was measured by bioluminescence as instructed in the LKB Wallac ATP monitoring kit. Glutathione levels were evaluated using the method of Saville (1958) and DNA content of cell cultures was determined as previously described (Karsten & Wollenberger, 1972). Identity of the endothelial cells was confirmed by immunocytochemical analysis using an antibody to human factor VIII related antigen.

Both HUAEC3 and HUAEC21 exhibited the typical cobblestone morphology of endothelial cells for at least 4 passages. The growth rates of the cells under the two conditions are remarkably similar at passage 3. However there is a small decrease in the cell number in HUAEC3 at passage 4. The amount of glutathione present did not significantly differ between conditions (2.08±0.32; 3%O_2 vs 2.60±0.30; 21%O_2 nmoles/10^6 cells: n=4; values represent mean ± s.e.m.). ATP levels were found to be significantly lower in HUAEC3 than HUAEC21 (14.4±1.4 vs 21.6±2.0 nmoles/10^6 cells; n=4; p<0.05, unpaired t test). Increase in phosphoinositide turnover in response to 0.1mM histamine, 1mM ATP, 1U/ml thrombin or 20mM sodium fluoride were found to be 4.5±0.8, 3.1±0.4, 3.3±0.4 or 3.4±0.4 fold over basal levels (n=7) in HUAEC3 which were not significantly different from the responses obtained in HUAEC21. On exposure of HUAEC3 to histamine the mean log EC_{50} value was found to be -5.11 ± 0.16 in the absence and -3.70±0.18 in the presence of 100nM mepyramine. The apparent K_B value calculated from parallel shifts obtained with mepyramine, assuming competitive antagonism, was found to be 4.56±0.83nM (n=7) which was similar to that previouly observed under 21%O_2 conditions (Hawley *et al*, 1995).

The results of this study demonstrate that the endothelial cells of the human umbilical artery can be grown in culture at an oxygen tension which mimics that *in situ*. Furthermore these cells retain their ability to increase phosphoinositide turnover in response to several agonists.

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231P *IN VIVO* EFFECT OF DOPAMINE ON PLATELET AGGREGATION IN THE RABBIT

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Human platelets take up dopamine by a specific, temperature and energy dependent process (Dean and Copolov, 1989). Dopamine (40μg/ml) potentiates ADP induced aggregation of rabbit platelets in vitro via an alpha-adrenergic type receptor (Ahtee and Michal, 1972) and higher doses of dopamine inhibit aggregation induced by ADP, adrenaline and collagen (Braunstein et al, 1977) probably via dopaminergic D1-like receptors (De Keyser et al, 1988).

We have studied the effects of dopamine in vivo in a model of thromboembolism using continuous monitoring of ^{111}Indium-labelled platelets in the pulmonary and cerebral vasculature of male NZW rabbits (May et al 1990). Animals were anaesthetised with diazepam (4mg/kg i.p.) followed 10 min. later by Hypnorm (0.4ml/kg i.m.). Dopamine infusions were commenced 40 min. prior to thrombin (90U/kg i.c.) or ADP (20μg/kg i.v.) induced platelet accumulation in the cerebral and pulmonary vasculature respectively and continued for the duration of the recording period. Platelet accumulation is expressed as mean ± s.e. mean (n=4) of the maximum % increase in counts above baseline values. Control and experimental values were compared using an unpaired t-test.

Pre-treatment with dopamine (30μg-2mg/kg/min i.v.) for 40 min had no effect ($P>0.05$) on subsequent platelet aggregation in the pulmonary vasculature induced by ADP (20μg/kg i.v.).
Infusion of dopamine (100μg/kg/min i.c.) significantly ($P<0.05$) increased the maximum % increase in platelet counts in the head following administration of thrombin (90U/kg i.c.) from 70.5±4.9 with saline to 108.3±5.6. Dopamine (1mg/kg/min i.c.) significantly ($P<0.05$) reduced platelet accumulation in the head from 126.6±9.5 (saline treated control) to 63.9±9.5.

These results extend previous in vitro findings by demonstrating that in vivo dopamine can both potentiate and inhibit agonist induced platelet aggregation suggesting the involvement of at least two distinct mechanisms. The inhibitory effects of dopamine on platelet function have important potential clinical implications and both the inhibitory and potentiating effects may be important with regard to the current clinical uses of dopamine in the treatment of congestive heart failure, shock and acute renal failure.

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232P ZD2486: A POTENT AND SELECTIVE ANTAGONIST OF PLATELET FIBRINOGEN RECEPTORS (GLYCOPROTEIN IIb/IIIa)

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Glycoprotein IIb/IIIa (GPIIb/IIIa) is a member of the integrin family of adhesion molecules. It is comprised of two glycoprotein subunits (α and β) joined in a non-covalent complex which spans the platelet plasma membrane; GPIIb/IIIa, on activation functions as a receptor for fibrinogen and other adhesive proteins. Fibrinogen binding to GPIIb/IIIa is a prerequisite for platelet aggregation independent of the nature of the initiating stimulus, antagonism of this receptor produces ubiquitous platelet inhibition. Platelets play a pivotal role in acute arterial thrombotic disorders such as unstable angina, and reocculsion following thrombolytic therapy or emergency angioplasty. Recently a chimeric monoclonal antibody (c7E3), directed against the GPIIb/IIIa receptor, was reported to reduce the ischaemic complications of coronary angioplasty and atherectomy in man (Topol *et al.*, 1994). We now describe the pharmacological properties of a novel small molecule GPIIb/IIIa receptor antagonist which might also have utility in a wide range of arterial thrombo-occulsive disorders.

In a sandwich ELISA technique *in vitro*, based on the method of Charo *et al.*, (1990), ZD2486 ((R)- 3-methyl-4-{4-[4-(4-pyridyl)piperazin-1-yl]phenoxy}butanoic acid) (1 to 1000 nM) caused concentration dependent inhibition of biotinylated fibrinogen binding to immobilised human platelet GPIIb/IIIa, giving a pIC_{50} value (mean ± s.e. mean) of 7.65 ± 0.1, n=4. In contrast, the compound (100 μM) did not modify integrin mediated adhesion of K562 (erythroleukemic), Jurkat (T-cell leukaemic) or Molt-4 (lymphoblastic leukaemia) to immobilised human vitronectin or fibronectin.

Functional platelet aggregation was measured using a Biodata aggregometer as previously described (Brownlie et al., 1994). ZD2486 (0.1 to 0.5 μM) caused concentration dependent inhibition of adenosine di-phosphate (ADP) induced aggregation of citrated human platelet rich plasma (PRP) *in vitro* giving an apparent pA_2 of 7.3 ± 0.1, n=4. Concentrations of ZD2486 in excess of 0.5 μM caused insurmountable receptor blockade. In addition the compound inhibited aggregation of human PRP mediated by a thromboxane mimetic U46619 (Bundy, 1975), collagen, adrenaline and PAF *in vitro* giving apparent pA_2 values of 7.3 ± 0.1, 7.3 ± 0.1, 7.3 ± 0.1 and 8.3 ± 0.2 respectively. In washed human platelets ZD2486 also inhibited thrombin induced platelet aggregation, pA_2 7.9 ± 0.1. The compound did not however modify ristocetin induced agglutination of washed human platelets; a phenomenon mediated by Von Willebrand Factor association with Glycoprotein Ib/IX.

We conclude that in vitro ZD2486 is both a potent and selective antiplatelet agent which acts as a fibrinogen receptor antagonist.

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233P ZD9583: A DUAL ACTING THROMBOXANE A_2 SYNTHASE INHIBITOR/RECEPTOR ANTAGONIST SUPPRESSES NEOINTIMAL HYPERPLASIA IN THE RAT

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ZD9583 (4Z)-6-[(2S,4S,5R)-2-(1-[2-cyano-4-methylphenoxy]-1-methylethyl)-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid) is a potent and selective thromboxane synthase (TXA_2) inhibitor and receptor antagonist.

In vitro, ZD9583 caused concentration dependent inhibition of both rat platelet U46619 (Bundy, 1975) induced aggregation and collagen stimulated TXA_2 production with pA_2 and IC_{50} values (mean ± s.e. mean) of 8.8 ± 0.2 (n=6) and 0.02 ± 0.01μM (n=4) respectively.

The ex vivo antagonist activity of ZD9583 was determined in rats by measuring U46619 stimulated aggregation of heparin treated platelet rich plasma obtained from rats orally dosed with ZD9583 or vehicle. TXA_2 synthase activity was assessed by measuring collagen stimulated TXA_2 production ex-vivo in whole blood withdrawn from the same animals. All data are expressed as mean ± s.e. mean and analysed using an unpaired students t-test.

Oral administration to rats of ZD9583 (1,3,5 and 10mg/kg) caused peak dose dependent inhibition of ex vivo stimulated TXA_2 production at 1 hour post dosing of : 38 ± 13%, 92 ± 2.5%, 97 ± 0.8% and 98 ± 0.5% respectively. Pronounced inhibition of platelet receptors (concentration ratio >140), and inhibition of TXA_2 synthase (>50%) persisted for 12 hours with all doses in excess of 1mg/kg.

In a rat model of left carotid endothelial cell denudation (Clowes *et al.*, 1983), ZD9583 (20mg/kg bid) dosed orally for 16 days caused profound inhibition of collagen stimulated TXA_2 production 91 ± 8.9% (n=8) and U46619 induced platelet aggregation (concentration ratio>100 n=4). This antiplatelet effect was associated with a significant ($p<0.05$) prolongation of rat tail bleeding time in ZD9583 (7.5 ± 0.07 min n=4) treated animals, when compared with vehicle (4.5 ± 0.2 min n=4) controls.

ZD9583 caused significant ($p<0.001$) inhibition of neointimal hyperplasia in de-endothelialized left carotid arteries. Intimal/medial ratios from the left carotid arteries of ZD9583 and vehicle dosed animals were (0.23 ± 0.038 mm^2 n=7) and (0.6 ± 0.042 mm^2 n=7) respectively. There was no discernible change in medial cross-sectional areas of either the ZD9583 or vehicle dosed groups.

These results indicate that manipulation of arachidonic acid metabolism by simultaneous TXA_2 synthase inhibition and TP receptor antagonism with ZD9583, is effective in the prevention of intimal hyperplasia in a rat model of endothelial denudation.

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234P ANALYSIS OF ANTAGONISM OF PHENYLEPHRINE-MEDIATED CONTRACTION OF THE RAT SMALL MESENTERIC ARTERY

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Previously, we reported that noradrenaline (NA), in addition to its α_1-adrenoceptor-mediated contractile effect, may have a relaxant action in the rat isolated small mesenteric artery (s.m.a.) assay in order to account for steep Schild plots obtained with compounds classified as α_1-adrenoceptor antagonists (Van der Graaf *et al.*, 1995a). Subsequently, we exposed a relaxant action of NA in this assay (Van der Graaf *et al.*, 1995b) which appeared to be mediated by dopamine D_1 receptors (Van der Graaf *et al.*, 1995c). Because we found that the selective α_1-adrenoceptor agonist, phenylephrine (PE), did not produce relaxation of precontracted s.m.a. (Van der Graaf *et al.*, 1995c), we used this agonist for a further study of the effects of α_1-adrenoceptor antagonists in this tissue.
S.m.a.'s (internal diameter 100-300μm) from male Wistar rats (225-350g) were mounted as 2mm ring segments in a myograph (37°C, gassed with 95%O_2/5%CO_2) as described before (Van der Graaf *et al.*, 1995a). The endothelium was removed, as confirmed by the lack of response to 10μM of the acetylcholine M-receptor agonist, 5-methylfurmethide, after precontraction with a single concentration of 10μM NA. After a 15min washout period, tissues were incubated for 90min with 30μM cocaine, 6μM timolol and antagonist or the appropriate vehicle. Single PE concentration-effect (E/[A]) curves (n=4-5) were obtained by cumulative dosing.
The α_1-adrenoceptor antagonists (see Van der Graaf *et al.*, 1995a), prazosin (10-300nM), tamsulosin (0.3-10nM) and HV723 (10-300nM) produced concentration-dependent, parallel, rightward shifts of the PE E/[A] curves and Schild analysis yielded slope parameters (b) not significantly different from unity (Table 1), characteristic of simple competitive antagonism. The pK_B estimates obtained for prazosin and tamsulosin were not significantly different from the pA_2 estimates obtained when NA was used as agonist (Table 1). This suggests that the antagonist affinity estimates obtained previously were not distorted by the steep Schild plots. The affinity estimate for HV723, a ligand which, in contrast to prazosin and tamsulosin, behaved as a competitive antagonist of NA (Van der Graaf *et al.*, 1995a), was significantly (~3-fold) lower in the case of PE (Table 1). This may be indicative of the presence of a heterogeneous receptor population, which, considering the low affinity for prazosin, has the characteristics of the α_{1L}-adrenoceptor class (Ford *et al.*, 1994).
In conclusion, prazosin, tamsulosin and HV723 behave as competitive antagonists of PE in the s.m.a. Because PE, in contrast to NA, does not exhibit a significant relaxant action in this assay (Van der Graaf *et al.*, 1995c), these results are consistent with our hypothesis that the steep Schild plots obtained previously (Van der Graaf *et al.*, 1995a) were due to an additional inhibitory effect of NA.

Table 1 Results of the analysis of antagonism of noradrenaline and phenylephrine in rat s.m.a.

	phenylephrine		noradrenaline[1]	
	pK_B±se	b±se	$pK_B(pA_2)$±se	b±se
prazosin	8.68±0.08	1.12±0.07	(8.5±0.1)	1.63±0.13[2]
tamsulosin	10.20±0.15	1.10±0.09	(9.8±0.2)	1.35±0.09[2]
HV723	8.46±0.15	0.96±0.09	8.96±0.08	0.98±0.06

[1]Data from Van der Graaf *et al.* (1995a)
[2]b significantly greater than unity ($P<0.05$)

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235P SELECTIVE SUPPRESSION OF ADENOSINE A_3 RECEPTOR-MEDIATED HYPOTENSIVE RESPONSES IN THE RAT BY DEXAMETHASONE

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The adenosine A_3 receptor agonist, N^6-2-(4-aminophenyl)ethyl-adenosine (APNEA), induces hypotension in the anaesthetized rat (Carruthers & Fozard, 1993). The response is associated with a widespread degranulation of tissue mast cells and a substantial increase in plasma histamine (Fozard *et al.*, 1995; Hannon *et al.*, 1995). Pharmacological evidence points to mast cell activation as the primary mechanism contributing to A_3 receptor-mediated hypotension in the rat (Hannon *et al.*, 1995). Recently, Ramkumar *et al.* (1995) demonstrated a marked increase in both the number of A_3 receptors and the functional response to their activation in rat basophilic leukemia (RBL-2H3) cells exposed to dexamethasone *in vitro*. It was therefore of interest to explore the effects of dexamethasone on the mast cell-dependent hypotensive response to A_3 receptor activation in the rat.

Male Sprague-Dawley rats (196-410 g) were anaesthetized with pentobarbitone sodium, 60 $mgkg^{-1}$ i.p., and set up for recording blood pressure (BP) and heart rate as previously described (Carruthers & Fozard, 1993). APNEA, N^6-cyclopentyladenosine (CPA), 2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethyl-carboxamidoadenosine (CGS 21680) or compound 48/80 were injected i.v. and dosing was cumulative. Responses to APNEA were obtained in animals pretreated with 8-(p-sulphophenyl) theophylline, 40 $mgkg^{-1}$ i.v. in order to "isolate" the A_3 receptor-mediated hypotensive response. Dexamethasone 21-phosphate disodium salt was dissolved in saline and injected s.c.

24 h pretreatment with dexamethasone, 1 $mgkg^{-1}$, induced significant but surmountable blockade of the hypotensive response to APNEA (1-30 µg/kg). The doses of APNEA which reduced BP by 30 mmHg (ED_{30}) were 6.5 ± 2.2 (n=5) and 17.7 ± 2.0 (n=5) μgkg^{-1} for animals given vehicle, or dexamethasone, respectively. A dose of 0.3 mg/kg was without effect (ED_{30}, 6.3 ± 0.3 μgkg^{-1}, n=4) and no further blockade could be achieved by increasing the dose of dexamethasone to 3 $mgkg^{-1}$ (24 h pretreatment: ED_{30}, 21.3 ± 6.0 μgkg^{-1}, n=4) or following 1 $mgkg^{-1}$ given on three successive days (ED_{30}, 23.2 ± 2.3 μgkg^{-1}, n=4). A 3 h pretreatment with dexamethasone, 1 $mgkg^{-1}$, was without effect (ED_{30}, 9.8 ± 2.2 μgkg^{-1}, n=3). Dexamethasone, 1 $mgkg^{-1}$ (24 h pretreatment) or 1 $mgkg^{-1}$ given on three successive days did not affect the BP fall induced by the mast cell degranulating agent, compound 48/80 (10-300 μgkg^{-1}, n=4). Similarly, the cardiovascular responses to the A_1 receptor agonist, CPA (0.3-10 μgkg^{-1}, n=4) or the A_{2A} receptor ligand, CGS 21680 (0.3-30 μgkg^{-1}, n=4), were unaffected by pretreatment with dexamethasone 1$mgkg^{-1}$ for 24 h.

The selective suppression by dexamethasone of A_3 receptor-mediated, mast cell-dependent hypotensive responses to APNEA in the rat, contrasts markedly with data from the RBL-2H3 cell line (Ramkumar *et al.*, 1995) where an increase in A_3 receptor responsiveness was seen following exposure to dexamethasone. Experiments to define further the mechanism of A_3 receptor-mediated response suppression by dexamethasone *in vivo* are in progress.

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236P THE NITRIC OXIDE SYNTHASE PRODUCT, L-CITRULLINE AND THE NITRIC OXIDE DONOR, SIN-1 ARE CONVULSANT, WHILST L-ARGININE IS ANTICONVULSANT IN RODENTS WITH INBRED REFLEX EPILEPSY

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The role of nitric oxide in epilepsy is equivocal and is complicated by studies using nitric oxide synthase inhibitors which inhibit the vascular form of the enzyme. We have previously shown that the neuronal selective nitric oxide synthase inhibitor, 7-nitroindazole is anticonvulsant after systemic administration in two animal models of reflex epilepsy, namely sound-induced seizures in DBA/2 mice and in genetically epilepsy-prone (GEP) rats (Smith et al., 1995). Yet penicillin-induced epileptiform activity is reduced in rats by the nitric oxide donor, S-nitrosopenicillamine (Marangoz et al., 1994). We have now extended our earlier findings by studying the effects of the nitric oxide synthase substrate, L-arginine, and product, L-citrulline, and of the nitric oxide donor 3-morpholinylsydnoneimine (SIN-1) in DBA/2 mice and in GEP rats.

DBA/2 mice, briefly anaesthetised with halothane, received intracerebroventricular (i.c.v.) administration of vehicle, L-arginine (0.14-4.7 µmole), D-arginine (1.4 µmole), L-citrulline (0.47-4.7 µmole), or SIN-1 (0.1-0.8 µmole) and were observed for up to 1 h for signs of seizure activity (n=7-10). If no seizure activity was observed the animals were exposed to sound of 100-110 dB at 12-16 kHz for up to 60s. GEP rats (200-400 g) received vehicle, L-arginine (0.5-5 g/kg), D-arginine (0.5-2.5 g/kg), or L-citrulline (0.05-5 g/kg) (i.p.) and were evaluated for anticonvulsant effect against sound-induced seizures at 0.25-8h after administration (n=7-8).

In DBA/2 mice, L-citrulline (1.4-4.7 µmole) or SIN-1 (0.3-0.8 µmole) induced clonic seizures with CD_{50} values (95% confidence limits) (µmoles i.c.v.) of 1.6 (0.2-13.7) and 0.5 (0.4-0.7) respectively (see fig 1A). L-arginine (but not D-arginine) reduced sound-induced clonic seizure with an ED_{50} value at +15 min of 0.5 (0.3-1.0) µmole (see fig 1B). In GEP rats, L-citrulline (0.05-5 g/kg i.p.) was not proconvulsant or anticonvulsant. L-arginine was more potent as an anticonvulsant than D-arginine with ED_{50} values against sound-induced clonic seizure at +4h for L-arginine of 0.9 (0.5-1.7) g/kg and for D-arginine of 2 (1.5-2.6) g/kg (i.p.).

Figure 1A. Convulsant effects in DBA/2 mice

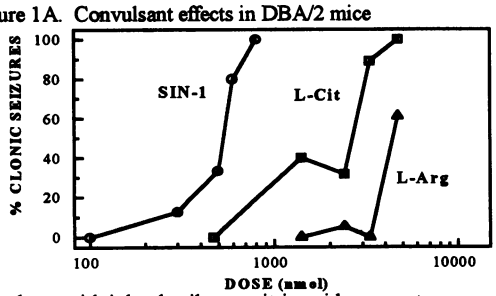
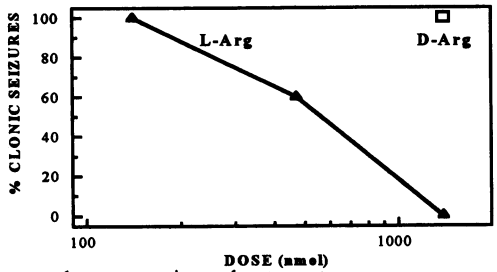


Figure 1B. Anticonvulsant effects in DBA/2 mice



In rodents with inbred epilepsy, nitric oxide may act as a proconvulsant rather than as an endogenous anticonvulsant agent.

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237P THE ROLE OF α AND β ADRENOCEPTORS IN A RODENT MODEL OF NEUROPATHIC PAIN

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Peripheral nerve or soft-tissue injury can lead to chronic pain syndromes, such as causalgia and reflex sympathetic dystropy, which are often dependent on the sympathetic nervous system (Bonica, 1970). While the role of the symapthetic nervous system in producing or maintaining chronic pain states has been widely studied, the role of adrenoceptor sub-types is not well defined. We have therefore examined the effects of α_1, α_2 and β adrenoceptor agonists and antagonists on tactile allodynia in a rodent model of painful peripheral neuropathy.

Male Sprague-Dawley rats (150 - 200g) were subjected to unilateral tight ligation of spinal nerves L5 and L6 just distal to the dorsal root ganglia (Kim & Chung, 1992). The animals were allowed to recover for 7-14 days before testing for tactile allodynia. Allodynia was measured as the hind paw withdrawal response to stimulation of the plantar surface with von Frey filaments (Chaplan *et al.*, 1994). A significant ($P<0.01$) reduction in the stimulus threshold (*i.e.* allodynia) was observed in the paw ipsilateral to the ligated nerves. The threshold for evoking withdrawal on the ligated side was 2.61 ± 0.12 g (mean ± s.e. mean, n=126) in comparison to ≥15 g on the contralateral side. Statistical analysis of the data used a Kruskal-Wallis non-parametric ANOVA test on ranked data followed by a post-hoc Fisher's LSD for multiple comparisons.

Systemic administration of the α_1 adrenoceptor antagonist, prazosin (0.1-1 mg kg^{-1}, ip), or the non-selective α adrenoceptor antagonist phentolamine (0.3-3 mg kg^{-1}, ip) did not significantly ($P>0.05$) alter tactile allodynia. Similarly, the α_1 adrenoceptor agonists methoxamine (0.1-3 mg kg^{-1}, ip) and phenylephrine (0.1-0.3 mg kg^{-1}, ip) did not significantly ($P>0.05$) affect tactile allodynia. In contrast, at 1hr post-dose, the non-α_2 sub-type selective adrenoceptor agonists clonidine (10-300 μg kg^{-1}, ip) or dexmedetomidine (DEX; 10-100 μg kg^{-1}, ip) resulted in a dose-dependent, reversible reduction in tactile allodynia. The minimal effective dose for DEX (mean ± s.e. mean ; % maximum possible effect (%MPE)) was 10 μg kg^{-1} (10.93 ± 2.74 % vs 2.05 ± 1.45 % for vehicle; $p<0.01$) and for clonidine was 60 μg kg^{-1} (28.23 ± 7.3 % vs 1.97 ± 2.91 % for vehicle; $p<0.01$). Moreover, DEX was more efficacious than clonidine as it produced a maximal amelioration of the tactile allodynia response at 100 μg kg^{-1} . In contrast, the %MPE for a maximally effective dose of clonidine (300 μg kg^{-1}) was 51.95 ± 3.65 %. The selective α_2 adrenoceptor antagonist RX 821002 (0.5 and 1.0 mg kg^{-1}, ip) (Berridge *et al.*, 1985), had no effect on tactile allodynia. However, the anti-allodynic actions of DEX (100 μg kg^{-1}, ip) were significantly attenuated ($p<0.01$) by RX 821002 (0.5 mg kg^{-1}, ip; % MPE= 92.88 ± 7.12 DEX alone vs. 18.53 + 9.27 for DEX plus RX 821002). This reversal supported an α_2 adrenoceptor-mediated mechanism of action. The β-adrenoceptor antagonist propranolol (4 and 8 mg kg^{-1}, ip) and the β-agonist isoproterenol (1 and 5 mg kg^{-1}, ip) did not significantly affect tactile allodynia.

In summary, the present findings are consistent with a role for α_2, but not α_1 or β, adrenoceptors in the modulation of tactile allodynia in a rat model of neuropathic pain.

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238P QUANTIFICATION OF CHEMOKINE-INDUCED CHANGES IN EXTRACELLULAR ACIDIFICATION RATE OF THP-1 CELLS USING A MICROPHYSIOMETER

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Chemokines are a family of small molecular weight (8-10 kDa) inducible cytokines which mediate the recruitment and activation of leucocytes at sites of inflammation. Chemokines exert their effects through the activation of G-protein coupled, seven transmembrane receptors. Receptor activation results in an increase in the metabolic activity of the cell with a subsequent elevation in H^+ ion production. Agonist-induced changes in the extracellular acidification rate (ECAR) can be measured in real time using the Cytosensor™ microphysiometer. Changes in ECAR of the monocytic cell line THP-1, in response to increasing concentrations (0.1 nM - 100 nM) of the C-C chemokines monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, macrophage inflammatory protein-1α (MIP-1α), MIP-1β and RANTES and the C-X-C chemokine interleukin 8 (IL-8) have been studied. For use in the microphysiometer, THP-1 cells were resuspended at $2x10^7$ cells/ml in low buffer RPMI-1640 containing 1% BSA, mixed with an agarose entrapment medium and placed into a microphysiometer capsule cup (150,000 cells/cup). Cells were perfused with low buffer/BSA media for 1 h prior to exposure to chemokines. THP-1 cells were then challenged with increasing concentrations of chemokine for consecutive 90 s periods, with the ECAR being measured over the final 30 s of each exposure. MCP-1 induced consistently a bell-shaped ECAR concentration-response curve (Figure 1). Concentration-related increases in ECAR were also observed in THP-1 cells exposed to MCP-2, MCP-3, MIP-1α, RANTES and IL-8. MIP-1β did not elicit a response at concentrations up to 100 nM. Maximal responses of 20 ± 1%, 10 ± 1.5% and 7.5 ± 2.5% were obtained for MCP-1 (10 nM), MIP-1α (30 nM) and IL-8 (30 nM) respectively. From the present experiments it is not known whether the responses to 100 nM MCP-2, MCP-3 and RANTES were maximal. The EC_{50} values for MCP-1, MIP-1α and IL-8 were calculated as 0.7 ± 0.05 nM, 13 ± 6 nM and 9 ± 1 nM respectively. Based upon responses obtained with 100 nM MCP-2, MCP-3 and RANTES EC_{50} values are ≥ 11 ± 0.03 nM, 7 ± 3 nM and 9 ± 1 nM respectively.

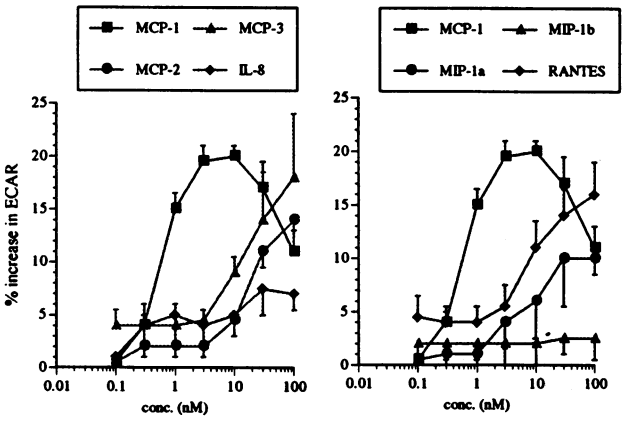

Figure 1. ECAR of THP-1 cells in response to challenge with increasing concentrations of chemokines. The same MCP-1 curve is represented on each graph for reference. Results are expressed as mean ± s.e.mean of four concentration-response curves.

In conclusion, these results indicate that under the conditions described, MCP-1 is the most potent of the chemokines tested in THP-1 cells. The potencies of these chemokines in inducing ECAR responses in THP-1 cells agree well with data reported previously for chemotactic responses in human monocytes (Uguccioni *et al.*, 1995).

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239P INDUCTION OF CYCLOOXYGENASE-2 BY LIPOTEICHOIC ACID IN BOVINE ENDOTHELIAL CELLS INVOLVES THE ACTIVATION FACTOR NF-κB

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Lipoteichoic acid (LTA) a cell wall-fragment of the gram-positive bacterium *Staphylococcus aureus*, causes the expression of the inducible form of nitric oxide synthase (iNOS) *in vitro* and *in vivo* (De Kimpe *et al.*, 1995). Here we investigate whether (i) LTA causes the expression of cyclooxygenase-2 (COX-2) in cultured bovine aortic endothelial cells (BAEC), and (ii) whether the induction of COX-2 by LTA involves the activation of NF-κB.

BAEC's were cultured in 96 or 6-well plates in culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4 mM) until confluent. LTA (10 μg/ml) was added to the cells to induce COX-2 activity. The accumulation of 6-keto-$PGF_{1\alpha}$, an indicator of COX activity, was measured 24 h later in the supernatant of BAEC's by radioimmunoassay (Salmon, 1978). Cell respiration an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mosmann, 1983).

Activation of BAEC with LTA (10 μg/ml) caused an increase in the concentration of 6-keto-$PGF_{1\alpha}$ in the medium from 30±9 ng/ml (n=14, baseline) to 118±13.8 ng/ml (n=12, $p<0.05$). Inhibition of the nuclear transcription factor NF-κB with pyrrolidine dithiocarbamate (PDTC, an antioxidant and a metal chelator; Baeuerle & Henkel,1994) or rotenone (an antioxidant which inhibits electron transport; Baeuerle & Henkel,1994.) significantly attenuated the increase in 6-keto-$PGF_{1\alpha}$ caused by LTA (Tab.1). Similarly, inhibition of IκB-protease, which is essential for the activation of NF-κB, by either L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) or calpain inhibitor-I (Lin *et al.)* also significantly reduced the increase in 6-keto-$PGF_{1\alpha}$ induced by LTA in BAEC (Table 1). Interestingly, neither LTA alone nor LTA in the presence of any of the above inhibitors of the activation of NF-κB (at the concentration used) caused a significant decrease in cell viability (data not shown). After LTA for 24 h there was also expression of COX-2 protein (70 kDa) as identified by Western Blot analysis. This expression was significantly attenuated by TPCK (n=9).

Table 1. Effect of inhibitors of NF-κB in LTA induced 6-keto-$PGF_{1\alpha}$ formation in BAEC's.

Inhibition of	Inhibitor	Concentration	6-keto-$PGF_{1\alpha}$ [ng/ml]	n
		Vehicle	30±9	14
-	-	LTA 10 μg/ml	118±13.8	12
NFκB activation	PDTC	20 μM	28±3.7*	9
NF-κB activation	Rotenone	30 μM	31±6.2*	12
IκB-protease	TPCK	10 μM	63±12.2*	12
IκB-protease	Calpain-I	30μM	35±12.2*	12

Data are given as mean ± s.e.mean; *$P<0.05$ vs LTA (unpaired Student's t-test)

Thus, the enhanced formation of 6-keto-$PGF_{1\alpha}$ by LTA in cultured BAEC's is due to the induction of COX-2 activity. In addition, our results suggest that the signal transduction mechanism leading to the induction of COX-2 protein and activity caused by LTA in BAEC involves the activation of the nuclear transcription factor NF-κB.

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240P THE EFFECT OF L-N^6-(1-IMINOETHYL)LYSINE ON ZYMOSAN-INDUCED OEDEMA IN RAT DORSAL SKIN

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L-N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor, attenuates inflammatory oedema (e.g. Hughes *et al.*, 1990). However, the involvement of inducible NOS (iNOS) in prolonged inflammatory oedema is unclear. We have recently shown that the iNOS inhibitor aminoguanidine (AG) inhibits oedema formation induced by i.d. zymosan over 0-4 h in rat (Ridger and Brain, 1995) and we now compare the effect of the iNOS inhibitor L-N^6-(1-iminoethyl)lysine (L-NIL), (Moore *et al.*, 1995). The dorsal skin of anaesthetised (pentobarbitone, 50 mg kg^{-1}, i.p.) male Wistar rats (220-250 g) was shaved. Prostaglandin production was inhibited with indomethacin (10 mg kg^{-1}, s.c., -30 min). L-NAME, AG & L-NIL were injected i.d. ± zymosan (10 μg $site^{-1}$, 0.1 ml $site^{-1}$). Plasma extravasation was measured over 0-45 min or 0-4 h by the accumulation of i.v. ^{125}I-albumin. Each animal was used as its own control. Tyrode (0.1 ml, i.d.) produced plasma extravasation of 24±2 (0-45 min) and 28.2±3 (0-4 h) μlsite^{-1}, mean±s.e.mean, n=4-8, which was not significantly altered by NOS inhibitors injected alone. L-NAME significantly inhibited zymosan-induced oedema over 0-45 min whereas AG and L-NIL did not. However, over 4 h, both AG and L-NIL significantly attenuated zymosan-induced oedema. iNOS is not expressed by 45 min after inflammatory insult (see Gross & Wolin, 1995). Therefore, any reduction in the plasma extravasation observed over the shorter time period of 45 min by L-NAME is not due to the inhibition of iNOS. By comparison, we suggest that AG and L-NIL are inhibiting oedema over 4 h as a consequence of inhibiting iNOS activity.

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Table 1. Modulation of zymosan-induced plasma extravasation. Results are expressed as μl plasma $site^{-1}$, mean ±s.e.mean, n= 4-8. *$p<0.05$, **$p<0.01$ significant inhibition of treated vs zymosan; ANOVA, followed by Student Newman-Keul's test.

Test agent		0-45 min		0-4 h	
		zymosan control	zymosan+test agent	zymosan control	zymosan+test agent
L-NAME	30 nmol $site^{-1}$	104±11.1	84.1±6.70	not done	not done
	100	104±11.1	68.4±10.3**	not done	not done
	300	104±11.1	69.2±8.10**	not done	not done
AG	30	101±11.8	93.8±14.4	182±20.7	135±16.3
	100	93.7±22.0	79.8±19.3	200±25.0	133±11.3*
	300	101±11.8	84.9±15.4	182±20.7	107±20.2*
L-NIL	30	93.7±22.0	93.6±20.6	200±25.0	128±15.3*
	100	93.7±22.0	89.9±19.5	200±25.0	136±18.3*

241P AUTORADIOGRAPHIC LOCALISATION OF [^{3}H]-(S)-ZACOPRIDE LABELLED 5-HT$_3$ RECEPTOR SITES IN THE PIG FOREBRAIN

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The technique of *in vitro* autoradiography has been used to investigate the distribution of 5-HT$_3$ (5-hydroxytryptamine$_3$) receptor recognition sites in the central nervous system of several species, using a variety of radioligands (for review see Laporte et al 1992). Following our report of the distribution of radiolabelled 5-HT$_3$ receptors in homogenates of pig brain (McWilliams et al 1995), we report the distribution of the binding of [^{3}H]-(S)-zacopride to 5-HT$_3$ receptor sites in pig forebrain, investigated using quantitative receptor autoradiography.

Pig brain tissue was obtained from a local abattoir and was cooled on ice within 30 min of death. Following dissection, brain regions were frozen at -80°C (within 2 hr of death). Frozen brain regions were cut using a cryostat, thaw mounted on to gelatin-coated glass slides, and stored at -80°C until use. Thawed (4°C) slide-mounted sections were pre-incubated in Tris/Krebs buffer (mM; Tris, 50; NaCl, 118.0; KCl, 4.75; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; glucose, 11.0; pH 7.4) for 30 min at 4°C, before being incubated in Tris/Krebs buffer (4°C) containing 0.4 nM [^{3}H]-(S)-zacopride in the absence (total binding) or presence of 1.0 μM granisetron (non-specific binding) for 60 min. The sections were washed twice for 5 min in ice-cold Tris/Krebs buffer, and rinsed for 1 sec in ice-cold distilled water. The sections were then rapidly dried in a stream of cold dry air before being exposed to tritium-sensitive film (Hyperfilm-[^{3}H], Amersham) along with tritium standards (Amersham) for 14 weeks. Developed autoradiographs were analysed and quantified (with reference to the tritium standards), using an image analysis system (MCID, Imaging Research Inc.). Total and non-specific binding (fmol mg^{-1} wet weight tissue equivalent) was determined for each area from 6-53 sections per animal, originating from 1-4 separate animals.

Specific binding (which represented between approximately 90-20% of total binding) of [^{3}H]-(S)-zacopride (0.4 nM) was differentially distributed throughout the forebrain of the pig. In contrast, non-specific binding was distributed homogenously. Highest levels of specific [^{3}H]-(S)-zacopride binding were detected in cerebral cortical areas of the pig brain (frontal cortex 4.75 (range 3.28-5.59, n=3), striate cortex 3.77 (n=1), parietal cortex 3.06 (range 2.04-4.07, n=2), temporal cortex 2.94 (range 2.59-3.32, n=3), occipital cortex 2.27 (range 1.80-2.73, n=2); mean fmol mg^{-1} tissue), with the binding being largely associated with the outer layers of the cortex. Lower levels were detected in other brain regions (hippocampus 1.13 (range 0.76-1.75, n=3), globus pallidus 0.91 (n=1), caudate putamen (0.71, range 0.63-0.78, n=4), cerebellum 0.41, n=1).

The distribution of radiolabelled 5-HT$_3$ receptor sites in the pig forebrain assessed using quantitative receptor autoradiography corresponds with our previous studies using homogenates of pig forebrain (McWilliams et al 1995), where specific [^{3}H]-(S)-zacopride binding was also highest in cortical areas of the pig brain.

We conclude that relatively high levels of [^{3}H]-(S)-zacopride labelled 5-HT$_3$ receptors are present in the pig cerebral cortex.

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242P EFFECT OF THE NK$_1$ RECEPTOR ANTAGONISTS, L-733,060 AND CP-99,994, AND MORPHINE IN THE RABBIT SPINAL REFLEX PREPARATION

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It has previously been suggested that NK$_1$ receptor antagonists may be useful in a number of indications including emesis, migraine and pain (for review see Longmore *et al.*, 1995). In this study the effects of the NK$_1$ receptor antagonists, CP-99,994 and L-733,060 ((2S,3S)-3-((3,5-bis(trifluromethyl)phenyl)methoxy)-2-phenyl piperidine) and their respective enantiomers CP-100,263 and L-733,061 (which both have a low affinity for NK$_1$ receptors) were tested in an electrophysiological model of nociception. The effect of morphine alone, as a positive control, and its interaction with CP-99,994 were also examined.

In this series of experiments rabbits were used since they have been shown to have NK$_1$ receptors with human type pharmacology (Beresford *et al.*, 1991). Animals were surgically prepared as previously reported for rats, (Laird *et al.*, 1993) but were in addition decerebrated. Electrophysiological recordings were made from motor units of the semitendinosus muscle of the hind limb following electrical stimulation of the skin of the foot in an area corresponding to their receptive fields. In all treatment groups n=4.

L-733,060, CP-99,994 and morphine (i.v) produced dose-dependent reductions in the facilitation and wind-up of the nociceptive flexor reflex in decerebrate, spinalised rabbits. Whilst there was some reduction in baseline response this was not as great as wind-up or facilitation. Pre-treatment with morphine (i.v.) had no effect on the CP-99,994 dose response curve. L-733,061 and CP-100,263 (i.v.) did not affect any of the measured parameters (see Table 1).

The enantiomeric selectivity of L-733,060 and CP-99,994 against the facilitation and wind-up of the nociceptive reflex indicates that NK$_1$ receptor antagonists may have central analgesic effects with lesser disturbances of normal baseline protective reflexes compared to morphine which appeared to affect all parameters at similar doses.

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Table 1 : Effect of NK$_1$ receptor antagonists and morphine on baseline, facilitation and wind-up in the spinalised, decerebrate rabbit

		ID$_{50}$ (μg/kg)				ID$_{50}$ (μg/kg)	
Drug	Baseline	Facilitation	Wind-up	Drug	Baseline	Facilitation	Wind-up
CP-99,994	50	6	10	L-733,060	~1000	30	200
CP-100,263	>1000	>1000	>1000	L-733,061	>10000	>10000	>10000
Morphine	500	300	500	Morphine (300μg) + CP-99,994	100	8	5

243P [^{3}H]EPIBATIDINE: A NOVEL HIGH AFFINITY LIGAND AT NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

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The novel (chloropyridyl) azabicycloheptane derivative epibatidine, originally extracted from the skin of the poison arrow frog *Epipedobates tricolor*, has potent analgesic effects following systemic administration in rodents (Spande *et al*, 1992), an effect attributed to an activity at nicotinic acetylcholine receptors (nAChR, Rupniak *et al*, 1994). Here we report the pharmacological characterisation of [^{3}H]epibatidine binding in rat brain.
Brains from male Sprague-Dawley rats (250g) were homogenised in 0.32M sucrose (1:10w/v) and membranes spun at 3000 rpm for 10min. The resulting supernatant was spun at 13,000rpm for 20min to yield a crude P_2 pellet. Pellets resuspended in 20mM HEPES pH7.4. (300μg) were incubated for 1hr at 23°C before termination by rapid filtration over GF/C filters presoaked in 0.3% polyethyleneimine (PEI). Non specific binding was determined using 1mM carbachol. In parallel studies [^{3}H]nicotine binding to rat brain (0.05-2nM) was determined using the same assay procedure and non specific displacer (1mM carbachol).
[^{3}H]epibatidine (0.008-2nM,Amersham Int. 25Ci/mmol) binding to rat brain membranes was saturable, reaching equilibrium within 60min. Scatchard analysis analysis demonstrated high affinity binding with 27 fold higher affinity to that seen with [^{3}H]nicotine (NEN 80 Ci/mmol, Table 1) and a 2 fold higher binding capacity. Subsequent pharmacological displacement of [^{3}H] epibatidine binding (0.05nM) showed a profile similar to [^{3}H]nicotine (0.3nM) with high affinity displacement by known nAChR agonists and competitive antagonists such as dihydro-β-erthyriodine (Table 1), but not so by the non competitive ganglion blocker mecamylamine.

Table 1. Binding characteristics of [^{3}H] epibatidine in rat brain.

Compound	[^{3}H]epibatidine	[^{3}H]nicotine
K_D (nM)	0.027 (0.020;0.038)	0.73 (0.56;0.96)
B_{max} (fmol/mg)	91 ± 12	55 ± 6.1
	K_i (nM)	K_i (nM)
(-)nicotine	4.4(4.0;5.6)	0.87(0.50;1.5)
(+)nicotine	44(27;71)	4.8(2.0;11)
Carbachol	180(140;220)	33(20;55)
Dihydro-βerythroidine	14(12;16)	3.1(1.6;5.9)
Mecamylamine	>400(0.5%)	>710(17%)

K_D and K_i values are geometric means. Values in parentheses are high and low errors of this mean. B_{max} values are arithmetic means ± s.e.m. (n = 5, Hill slopes close to unity).

These studies suggest that epibatidine is a high affinity ligand for central nAChR believed to be predominantly α-4, β-2 nACh receptors. The higher number of binding sites observed with epibatidine compared to nicotine may represent a population of receptors containing the α-3 subunit which have previously been shown to be present in rat striatum and ganglia (Sullivan *et al*, 1994).

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244P ATTENUATION OF α_2 ADRENCEPTOR-MEDIATED NOCICEPTION IN DIABETIC RATS

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Abnormalities in central noradrenergic systems, which include a decrease in activity of tyrosine hydroxylase and turnover rate of noradrenaline, and an increase in the densities of alpha-1 and beta-2 adrenoceptors have been described in models of diabetes mellitus (Bitar & DeSouza, 1990). Noradrenergenic systems have a well established role in nociception and alpha-2 adrenoceptor agonists elevate nociceptive thresholds (Monasky et al., 1990). We have studied the effects of clonidine and yohimbine on nociceptive responding during streptozotocin-diabetes in rats.

Adult female Sprague Dawley rats (200-220g) were made diabetic by an injection of STZ (55mg/kg, i.v.). Control animals received vehicle only. Diabetic rats were randomly divided into two groups; one received insulin (s.c.) daily and the other saline. To permit i.t. administration of drugs rats were catheterized according to the procedure of Yaksh & Rudy, (1976). Nociceptive thresholds were determined by measuring withdrawal latencies in the tail-immersion test at 48°C. In all tests there were 10-12 rats per group. Statistical analysis was made by one-way analysis of variance followed by t-tests.

Nociceptive thresholds increased progressively with the duration of diabetes, reaching 22% above control values ($p<0.05$) at two weeks and 62% at 12 weeks ($p<0.05$). In contrast, tail-withdrawal latencies of insulin-treated diabetic rats were significantly lower than those of diabetic and corresponding controls, falling to 80% ($p<0.05$) and 51% ($p<0.05$) respectively. In control rats clonidine (s.c.), administered 30 min prior to testing, produced a dose-dependent increase in latencies reaching 85% above pre-drug levels at 1.0 mg/kg, the highest dose examined. The analgesic effect of clonidine was markedly reduced in diabetic animals; for example, at 400 μg/kg the drug was without effect whilst in controls latencies were increased by 60% ($p<0.05$). A similar attenuation of clonidine's analgesic effect was obtained following its i.t. administration (10μg in 10μl). In contrast, the insulin-treated diabetic group showed a greater analgesic response to clonidine, particularly at the lower doses, when 100 and 200 μg/kg raised thresholds by 48% (versus 10% in control) and 55% (versus 36% in control) respectively. Acute hyperglycaemia induced by administration of D-glucose (20 mmole, i.p.) did not affect the analgesic action of clonidine. Yohimbine alone produced a dose-dependent hyperalgesia in control rats, reducing nociceptive thresholds by 31% ($p<0.05$) at 5.0 mg/kg, the highest dose used. However, there was no significant effect of the antagonist in acute or chronic diabetic animals. Yohimbine at 1.25 mg/kg (i.p.), a dose that does not alter tail-withdrawal latencies, completely prevented the analgesic effect of clonidine (0.2 mg/kg), when administered 15 min prior to the agonist.

In conclusion, these findings indicate that clonidine's antinociceptive effect is markedly attenuated in chronically diabetic rats and suggest that this results from an alteration in central alpha-2-adrenoceptor function.

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245P NOCICEPTION AND INFLAMMATORY HYPERALGESIA IN B_2 BRADYKININ RECEPTOR KNOCKOUT MICE

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Interpretation of the relative contributions of bradykinin (BK) B_1 and B_2 receptors in pain and inflammation is complicated by the narrow active dose windows of peptide antagonists in animal assays, and by the possibility that carboxypeptidases may transform peptide antagonists from B_2 to B_1 receptor selectivity *in vivo* (Regoli et al, 1986). Transgenic mice with disruption of the gene encoding the B_2 receptor (*Bk2r*$^{-/-}$) were developed to examine the contribution of B_2 receptors in nociception and inflammation without using pharmacological antagonists, and explore the role of the B_1 receptor in the absence of the B_2 receptor.

Bk2r$^{-/-}$ mice were developed as described by Borkowski et al (1995). Both sexes (20-30g) were used in this study and were either hybrids of the C57 x J129 strains (BK experiment only) or inbred J129 strain. Control animals were matched as closely as possible for genetic background and age.

Chemonociception: In wild-type (WT) control mice, intraplantar (i.pl.) injection of the B_2 receptor agonist BK (10 nmol in 20 µl) elicited a short-lasting (<15 min) nociceptive response of flinching (mean number ± s.e.m.=64±12) and leg raising (duration=84±29s, n=7-8, P<0.05 ANOVA) during the 15 min observation period. This response was absent in *Bk2r*$^{-/-}$ mice (flinching=10±4; leg raising=6±4s, P>0.05 ANOVA, n=5). In WT mice, i.pl. injection of formalin (20 µl of 2.5 % solution) induced a biphasic nociceptive response of leg raising and licking (early phase 0-10 min: 145±23s, late phase 10-35 min:179±40s). This response was similar in *Bk2r*$^{-/-}$ mice (early phase:121±28s, late phase:209±60s, P>0.05 ANOVA, n=7). In *Bk2r*$^{-/-}$ mice, i.pl. co-injection of the B_1 receptor antagonist, des-Arg^9[Leu^8] BK (0.3 nmol) markedly attenuated the late, but not the early, phase response to formalin by 73% (P<0.05 ANOVA, n=9).

Thermal hyperalgesia: Nociceptive thresholds to a noxious thermal stimulus, determined using a paw flick test (Hargreaves et al, 1988), was not different in WT (14.3±0.8s, n=10) and *Bk2r*$^{-/-}$ mice (14.7±0.8s, n=12, P>0.05 ANOVA). Following i.pl. injection of carrageenan (6 mg in 20 µl, 3h previously) marked paw oedema and thermal hyperalgesia was observed (reduction in paw withdrawal latency [RPWL] in WT mice=5.6±0.7s, n=5, P<0.05 ANOVA followed by Newman-Keuls [N-K] test). In contrast, thermal hyperalgesia was not induced by carrageenan in *Bk2r*$^{-/-}$mice (RPWL=2.5±0.6s; n=5-6, P>0.05 N-K test). However, i.pl. injection of complete Freund's adjuvant (30 µl of 0.1% solution, 3 days previously) caused reddening and swelling of the paw and induced thermal hyperalgesia of similar magnitude in both WT (RPWL= 7.9±0.8s) and *Bk2r*$^{-/-}$ mice (RPWL=7.4±1.6s; n=5, P>0.05 N-K test).

Overall these findings are consistent with studies using peptide B_1 and B_2 receptor antagonists that show activation of B_2 receptors to be involved in the acute nociceptive responses to certain agents (Dray & Perkins, 1993). The ability of the peptide B_1 receptor antagonist des-Arg^9[Leu^8]BK to attenuate the late phase formalin response in the *Bk2r*$^{-/-}$ mouse demonstrates that its antinociceptive activity is not dependent on B_2 receptor mediated events.

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246P 4-METHYLCATECHOL INDUCES MECHANICAL HYPERALGESIA IN NAÏVE ADULT RATS BY A NERVE GROWTH FACTOR-DEPENDENT MECHANISM

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Elevation of peripheral levels of nerve growth factor (NGF) is known to produce thermal and mechanical hyperalgesia in adult rats (Lewin, et al., 1993).

In the present study, the algesic effects of the NGF-inducing compound, 4-methylcatechol (4MC) (Furukawa et al., 1990), were assessed in male Sprague-Dawley rats (230-250g, n=6/group). Changes in mechanical sensitivity were determined using an Ugo Basile Analgesymeter; a reduction in withdrawal threshold being used as a measure of hyperalgesia. To test for the presence of mechanical allodynia, calibrated nylon von Frey filaments were employed; a reduction in the threshold for eliciting a flexion withdrawal reflex being taken as a measure of allodynia.

An intraperitoneal injection of 25µg 4MC produced mechanical hyperalgesia within 6 hours, giving a reduction in pain threshold from 100.4 ± 3.3g to 73.3 ± 1.7g (P<0.001). This effect persisted for at least 24 hours (70.0 ± 3.8g; P<0.01). The same dose of 4MC produced a reduction in von Frey threshold from 89.2 ± 6.3g to 53.7 ± 3.8g after 4 hours (P<0.001), but there was no significant reduction after 24 hours. Data are presented as mean ± sem and the values compared using Student's t-test.

From dose response studies, a dose of 2.5µg was selected for intraplantar administration. Mechanical hyperalgesia was produced after 4 hours, with a reduction in pain threshold from 124.2 ± 5.6g to 66.25 ± 2.2g (P<0.001) that persisted for at least 48 hours.

The mechanical hyperalgesia was prevented for at least 4 hours by a 30 minute pretreatment with an intraplantar injection of 100µl monoclonal anti-NGF (Predose: 128.8 ± 6.4g; 2h :128.3 ± 8.6g; 4h: 128.5 ± 10.3g). This same dose of antibody also blocked the mechanical hyperalgesia evoked by an intraplantar application of 200ng NGF for up to 4 hours (Predose: 132.5 ± 4.1g; 2h: 125.8 ± 5.4g; 4h: 126.3 ± 3.5g).

These findings demonstrate the systemic or local administration of 4-methylcatechol induced mechanical hyperalgesia and allodynia. This hyperalgesia was dependent on NGF as it could be blocked by pretreatment with an antibody to NGF. This data provides further support for the rôle of NGF in nociceptive processes.

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247P 4-METHYLCATECHOL, AN INDUCER OF ENDOGENOUS NERVE GROWTH FACTOR (NGF) SYNTHESIS, HAS ANALGESIC ACTIONS IN A MODEL OF NEUROPATHIC PAIN

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A constant source of NGF is required for the development and maintenance of sympathetic and sensory neurones. There is good evidence to suggest that NGF and other neurotrophic factors might be efficacious in the treatment of peripheral neuropathies.

The effects of 4-methylcatechol (4MC), a potent inducer of NGF synthesis *in vitro* and *in vivo* (Kaechi *et al*, 1993), were examined in a model of neuropathic pain.

Neuropathic pain was produced, under enflurane anaesthesia, by partial ligation of the left sciatic nerve (Seltzer *et al*, 1990) of male Sprague-Dawley rats (120-140g; n = 6/group). In this model the rats develop mechanical allodynia and a hyperalgesia to mechanical and thermal stimuli in the paw on the ligated side. Mechanical sensitivity was assessed with an Ugo Basile Analgesymeter; a reduced withdrawal threshold being used as a measure of hyperalgesia. Data are presented as mean ± sem and the values compared using Student's t-test.

From dose-response studies with 4MC, a single intra-peritoneal dose of 25µg was selected. Animals injected with 4MC two weeks after surgery demonstrated a significant increase in the pain threshold of the ligated paw to mechanical stimulation (38.3 ± 2.1g in control rats, 53.3 ± 2.5g in 4MC treated rats; $p < 0.001$).

Likewise, an intra-plantar injection of 200ng NGF increased mechanical pain thresholds (36.7 ± 2.8g in control rats, 55.8 ± 4.4g in NGF-treated rats; $p < 0.01$). The effects of both NGF and 4MC follow similar time courses; both demonstrating peak analgesic effects at 4-6 hours post-dose.

The increase in pain threshold in 4MC-treated animals (53.3 ± 2.5g threshold) was blocked by a 30 minute pre-treatment with an intra-plantar injection (100µl) of monoclonal anti-NGF (34.2 ± 2.0g threshold; $p < 0.001$). This dose of anti-NGF had previously been shown to attenuate the analgesia induced by 200ng intra-plantar NGF (threshold 55.8 ± 4.4g compared to 43.3 ± 1.1g in anti-NGF pre-treated animals; $p < 0.05$).

In the paw on the non-ligated side there was no change in the pain threshold in 4MC-treated animals compared to control animals (95.8 ± 0.8g and 93.3 ± 1.7g respectively).

These results suggest that 4MC stimulates the de novo synthesis of NGF which can cause a reversal of the mechanical hyperalgesia associated with neuropathic pain and provide further evidence of a role for neurotrophins in the pathogenesis of neuropathic pain.

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248P A MODEL FOR THE *IN VIVO* EVALUATION OF TACHYKININ NK_1 RECEPTOR ANTAGONISTS USING CARRAGEENAN-INDUCED HYPERALGESIA IN THE GUINEA-PIG PAW

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From molecular biology studies and the pharmacological profile of selective NK_1 receptor antagonists, it is known that species homologues of the NK_1 receptor exist (Hall *et al*, 1993). The NK_1 receptor in human, guinea-pig, gerbil, hamster and rabbit appears to be distinct from that in rat and mouse (Beresford *et al*, 1991). Thus models in guinea-pig and gerbil may be preferable to rat or mouse as predictors of activity in man..

The use of a model of carrageenan-induced inflammatory hyperalgesia in the guinea-pig as an assay for tachykinin NK_1 receptor antagonists is described. Carrageenan (1%, Viscarin) was injected intra-plantar (100µl) into one hind paw of male or female Dunkin-Hartley guinea-pigs (220-250g; n = 6/group). Mechanical hyperalgesia was assessed with an Ugo Basile Analgesymeter; withdrawal threshold being determined as the first sign of pain response (struggling or paw withdrawal). Thermal hyperalgesia was measured using an Ugo Basile Plantar Test (Hargreaves Method) apparatus and the latency to paw withdrawal measured. Withdrawal thresholds were measured in both the inflamed and non-inflamed paws. Drug effects were expressed as the mean ± sem percentage reversal of hyperalgesia compared by Student's t-test.

Carrageenan produced a marked mechanical and thermal hyperalgesia which peaked at 4 hours and persisted for over 24 hours. All drug effects were studied 24 hours after carrageenan injection. Both aspirin and morphine dose-dependently reduced the hyperalgesia. Aspirin typically inhibits the hyperalgesia by 40-45% at 300mg/kg p.o. Morphine at 10mg/kg s.c. produces 70-80% reversal of both mechanical and thermal hyperalgesia, with ED_{50} values of 1.85mg/kg and 2.51mg/kg respectively. All 4 NK_1 antagonists when given orally produced a dose-related but incomplete reversal of mechanical hyperalgesia. (see table 1). Thermal hyperalgesia was similarly reduced by CP 99,994 (30mg/kg) and FK 888 (10mg/kg) p.o..

Table 1 Maximum reversal of mechanical hyperalgesia as measured 1h (SR 140333) or 3h (CP 99,994, FK 888 and RPR 100893) after drug administration.

Compound	Dose	% Reversal	Significance
CP 99,994	30mg/kg	34.6 ± 3.4	$p < 0.001$
FK 888	10mg/kg	40.0 ± 9.5	$p < 0.01$
RPR 100893	100mg/kg	30.9 ± 3.5	$p < 0.001$
SR 140333	100mg/kg	26.5 ± 5.7	$p < 0.05$

This model therefore provides a useful functional model for the assessment of the analgesic activity of NK_1 antagonists which show selectivity for the guinea pig and human receptor (Beresford *et al*, 1991).

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249P THE EFFECT OF HYPERTHYROIDISM ON β-ADRENOCEPTORS IN RAT ISOLATED THORACIC AORTAE

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Hyperthyroidism is known to be associated with several cardiovascular alterations. Significant changes in the pharmacodynamic behaviour of the cardiovascular system may be expected, although this matter has not been studied in detail.We have investigated the response to various compounds in isolated aortae obtained from chronic hyperthyroid Wistar rats.

Hyperthyroidism was induced by feeding with a chow containing 5 mg L-thyroxine (T4) per kg, for 4 weeks.The rats were anaesthetized with pentobarbital (50 mg/kg ip) and subjected to artificial respiration via a tracheal cannula. Heparin (500 IU) was administered via a cannulated left carotid artery. The carotid blood pressure was measured by means of a Statham P23 pressure transducer and recorded on a Maclab/8 system. Heart rate was obtained via the recordings of the carotid blood pressure. The thoracic aortae were excised and suspended in an organ bath with a physiological salt solution, of the following composition (mM): NaCl (136); KCl (2.5); $MgCl_2$ (0.5); $CaCl_2$ (1.8); NaH_2PO_4 (0.42); $NaHCO_3$ (11.9); and glucose (5), at 37°C, and gassed with 95% O_2 and 5% CO_2. Isometric force was measured and recorded. After 1 h of equilibration at a resting tension of 10 mN the preparation was subjected to a depolarising potassium salt solution (dpss) (containing 40 mM K^+), phenylephrine (Phe 10^{-6}M), and again to dpss, with intervals of 20 min. Cumulative concentration response curves (CRC) were constructed for: α_1-adrenoreceptor agonists (phenylephrine, methoxamine, cirazoline), ß-adrenoreceptor agonists (isoproterenol, salbutamol, terbutaline), forskolin, and dibutyryl cAMP. CRC for vascular relaxation were constructed after precontraction with Phe 10^{-6}M. Schild analysis was performed with the ß-adrenoceptor antagonists ICI 118,551 ($ß_2$) and CGP 20712A ($ß_1$), using Phe for precontraction and isoproterenol as the ß-agonist. Controls were aortae from euthyroid animals. The plasma thyroxine values were 57 ± 2 for controls and 229 ± 4 for the hyperthyroid animals (n=65). The results are enumerated in table 1. With α_1-adrenoreceptor agonists there were hardly any differences; using ß-adrenoreceptor agonists there was an enhanced vasodilatation wich prompted further Schild analyses. In the presence of the $ß_2$-adrenoceptor antagonist ICI 118,551 the Schild plot became biphasic when constructed for aortae from hyperthyroid rats, with a pK_b of 8.47 for the high affinity component, and pK_b of 7.02 for the low affinity part. $ß_1$-Adrenoceptor antagonism with CGP 20712A did not influence the Iso CRC of control preparations, whereas in those of T4-treated animals, a concentration-dependent rightward shift occurred. The Schild analysis revealed a pK_b of 8.83.

In conclusion, hyperthyroidism appears to be associated with changes at the receptor level, including the appearance of functional $ß_1$-like adrenoceptors. The second messenger system, however, was not affected by hyperthyroidism.

Table 1.	control	T4-treated	control	T4-treated
	-log EC_{50}		E_{max} (mN)	
phenylephrine	6.7 ± 0.1	6.8 ± 0.1	8.1 ± 0.9	6.9 ± 0.8
methoxamine	4.8 ± 0.2	5.2 ± 0.0*	8.8 ± 0.9	5.3 ± 0.5*
cirazoline	6.9 ± 0.1	7.0 ± 0.1	2.7 ± 0.4	2.1 ± 0.4
	-log EC_{50}		E_{max} (%)	
isoproterenol	5.9 ± 0.1	6.4 ± 0.1*	81 ± 5	100 ± 0*
salbutamol	5.9 ± 0.1	6.2 ± 0.2	89 ± 4	98 ± 2
terbutaline	5.2 ± 0.1	6.0 ± 0.1*	87 ± 2	100 ± 0
forskolin	7.2 ± 0.0	7.3 ± 0.1	100 ± 0	100 ± 0
dibutyryl cAMP	4.3 ± 0.0	4.4 ± 0.0	100 ± 0	100 ± 0

* Indicates a significant ($p < 0.05$) difference from control.

250P FURTHER INVESTIGATION INTO AMPLIFYING INTERACTIONS BETWEEN 5-HT_{1D} AND ANGIOTENSIN AT_1 RECEPTORS IN VASCULAR SMOOTH MUSCLE

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Previous studies have shown that 5-HT and various vasospastic agents can act synergistically to produce vascular contractions (MacLennan & Martin, 1992), but the mechanism of this phenomenon still remains unclear. We have investigated the synergistic effect of the vasospastic agent angiotensin II (AII) on 5-HT_{1D} receptor-mediated contractions of rabbit femoral artery rings.

Contractions to 5-HT were measured as isometric force changes in rabbit femoral artery rings (RbFA) prepared as detailed elsewhere (MacLennan & Martin, 1992). The Krebs buffer contained 0.3μM spiperone, mepyramine and prazosin to block actions at 5-HT_2, histamine-H_1 and α_1-adrenergic receptors respectively. Tissues were exposed to pargyline (500μM, 30min) to inactivate MAO, and viability assessed by depolarisation with 80mM KCl. After washout tissues were 'primed' by addition of AII to produce a steady-state contraction ~45% of the KCl maximum, after which concentration-effect curves to 5-HT were constructed. Results are expressed as mean ± s.e. mean, n ≥ 4, of data expressed as % 80mM KCl.

AII produced powerful concentration-dependent contractions of RbFA ($p[A_{50}]$ 9.86 ± 0.06; max 169 ± 3.6%) that were blocked by losartan in a simple competitive manner (pK_B = 8.90 ± 0.04, 19d.f.), consistent with activation of AT_1 receptors. On the other hand, responses to 5-HT (mediated by 5-HT_{1D} receptors) were small in amplitude, achieving only 13.3 ± 1.2% of the KCl maximum ($p[A_{50}]$ 7.86 ± 0.03). However, in the presence of AII (0.03- 0.6nM: ~45% KCl max), 5-HT contractions were potentiated ($p[A_{50}]$ 8.45 ± 0.05) and substantially augmented (max 84.8 ± 2.7%), exhibiting super-addition of effect over the range of 5-HT concentrations used.

Synergy between 5-HT_{1D} and AT_1 receptors did not result from mechanical (e.g. length-tension relationship) changes in the tissue, since augmented 5-HT effects were unaffected by addition of the phosphodiesterase inhibitor IBMX to restore resting tissue tension (max -IBMX 90.4 ± 5.5% c.f. max +IBMX 105.0 ± 6.6%). Similarly, synergy was unaffected if the AII contracture was allowed to 'fade' over 180min (control max 83.2 ± 3.2% c.f. post-fade max 79.0 ± 9.5%). However, synergy was not observed when tissues were exposed to losartan (0.1μM) 60min prior to addition of AII (5-HT $p[A_{50}]$ 7.76 ± 0.10, max 14.0 ± 2.8%). Indeed, synergy was prevented even when tissues were first exposed to AII and the steady-state contracture reversed by addition of excess losartan (0.1μM) (5-HT $p[A_{50}]$ 7.86 ± 0.07; max 7.6 ± 1.5%).

These data show that, in RbFA, 5-HT_{1D} receptor-mediated contractions exhibit super-addition with low concentrations of AII. This effect is independent of the contractile status of the tissue, but appears to be an AT_1 receptor specific phenomenon. Furthermore, it is dependent on the continued activation of receptors, even when the contractile response has 'faded'. This phenomenon of receptor cross-talk, to produce super-addition of effect, is not only seen in isolated tissue systems (MacLennan & Martin, 1992) but also in cell-based systems where synergy between adenosine A_1 and P_2-purinoceptors (Megson *et al.*, 1995) has been observed. These data emphasise the potential importance of the amplifying interactions between receptors in the pathophysiology of hyperreactive conditions.

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251P VASCULAR REACTIVITY OF RENAL ARTERIES FROM SPONTANEOUSLY HYPERTENSIVE RATS WITH STREPTOZOTOCIN-INDUCED DIABETES MELLITUS

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Diabetes mellitus and hypertension frequently coincide. Since both diseases are known to cause renal damage the aim of the present study was to investigate whether hypertension and diabetes mellitus show additive effects on the renal vascular reactivity with respect to contractile and relaxant stimuli.

We used the model of the spontaneously hypertensive rats (SHR) with streptozotocin (STZ)-induced diabetes mellitus. At an age of 21 weeks and 8 weeks after the injection of streptozotocin (60 mg/kg) or vehicle, the SHR and the normotensive Wistar Kyoto control rats were sacrificed. On the basis of the two parameters, blood pressure and blood glucose level, four separate subpopulations were obtained: normotensive, non-diabetic (CW) and diabetic (DW) Wistar Kyoto rats, and spontaneously hypertensive non-diabetic (CS) and diabetic (DS) rats, respectively. In a constant flow setup the isolated left kidneys of these animals were perfused with 6 ml of oxygenated Tyrode's solution per minute at 37°C. All drugs were applied via the perfusate in order to obtain equilibrium effects.

The kidney/body weight ratios of the four groups as well as the initial perfusion pressures are enumerated in Table 1. An increased renal mass was observed in both groups of diabetic animals (DW, DS). However, the passive renal vascular resistance, as reflected by the differences in the initial perfusion pressure, seems not to be related to the renal mass. The hypertensive state increased, whereas the diabetic state decreased the passive and the agonist-stimulated, active renal vascular resistance. In all four groups neither methacholine, which is known to stimulate the release of vasorelaxant endothelial factors nor sodium nitroprusside, an endothelium-independent NO-donor, could completely relax the phenyleprine (10 μM)-induced increase of vascular tone. No diabetes- or hypertension-related differences in the potency or efficacy of these to vasodilators could be detected.

We conclude that hypertension per se leads to an increased vascular reactivity, whereas diabetes mellitus tend to decrease the passive and agonist-stimulated active vascular resistance. In the model used hypertension and diabetes mellitus do not impair the endothelium-dependent or -independent vasorelaxation.

Table 1. Kidney to body weight ratio (KW×100/BW), initial perfusion pressure (PP_i), and concentration-response parameters (Emax, in mmHg increase or % relaxation, above; pD2, of molar concentration, below; n=5-7) of the four groups of rats. Data presented as mean ± s.e.mean. †:$p<0.05$ *vs.* normoglycemic group; *:$p<0.05$ *vs.* normotensive group.

	KW×100/BW	PP_i (mmHg)	Phenylephrine	5-HT	Angiotensin II	Methacholine	Nitroprusside
CW	0.36 ± 0.002	37.5 ± 2.2	169.9 ± 6.0	201.3 ± 13.8	141.7 ± 19.4	44.4 ± 5.3	49.1 ± 3.7
	(n=35)	(n=39)	6.48 ± 0.04	7.04 ± 0.01	9.00 ± 0.05	6.32 ± 0.06	4.68 ± 0.13
DW	0.63† ± 0.007	28.7† ± 1.5	158.4 ± 7.8	175.1† ± 6.6	128.1 ± 17.8	60.8 ± 6.91	48.2 ± 3.2
	(n=37)	(n=38)	6.61 ± 0.01	6.94 ± 0.01	9.25 ± 0.14	5.95 ± 0.19	4.64 ± 0.36
CS	0.38* ± 0.002	43.3* ± 1.8	245.9* ± 12.5	227.4 *± 14.5	211.8* ± 11.0	47.2 ± 7.3	52.6 ± 4.2
	(n=38)	(n=38)	6.26 ± 0.02	7.06 ± 0.01	9.50 ± 0.14	6.79 ± 0.03	4.90 ± 0.16
DS	0.67†* ± 0.005	39.5* ± 1.7	184.6† ± 8.3	192.5† ± 8.7	151.5† ± 10.1	54.8 ± 6.0	49.6 ± 7.5
	(n=42)	(n=43)	6.57 ± 0.01	7.10 ± 0.01	9.35 ± 0.10	6.56 ± 0.20	4.43 ± 0.37

252P BLUNTED INHIBITORY EFFECTS OF CHOLINERGIC AGENTS IN ISOLATED HEARTS FROM HYPERTENSIVE-DIABETIC RATS

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In the present study we investigated the cardiac responsiveness to cholinergic agents in isolated perfused hearts taken from spontaneously hypertensive rats (SHR) with simultaneous diabetes.

SHR and normotensive Wistar Kyoto rats (WKY) of 12 weeks of age were made diabetic by an injection of streptozotocin (55 mg/kg i.v). 8 Weeks later the inhibitory effects of carbachol and oxotremorine were assessed in paced (6Hz) Langendorff hearts (37°C), expressed as left ventricular pressure (LVP in mmHg, measured via a balloon inserted into the left ventricle) and coronary flow (cf in ml/minute/gram wet heart weight). The balloon filling pressure, corresponding to the diastolic pressure was kept at 10 mmHg.

The basal left ventricular pressure in hearts from control WKY (CW) (LVP in mmHg, mean values ± SEM, $n \geq 15$, CW: 75 ± 4) and control SHR (CS: 68 ± 2) were significantly ($p<0.05$) higher compared to the LVP in hearts from both diabetic WKY (DW: 49 ± 2) and diabetic SHR (DS: 41 ± 2). LVP of the DS was lower ($p<0.05$) compared to the LVP in hearts obtained from DW. As reflected by concentration response curves (CRC), hearts from both groups of hypertensive rats developed an impaired ($p<0.05$) negative inotropic response to oxotremorine (10^{-8} - 10^{-5} M) compared to hearts from both CW and DW (maximally developed decrease in LVP (%), $n \geq 6$, CW: 47 ± 4, DW: 37 ± 4, CS: 18 ± 5, DS: 22 ± 6). Similarly, the decrease in the cf (maximal decrease in cf (%), $n \geq 6$) was less ($p<0.05$) in both CS- (24 ± 7) and DS hearts (27 ± 3) compared to the reduction in cf in CW- (46 ± 3) and DW organs (40 ± 2). A complicating factor, however, was that the CS hearts developed ventricular fibrillation at a concentration of 10^{-7} - 10^{-6} M oxotremorine. Consequently, interpretation of the differences occurring in the upper part of the CRC were difficult. The presence of diabetes in the SHR prevented the occurrence of this arrhythmia. When CRC's were made for carbachol (10^{-8} - 10^{-5} M), the decrease in LVP was blunted ($p<0.05$) in hearts from DW (maximal decrease in LVP (%), $n \geq 9$, DW: 49 ± 4) compared to those from CW (76 ± 3). The maximal decrease of LVP was less ($p<0.05$) in hearts from CS (57 ± 5) compared to CW, whereas the negative inotropic response in hearts from DS (46 ± 4) did not differ from that in DW-organs. Impairment in coronary flow (maximal decrease in cf (%), $n \geq 9$) in hearts from both DW (49 ± 4) and DS (49 ± 4) was decreased ($p<0.05$) compared to the reduction of cf in CW (66 ± 2) and CS (62 ± 4), respectively. The occurrence of ventricular fibrillation, observed for oxotremorine in hearts from control SHR was not observed after administration of carbachol.

These results indicate that the cholinergic receptor mediated cardiac responsiveness (decrease in left ventricular pressure and coronary flow) is significantly depressed in hearts obtained from diabetic and hypertensive rats.

253P THE INFLUENCE OF HYPERTHYROIDISM ON THE NUMBER OF β-ADRENOCEPTORS IN LEFT VENTRICLE AND KIDNEY IS TRANSIENT AND TIME-DEPENDENT

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Several investigators have shown by means of radioligand binding studies that thyroid hormones can modulate the number of cardiac ß-adrenoceptors (Williams and Lefkowitz, 1977). We investigated a possible time dependency of the influence of hyperthyroidism on ß-adrenoceptor density in rat left heart ventricle and renal cortex.

Hyperthyroidism was induced in male Wistar rats by feeding the animals with 5 mg/kg L-thyroxine(T4)-containing chow during 1, 4 and 8 weeks, respectively. In another group of rats hyperthyroidism was assessed by subcutaneous injection of 750 µg/kg T4 for 7 days. Cardiac left ventricle and renal cortex tissue were used for the assessment of ß-adrenoceptor density by using (-)-[^{125}I]-iodocyanopindolol (ICYP). The ratio of $ß_1$:$ß_2$-adrenoceptors was determined by the inhibition of ICYP binding using the selective $ß_2$-adrenoceptor antagonist ICI 118,551.

T4 treatment caused significant increases in serum T4 levels (nmol/l): 294±16 (1 week), 266±13 (4 weeks) and 170±6.4 (8 weeks) compared to 56±2 of control animals (n=8; P<0.05). In T4-injected animals (1 week) a T4-level of 136±7 nmol/l (n=9; P<0.05) was measured. A pronounced effect on heart rate was observed, which was increased by approximately 100 beats/min in all T4-treated animals (451±15, 1 week T4-fed; 458±20, 1 week T4-injected; 444±8, 4 weeks T4-fed; 458±13, 8 weeks T4 fed; beats/min, n=7-9) versus control animals (344±9; beats/min, n=9; P<0.05). T4-treatment of the animals for 1 week enhanced the number of ß-adrenoceptors in the left ventricle both in T4-injected (69.7±3.6 fmol/mg, n=8) and in T4-fed animals (69.1±2.9 fmol/mg, n=7) compared to control animals (49.5±6.7 fmol/mg, n=6; P<0.05). After 4 (51.1±3.4 fmol/mg, n=8) and 8 weeks (49.8±2.9 fmol/mg, n=7) of hyperthyroidism the number of ventricular ß-adrenoceptors returned to control levels (49.5±6.7 fmol/mg, n=6). The dissociation constant (K_D) was slightly enhanced after 8 weeks of hyperthyroidism (61±8 versus 34±8 pmol/l, n=6-8; P<0.05). The other treatment-regimens did not affect K_D-values. Hyperthyroidism did not influence the ratio $ß_1$:$ß_2$-adrenoceptors in left ventricular tissue. Both 1 week of T4-treatment via injections (66.2±8.5 : 33.8±8.5; $\%ß_1$:$\%ß_2$, n=6) and 4 weeks of T4-treatment via rat-chow (77.3±3.2 : 22.7±3.2; $\%ß_1$:$\%ß_2$, n=6) resulted in a similar ratio of $ß_1$:$ß_2$-adrenoceptors in the left ventricle with respect to control values (73.8±1.3 : 26.2±1.3; $\%ß_1$:$\%ß_2$, n=6). In the renal cortex hyperthyroidism both for 1 (T4-fed: 86.7±5.9 fmol/mg, n=8; T4-injected: 77.9±6.0 fmol/mg, n=8) and 4 weeks (69.8±3.4 fmol/mg, n=8) enhanced the number of ß-adrenoceptors compared to control animals (48.9±3.8 fmol/mg, n=4; P<0.05). This rise disappeared after 8 weeks of T4-treatment (38.8±7.1 fmol/mg, n=7). K_D-values of ß-adrenoceptors in renal cortex were not affected by any hyperthyroid state of the animals. The $ß_1$:$ß_2$-adrenoceptor ratio in renal cortex had increased after 1 week of T4-injections (79.5±4.5 : 20.5±4.5; $\%ß_1$:$\%ß_2$, n=7) compared to control animals (65.3±4.3 : 34.7±4.3; $\%ß_1$:$\%ß_2$, n=6; P<0.05). T4-treatment during 4 weeks did not affect the $ß_1$:$ß_2$-adrenoceptor ratio.

In conclusion, the effect of hyperthyroidism on the characteristics of ß-adrenoceptor binding is transient and time-dependent, showing a maximal effect after 1 week followed by a subsequent decrease towards normal values. The time course of the rise in ß-adrenoceptor density also depends on the investigated tissue.

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254P INFLUENCE OF THE HYPERTENSIVE STATE ON THE PHARMACOLOGICAL AND MORPHOLOGICAL CHARACTERISTICS OF RAT ISOLATED CORONARY ARTERIES AND AORTAE

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Important morphological and functional changes in the vascular bed are known to occur in experimental and human hypertension. However, the severity of the defects and the mechanisms involved among different vascular beds and the various models of hypertension are clearly heterogeneous.

Accordingly, we evaluated the contractile response of phenylephrine and the endothelium-dependent relaxation induced by methacholine in isolated thoracic aortic rings obtained from 30 and 52 weeks' old SHR and WKY rats, respectively. In coronary arteries taken from 22 weeks old WKY and SHR we investigated the response to serotonin (5-HT) and methacholine. In addition, we quantitatively studied the morphology of the coronary arteries and thoracic aortae taken from 4, 30 and 52 weeks' old SHR and WKY, respectively.

In the coronary arteries the maximal effect of 5-HT was significantly (P<0.05) enhanced in the SHR vessels (2.8 ± 0.4 mN/mm) compared to the maximal effect in the WKY preparations (0.9 ± 0.2 mN/mm), and the concentration-response curves in the SHR-arteries were significantly (P<0.05) shifted leftward (pD_2= 6.19 ± 0.03 and 6.77 ± 0.06 for WKY and SHR, respectively). The methacholine-induced relaxation was not influenced by hypertension of the donor animals. The pD_2- and maximal effect values of the concentration-response curves of phenylephrine were the same in the aortic rings taken from age-matched WKY (6.35 ± 0.06, 4.3 ± 0.4 mN) and SHR (6.52 ± 0.07, 3.8 ± 0.4 mN). The methacholine-induced responses (pD_2 and maximal relaxation) were only significantly (P<0.05) impaired in 52 weeks' old SHR (6.97 ± 0.10, 58.3 ± 6.4%) when compared with the age-matched WKY (7.29 ± 0.08, 87.3 ± 3.1%).

In coronary arteries taken from the SHR the media/lumen ratio values were significantly (P<0.05) increased compared to those obtained from the age-matched WKY. The media/lumen ratio for WKY and SHR are 0.66 ± 0.06 and 0.80 ± 0.10 (4 weeks old), 0.65 ± 0.07 and 1.24 ± 0.09 (30 weeks old) and 0.79 ± 0.12 and 1.51 ± 0.18 (52 weeks old), respectively. Both the media- and lumen- area of the aortae taken from the SHR were increased when compared with those from the age-matched WKY rats. Therefore, no difference in media/lumen ratio between the aortae from the WKY and SHR (0.39 ± 0.02 and 0.37 ± 0.01, respectively for 52 weeks old rats) were observed.

In conclusion, the sequelae of elevated blood pressure may strongly influence both the drug-induced functional properties and the morphological characteristics of the vascular system. In addition, the responsiveness and morphological structure of different vascular beds during hypertension are clearly heterogeneous.

255P IMPLICATION OF ENDOTHELIAL AND NONENDOTHELIAL CYCLOOXYGENASE PRODUCTS IN RESPONSES TO ANGIOTENSIN II OF RESISTANCE ARTERIES FROM SPONTANEOUSLY HYPERTENSIVE RATS

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The mechanisms underlying contractile responses of resistance arteries to angiotensin II (Ang II) were compared in small mesenteric arteries from spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats.

Branch II or III of superior mesenteric arteries were dissected from 12-14 week old male SHR and WKY rats. Arterial segments (1.6-2.0 mm) were removed and mounted on a myograph filled with physiological salt solution (PSS; composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 0.4, $NaHCO_3$ 14.9, $MgSO_4$ 1.17, $CaCl_2$ 2.5, glucose 5.5) continuously kept at 37 °C and gassed with a mixture of 95 % O_2, 5 % CO_2 (pH 7.4). In some experiments, the endothelium was removed immediately after dissection by infusing PSS containing 0.5 % 3-[(3 Cholamidopropryl) dimethylammonio]-1-propanesulfonate (CHAPS). Ang II concentration-effect curves were constructed by successive non cumulative additions of increasing concentrations of the peptide in 25 mM KCl-PSS, each separated by 1 h washout period. The 25 mM KCl-PSS was used to prevent the effect of the endothelial hyperpolarizing factor. When used, inhibitors were incubated 30 min prior the addition of Ang II. Unpaired Student *t* test was used for statistical analysis.

The results are presented in Table 1. In endothelium intact arteries, the contractile responses to Ang II were not significantly different up to 10 nM in the two strains. However, the contraction induced by 100 nM Ang II was higher in SHR than in WKY vessels. Endothelium removal differentially enhanced responses to Ang II in arteries from the two strains. In WKY, it only increased responses to low concentrations (1 and 3 nM) of Ang II whereas in SHR, it enhanced responses to Ang II at any concentration studied. This unmasked greater responses to Ang II in endothelium-denuded SHR compared to WKY arteries. In endothelium intact vessels, inhibition of nitric oxide production using the specific nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 µM), increased the Ang II-induced responses in arteries from both SHR and WKY rats. However, no significant difference was observed between the two strains in the presence of L-NAME. The cyclooxygenase inhibitor, indomethacin (Indo, 10 µM) reduced the Ang II responses in endothelium intact and denuded vessels from both strains. However, in endothelium denuded vessels the inhibitory effect of Indo was more pronounced in SHR than in WKY arteries. In vessels without endothelium from both strains, responses to maximally active concentration of Ang II (100 nM) were affected neither by the inhibitor of phospholipase A_2, quinacrine (0.3 µM), nor by the specific thromboxane A_2 receptor antagonist, GR 32191B (3 µM). The responses to 100 nM Ang II were 1.7 ± 0.4 and 1.0 ± 0.2 mN/mm (n = 5) in the presence of quinacrine and 2.4 ± 0.5 and 0.9 ± 0.3 mN/mm (n = 5) in the presence of GR 32191B for SHR and WKY vessels, respectively.

These results show that the endothelium influenced the response to Ang II in the two strains via the opposing effects of nitric oxide and of a vasoconstrictor cyclooxygenase product. In addition, they indicate that Ang II-induced vasoconstriction is partially mediated by another nonendothelial cyclooxygenase product, which accounts for increased responsiveness to Ang II of endothelium-denuded resistance arteries. The latter contractile factor is not thromboxane A_2 and does not involved the phospholipase A_2 pathway.

Table 1. Contractile response (in mN/mm) of small mesenteric resistance arteries from SHR and WKY rats, in vessels with intact and denuded endothelium

	SHR [Ang II] (nM)				WKY Ang II] (nM)			
	1	3	10	100	1	3	10	100
Intact vessels								
Control	0.3	0.6	1.0	1.4*	0.3	0.4	1.0	1.0
	±0.04	±0.1	±0.1	±0.12	±0.04	±0.1	±0.1	±0.1
	(7)	(6)	(12)	(13)	(17)	(8)	(21)	(18)
L-NAME	0.9‡	1.2‡	1.7	1.9	0.4	1.0‡	1.7‡	1.3
	±0.35	±0.28	±0.44	±0.43	±0.07	±0.3	±0.13	±0.2
	(5)	(6)	(5)	(6)	(5)	(5)	(8)	(5)
Indo	0.2	0.3‡	0.3‡	0.3‡	0.2	0.2‡	0.4‡	0.4‡
	±0.07	±0.04	±0.1	±0.03	±0.06	±0.08	±0.1	±0.15
	(5)	(5)	(5)	(5)	(5)	(5)	(9)	(6)
Denuded vessels								
Control	0.7*†	1.2†	1.8 *†	2.2*†	0.4†	1.0†	1.1	1.2
	±0.1	±0.3	±0.15	±0.2	±0.07	±0.16	±0.15	±0.14
	(14)	(8)	(16)	(17)	(10)	(6)	(11)	(11)
Indo	0.4‡	0.6‡	1.0‡	1.6	0.4	0.9	0.8	0.7‡
	±0.1	±0.01	±0.2	±0.3	±0.07	±0.25	±0.14	±0.06
	(6)	(8)	(5)	(5)	(6)	(6)	(7)	(7)

The results are mean ± s.e.mean. of n experiments (between parenthesis). * $P < 0.05$ versus WKY; † $P < 0.05$ versus intact vessels; ‡ $P < 0.05$ versus control.

256P CHARACTERIZATION OF CALCIUM STORES AND INFLUX MECHANISMS INVOLVED IN THE CONTRACTION INDUCED BY NORADRENALINE IN RAT RESISTANCE ARTERIES

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The contractile response of blood vessels to noradrenaline (NA) is produced via Ca^{2+} release from intracellular stores and Ca^{2+} entry. This latter phenomenon is partially controlled by the Ca^{2+} content of intracellular stores and is called "capacitative entry" (Putney, 1990). However the mechanisms underlying both Ca^{2+} release and entry pathways are still under debate. Therefore, the aim of the present study was firstly to characterize the nature of the intracellular Ca^{2+} stores released by NA and secondly to investigate the mechanism of the Ca^{2+} entry after depletion of these Ca^{2+} stores within rat small mesenteric resistance arteries.

Branch II or III of superior mesenteric arteries were dissected from male Wistar rats (250-350 g). Arterial segments (1.5-2 mm) were removed and mounted in a myograph filled with physiological solution (PSS) of the following composition, in mM: NaCl 119, KCl 4.7, KH_2PO_4 0.4, $NaHCO_3$ 14.9, $MgSO_4$ 1.17, $CaCl_2$ 2.5, glucose 5.5. In Ca^{2+}-free PSS, calcium was omitted and 0.5 mM EGTA was added. PSS was continuously kept at 37°C and gassed with a mixture of 95% O_2, 5% CO_2 (pH 7.4). In some experiments, changes in contraction and intracellular Ca^{2+} ions ($[Ca^{2+}]_i$) were determined simultaneously using the calcium-indicator, fura-2. All the experiments were carried out on vessels without endothelium, which was removed by intraluminal perfusion with 0.5% 3-[(3 Cholamidopropryl) dimethylammonio]-1-propanesulfonate (CHAPS) for 30 s. All the inhibitors were used at maximally active concentrations. Contractile response was expressed as percentage of the maximal contractile response obtained with 10 µM NA in normal PSS. Results are expressed as mean ± s.e.mean of *n* experiments. Unpaired Student *t* was used for statistical analysis.

In Ca^{2+} free medium, NA (10 µM) produced a transient increase in both $[Ca^{2+}]_i$ and contraction (57 ± 2.9 %, n=12, $P<0.001$). Under the same condition, caffeine (10 mM) produced fast transient increases in $[Ca^{2+}]_i$ and tension (43 ± 2.1 %, n=6) and it abolished response to subsequent addition of NA (10 µM). Also, in Ca^{2+}-free medium, the inhibitors of calcium ATPase, thapsigargin (1 µM) and cyclopiazonic acid (20 µM), had no effect on either $[Ca^{2+}]_i$ or contraction when added to unstimulated vessels, but they reduced significantly the responses to 10 µM NA. The NA-induced contraction was decreased by 60 ± 4.5% (n=6, $P<0.001$) in the presence of thapsigargin and by 63 ± 3.2% (n=6, $P<0.001$) in the presence of cyclopiazonic acid. After depletion of intracellular calcium stores with NA (10 µM) in Ca^{2+}-free medium, addition of exogenous $CaCl_2$ (2.5 mM) produced a large contraction in the continuous presence of NA (99.6 ± 2.4 %, n=22). Under the same condition, the response to exogenous $CaCl_2$ was significantly decreased by Ca^{2+} entry blockers (nitrendipine (1 µM) (by 50 %, n=9, $P<0.001$), SK&F 96365 (30 µM) (by 80 %, n=5, $P<0.001$) and by tyrosine kinase inhibitors genistein (30 µM) (by 60 %, n=6, $P<0.001$) and tyrphostins (100 µM) (by 40 %, n=6, $P<0.01$). In addition, it was abolished by an inhibitor of sodium-calcium exchange, amiloride (1 mM; n=5). However, the calcium-induced contractile response was affected neither by 300 µM N^G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase nor by 30 µM Rp-8-bromoguanosine 3', 5'-cyclic monophosphorothioate, a selective inhibitor of cGMP protein kinase. These results suggest that NA releases two intracellular Ca^{2+} stores in rat small mesenteric resistance arteries. These stores are both caffeine-sensitive, but only one of them is sensitive to thapsigargin and cyclopiazonic acid. The Ca^{2+} influx produced by NA after depletion of intracellular stores, so called "capacitative entry", is mediated by both dihydropyridine-sensitive Ca^{2+} channels and Na^+-Ca^{2+} exchange. This phenomenon involves the activation of tyrosine kinase pathway. By contrast to data obtained in rat pancreatic acini cells (Xu et al., 1994), nitric oxide synthase-cGMP-cGMP kinase pathway seems not to be implicated in the regulation of Ca^{2+} influx in endothelium denuded rat resistance arteries.

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257P DIADENOSINE-PENTAPHOSPHATE CONTRACTS AND RELAXES RAT RESISTANCE ARTERIAL SMOOTH MUSCLE THROUGH P_{2x}-PURINOCEPTORS

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Diadenosine phosphates have been suggested to participate in the control of blood pressure (Schluter et al, 1994). We evaluated whether diadenosine-pentaphosphate (AP5A) directly influences the contractile reactivity of resistance arterial smooth muscle and whether P_{2x}-purinoceptors are involved herein.
From the mesentery of adult male Wistar rats (≈300 g; n=16), resistance artery segments were isolated, depleted of neuropeptides (1 μM capsaicin, 20 min), chemically sympathectomized (1.5 mM 6-OHDA, 10 min), mechanically denuded of endothelium (Osol et al, 1989) and mounted in myographs for recording of isometric tension development (Boonen et al, 1993). Effects of 10 μM AP5A were compared to those of 10 μM adenosine, ATp and α,β-methylene ATP (α,β-mATP) before and after blockade of P2x responses by pretreatment with α,β-mATP or presence of 10 μM pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). They were expressed as % of the maximal contractile response to high potassium, phenylephrine plus vasopressin and are shown as mean±SEM.
At basal tone, ATP, AP5A and α,β-mATP induced transient (t½ < 2 min) contractions (7±2, 34±5, 59±6%). Pre-exposure to α,β-mATP prevented the response to AP5A, while PPADS reduced the contractile action of AP5A (16±3%) and blocked that of α,β-mATP.
During contraction induced by 10 μM phenylephrine (87±3%) adenosine and ATP caused a small relaxation (-17 and -20%), while AP5A and α,β-mATP induced a profound relaxation (-86 and -97%) after a transient further increase in tension. This relaxing effect of AP5A was poorly reversible (t½ > 20 min) and was markedly reduced (-18 and -21%) by PPADS and by pretreatment with α,β-mATP.
During contraction induced by 35 mM potassium (71±5%), AP5A induced, after a transient increase in tone, a small relaxation (-23±4%), that was comparable to that noted with adenosine and ATP.
These findings indicate that AP5A can both contract and relax resistance arterial smooth muscle through a direct action that involves P_{2x}-purinoceptors. The relaxing effect seems to involve an hyperpolarizing mechanism that counteracts the contractile effect. That adenosine mimicked some of the effects of AP5A and that ATP was less potent than AP5A and α,β-mATP may result from degradation of the agonists in the tissue.

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258P POTENTIATING INTERACTIONS BETWEEN NORADRENALINE AND ANGIOTENSIN II IN RABBIT FEMORAL ARTERY ARE DEPENDENT ON ORDER OF AGONIST INCUBATION AND ASSAY CONDITIONS

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In rabbit femoral artery, preincubation with a fixed concentration of angiotensin II (AII) has been shown to potentiate the noradrenaline (NA) concentration-effect (E/[A]) curve (Purdy & Weber, 1988). Similarly, NA potentiated the AII E/[A] curve (Prins *et al.*, 1992). Having experienced difficulty in repeating these results, we compared our standard assay conditions with those of the above authors who, unlike ourselves, pre-exposed their assays to high K^+ concentrations. We now report the effect of the inclusion of such a sighter in our experimental design and of changing the order of agonist incubation.
Rings of femoral artery (3mm) were obtained from rabbits (New Zealand White, 2.5-3.0kg) killed by phenobarbitone sodium (≥80mg/kg i.v.). The endothelium was removed mechanically before mounting between wire hooks for isometric tension measurement. The organ-baths contained Krebs-Henseleit solution at 37°C, including 0.3μM desipramine and 30μM corticosterone, gassed with 95%O_2/5%CO_2.
Under our original assay conditions, where no sighter was used, we were unable to demonstrate a potentiating interaction, in the form of a leftward shift of the NA E/[A] curve in the presence of AII. However, a decrease in the slope of the curves indicated some form of interaction other than simple addition of effects. When the tissues were pre-exposed to a K^+ (80mM) sighter, which was thoroughly washed from the tissue, we found an AII concentration-dependent leftward shift of the NA E/[A] curves with a maximum log dose-ratio (d.r.) of -0.46±0.12 (10 d.f.). However, in contrast to Prins *et al.*, (1992) we found that AII E/[A] curves were not potentiated following preincubation with NA and, in fact, were shifted to the right (max. log d.r. +0.44±0.13, 11 d.f.).
In a randomised block experiment, we confirmed the potentiation of NA by AII by comparing the effect of 3nM AII on NA E/[A] curves in control tissues (log d.r. +0.20±0.19, 12 d.f.) or following a K^+ sighter (log d.r. -0.55±0.18, 9 d.f.). To determine whether this was a specific action of K^+ or a general consequence of the precontraction of the tissue, a similar experiment was performed using 10μM NA as sighter. In neither case did the sighter have a significant effect on the location of the NA control curve (log d.r.'s K^+: +0.34±0.21, 11 d.f. & NA: +0.20±0.11, 10 d.f.). In this second experiment, as expected, the response to NA was not potentiated by AII in the absence of the sighter (log d.r. -0.18±0.18, 10 d.f.). However, when the NA sighter was used, AII still did not potentiate NA (log d.r. +0.01±0.16, 10 d.f.).
In conclusion, potentiation was found to be dependent on the order of agonist incubation. Preincubation of AII potentiated the response to NA but NA did not potentiate AII responses. In addition, potentiation of NA by AII was not observed unless the tissues had been pre-exposed to high K^+ concentrations. A possible explanatory model is currently under investigation.

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259P α_{1A}-ADRENOCEPTOR SUBTYPE MEDIATES TONIC CONTRACTIONS TO PHENYLEPHRINE IN RAT HEPATIC PORTAL VEIN

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The rat hepatic portal vein contains only α_1- and not α_2-adrenoceptors (Digges & Summers, 1983). Most recently electrophysiological studies have suggested there is a homogenous population of α_{1A}-adrenoceptors (Lepretre *et al*, 1994). In the present study the α_1-adrenoceptor subtype has been further characterised pharmacologically using antagonists demonstrated to have subtype selectivity.

A 15-20mm length of hepatic portal vein from male Sprague-Dawley rats (350-550g) was set up longitudinally in a modified Krebs solution at 37°C and gassed with 95:5% O_2:CO_2.The Krebs solution contained 50mM K^+ to largely suppress spontaneous phasic activity. Antagonists were equilibrated for 30 min.

Cumulative phenylephrine (10^{-8}-$3x10^{-5}$M) dosing evoked tonic contractions (max. 0.22±0.01g, mean±s.e.m.) through α_1-adrenoceptors as they were antagonised by prazosin (pA_2 9.2, slope of Schild plot 0.84±0.16). The contractions were also competitively antagonised by WB4101 (pA_2 9.4, slope 0.89±0.16), 5-methyl urapidil (pA_2 8.6, slope 1.02±0.19), indoramin (pA_2 8.4, slope 0.87±0.15) and BMY7378 (pA_2 6.5, slope 1.02±0.23). Correlation of these values with those against noradrenaline contractions of the rat epididymal vas deferens and human prostate, α_{1A}-adrenoceptor-mediated responses (Burt *et al*, 1995; Marshall *et al*, 1995) gave correlation coefficients of 1.00 (slope 0.97) and 0.97 (slope 0.79) respectively. Correlation of the pA_2 values from the portal vein with published pK_i values on cloned α_1-adrenoceptor subtypes from a number of different studies (see Burt *et al.*, 1995; Marshall *et al.*, 1995) gave correlation coefficients of 0.97 (slope 0.94), 0.53 (slope 0.54) and -0.09 (slope -0.08) with the α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors respectively.

The affinity of the antagonists, including the α_{1A}-selective indoramin and the α_{1D}-selective BMY7378, were very similar to those from two tissues where the contraction is mediated through α_{1A}-adrenoceptors. In addition the highest correlation was obtained between the functional pA_2 values on the portal vein and published pK_i values from binding studies for the cloned α_{1a}-adrenoceptor subtype. Therefore, in agreement with Lepretre *et al.* (1994), α_{1A}-adrenoceptors appear to mediate tonic contractions of the rat portal vein.

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260P THE EFFECTS OF GTN, GSNO, SNAP, SIN-1 AND L-ARGININE ON CONTRACTILITY OF ISOLATED HUMAN PREGNANT MYOMETRIUM

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There is evidence for the L-arginine-nitric oxide (NO) pathway modulating myometrial contractility. In the rat, L-arginine and sodium nitroprusside inhibited contractions of the isolated myometrium (Yallampalli *et* al, 1993; 1994) and recently glyceryl trinitrate (GTN) was used in patients in premature labour (Lee *et al*, 1994). The aim of the present experiments was to study the effects of L-arginine and several NO-donor compounds on contractions of isolated myometrium from pregnant women.

Biopsies of human myometrium were obtained at caesarean section (with written informed consent and Joint UCL/UCH Ethical Committee approval)). Strips were set up (2g tension) in Krebs' solution at 37°C and gassed with 95:5% O_2:CO_2 (Morrison *et al*, 1993). After 2h equilibration, oxytocin (10^{-9}M) was added and at further 30 min intervals increasing concentrations of either GTN, GSNO (*S*-nitroso-L-glutathione), SNAP (*S*-nitroso-*N*-acetylpenicillamine) or SIN-1 (3-morpholinosydnonimine) were added (all from 10^{-8} to 10^{-3}M). In other strips oxytocin was not given but L-arginine (10^{-8} - 10^{-3}M) was added, concentration increasing at 30 min intervals. Tension was measured using Grass FT.03 transducers and recorded by Biopac Systems Inc.MP100WS. The average maximum tension and the average area-under-the-curve for each contraction was calculated for each 30 min period exposure to a given drug concentration.

The oxytocin-induced myometrial contractions were inhibited in a concentration-dependent manner and by 100% by all the NO-donor compounds. The pEC_{50} values for GTN, GSNO, SNAP and SIN-1 in reducing the maximum tension were 6.40±0.78 (mean±s.e.mean), 4.45±0.73, 4.80±0.38 and 5.72±0.70 respectively. These compounds appeared around 10-fold more potent in reducing the average area under the curve for each contraction (pEC_{50} values 7.41±0.82, 5.92±0.40 and 5.90±0.82 for GTN, GSNO and SNAP respectively) with the exception of SIN-1 which was no more potent (5.84±0.55). Intrinsic myometrial contractions were unaltered by the addition of L-arginine.

The results with L-arginine are at variance with those obtained using myometrium from another species, the rat (Yallampalli *et al*, 1993; 1994). The present observations in isolated tissues demonstrate relative potency differences between NO-donor compounds. This may be relevant to their potential clinical use in preterm labour.

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261P EVIDENCE FOR ATYPICAL β-ADRENOCEPTORS IN THE RAT VASCULATURE

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Atypical or β_3-adrenoceptors have been identified in adipocytes and a number of gastrointestinal smooth muscle preparations, e.g., guinea pig ileum (Bond & Clarke, 1987) and rabbit jejunum (Norman & Leathard, 1990). Also, the presence of atypical β-adrenoceptors has been observed in vasculature, i.e., rat mesenteric artery (Sooch & Marshall, 1995) and carotid artery (Oriowo, 1994). A common feature of atypical β-adrenoceptors is their resistance to blockade by most β-adrenoceptor antagonists. Furthermore, agonists selective for the β_3-adrenoceptor have been identified, e.g., ZD 7114, BRL 37344. The present study provides evidence for atypical β-adrenoceptors in the rat thoracic aorta and pulmonary artery.

Aortic and pulmonary arterial rings from male Sprague-Dawley rats (300-450g) were suspended under 0.5g tension in Krebs solution at 37^0C and oxygenated with 95%O_2/5%CO_2 for isometric tension recording. Rings were preconstricted with phenylephrine and cumulative concentration-relaxation curves to β-adrenoceptor agonists constructed. Antagonists were equilibrated for 30 min before a second dose-response curve was obtained. Results are expressed as mean±s.e.mean.

Isoprenaline (10nM-10μM) produced a concentration-dependent relaxation (pEC_{50} 7.4±0.1) of phenylephrine-induced tone (0.3μM) in rat thoracic aortic rings. Propranolol produced concentration-ratios of 8.1±1.8 and 7.3±0.2 at 0.1 and 1μM. In the rat pulmonary artery, isoprenaline (10nM-1μM) relaxed rings (pEC_{50} 8.0±0.1) with increasing concentrations of propranolol shifting control curves to the right (e.g., concentration-ratios of 20 and 96 at 0.01 and 0.1μM). However, the antagonism was non-competitive (Schild slope < 1). ZD 7114 (10nM-100μM; Holloway *et al.*, 1991) produced concentration-dependent relaxations of both thoracic aortic (pEC_{50} 5.4±0.1) and pulmonary arterial rings (pEC_{50} 6.2±0.1). Responses to ZD 7114 were not antagonized by propranolol (1μM) in either tissue. BRL 37344 (Arch *et al.*, 1984) relaxed both tissues producing approximately 72 and 75% relaxation in the rat thoracic aorta and pulmonary artery, respectively, at the highest concentration used (30μM). Relaxations to BRL 37344 were not antagonized by propranolol (1μM) in the thoracic aorta, but there was a small shift of the dose-response curve in the pulmonary artery.

In conclusion, isoprenaline-induced relaxations comprise a propranolol-sensitive and -insensitive component in the rat thoracic aorta. Although propranolol antagonized responses to a greater extent in the pulmonary artery, the antagonism was non-competitive. Furthermore, selective β_3-adrenoceptor agonists produced relaxations of both tissues. Together these data suggest that atypical β-adrenoceptors exist in the rat vasculature and mediate vasorelaxation.

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262P CALCITONIN GENE-RELATED PEPTIDE (CGRP) RECEPTORS IN RAT VAS DEFERENS, PULMONARY ARTERY, INTERNAL ANAL SPHINCTER AND THORACIC AORTA

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The CGRP receptor has been divided into type 1 (guinea pig atrium) and type 2 (rat vas deferens) based partly on the affinities of the linear human CGRP analogue [Cys(ACM)^{2,7}]hα CGRP and the antagonist hα $CGRP_{8-37}$ (Dennis *et al.*, 1989; 1990). Different affinities of the CGRP peptides may be due to degradation by peptidases (Longmore *et al.*, 1994).

The aim of the present studies was to examine the CGRP receptor in rat isolated prostatic vas deferens (vas), pulmonary artery, internal anal sphincter (IAS) and thoracic aorta.

Tissues were set up (0.5g tension) in Krebs' solution at 37°C (95% O_2/ 5% CO_2). Cumulative dose response curves to human (h) α CGRP and its analogues were produced in the absence and presence of the antagonists hα- and hβ $CGRP_{8-37}$ (10^{-5} or $3x10^{-5}$M; 20 min equil.) and peptidase-inhibitors (10^{-6}M of phosphoramidon, thiorphan, amastatin, bestatin and captopril; 30 min equil.).

Hα CGRP, hβ CGRP and rat β CGRP were of similar potency to each other for a single tissue although they were around 10 times more potent in the IAS (pEC_{50} around 8.8) compared with the aorta (pEC_{50} around 7.6). Rat amylin and [Cys(ACM)^{2,7}]hα CGRP were about 20- and at least 1000-fold less potent than the CGRP peptides in the vas, pulmonary artery and aorta. Hα $CGRP_{8-37}$ (10^{-5}M) and hβ $CGRP_{8-37}$ had a similar affinity against hα CGRP in the vas, pulmonary artery and IAS (pK_B value around 6.0) while in the aorta, hα $CGRP_{8-37}$ was less potent (pK_B around 4.8). In the vas, this antagonist (10^{-5}M) resulted in a pK_B value of 5.8±0.1 against both rat amylin and rat β CGRP. Peptidase-inhibitors (10^{-6}M) did not modify the pEC_{50} values of hα CGRP in the vas, pulmonary artery and aorta or the affinity of hα $CGRP_{8-37}$ (at least in the vas and pulmonary artery).

Unlike Longmore (1994), inhibition of peptidases did not alter either the potency of hα CGRP or the affinity of the antagonist hα $CGRP_{8-37}$. In the absence of peptidase-inhibitors, both hα- and hβ $CGRP_{8-37}$ shared similar antagonist activities against hα CGRP (at least in the vas, pulmonary artery and IAS). The subtype of the CGRP receptor appears to be a $CGRP_1$ type on the basis of the relative potency of [Cys(ACM)^{2,7}]hα CGRP (at least in the vas, pulmonary artery and aorta). However, this is contradicted by the low affinity of hα $CGRP_{8-37}$ (in all tissues) being consistent with a $CGRP_2$ receptor subtype. Therefore, these results, even in the presence of peptidase-inhibitors, do not fit with the proposed CGRP receptor classification.

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263P VASORELAXANT EFFECTS OF SR 47063 IN HUMAN SAPHENOUS VEIN AND RAT AORTA

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SR 47063 is a new potassium channel opener (KCO) which lowers blood pressure in animals and exhibits KCO-like activity in rat vasculature (Guiradou et al., 1991). Existing data suggest that SR 47063 is more potent than the structurally-related KCO levcromakalim (LK), however, little information is available regarding the *in vitro* effects of this drug in isolated blood vessels. We have previously shown that LK relaxes KCl-precontracted human saphenous vein (HSV) with an IC_{50} value of 9.7×10^{-8} M (Criddle & Soares de Moura, 1994). In the present study, we have examined the relaxant action of SR 47063 in HSV and additionally in rat aorta (RA).

HSVs were obtained from patients undergoing heart revascularisation (Institutional approval was granted) and RAs from male Wistar rats (250-350g). Rings of HSV or RA were mounted for the recording of isometric tension using conventional techniques and bathed in Krebs solution at 37°C. Rings were precontracted with either 20mM KCl or 10μM noradrenaline (NA) and, following a plateau response, relaxant cumulative concentration-response curves were obtained to SR 47063 (10^{-10} M - 10^{-6} M). Tissues were pre-treated with either 3μM glibenclamide (GLIB) or solvent equivalent for 15 minutes before addition of spasmogen.

In both RA and HSV, SR 47063 concentration-dependently relaxed KCl- and NA-precontracted vessels, with slightly greater potency against the former (table 1). Relaxant effects were inhibited greatly in the presence of GLIB. In addition, whereas in KCl-precontracted vessels a complete relaxation could still be induced by 1μM SR 47063 in the presence of GLIB, in NA-precontracted vessels 75% and 56% of the contraction still remained in HSV and RA, respectively. Thus in NA-contracted vessels IC_{50} values could not be obtained in the presence of GLIB.

Table 1. IC_{50} values for SR 47063-induced relaxation of HSV and RA.

	CONTROL	+ GLIB
HSV		
KCl	$8.10 \pm 0.28 \times 10^{-9}$ M (8)	$4.12 \pm 0.61 \times 10^{-7}$ M (8)
NA	$3.02 \pm 0.15 \times 10^{-8}$ M (8)	-
RA		
KCl	$3.86 \pm 0.76 \times 10^{-9}$ M (11)	$2.19 \pm 0.29 \times 10^{-7}$ M (9)
NA	$8.82 \pm 0.56 \times 10^{-9}$ M (6)	-

(values are mean ± s.e.mean, number of observations in parentheses)

Our study shows that SR 47063 potently relaxes HSV and RA, exhibiting a greater activity than that of the structurally-related drug LK. The effects of SR 47063 in HSV and RA are consistent with that of an opener of ATP-dependent potassium channels.

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264P SUMATRIPTAN EVOKES 5-HT_{1D}-LIKE RECEPTOR-MEDIATED CORONARY VASOCONSTRICTION AND MESULERGINE-SENSITIVE, NO-DEPENDENT, CORONARY VASODILATATION IN GUINEA-PIG ISOLATED HEART

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The anti-migraine drug sumatriptan (ST), a relatively selective 5-HT_{1D} receptor agonist (Pertouka & McCarthy 1989) has both vasodilator (Schoeffter & Hoyer 1994) and vasoconstrictor activity (Hoyer *et al.*, 1994) in coronary vasculature and is thus contraindicated in patients with, or at risk of, coronary artery disease. We have examined the effects of ST, alone and in the presence of the selective 5-HT_{1D} antagonist GR127935 (GR) and the mixed 5-HT_2 antagonist mesulergine (MS), on coronary flow (CF) and NO release.

Male Dunkin Hartley guinea-pigs (350-400g) were terminally anaesthetised with pentobarbitone (60 $mgkg^{-1}$ i.p.) and heparinised (250 iu sodium heparin i.p.). Hearts were excised and perfused under constant pressure perfusion (100cm H_2O) with modified Krebs solution containing (mM) KCl 4.0 and $CaCl_2$ 1.4 (pH 7.4, 37°C). Hearts were paced via the left ventricle (275 beats min^{-1}). CF was measured by timed collection of coronary effluent and the change in flow (ΔCF) was expressed as + or - ml $min^{-1}g^{-1}$ of ventricle. After 30 min of control perfusion, hearts (n= 6-8/group) received, cumulatively, ST (0.1nM-1μM), or ST in the presence of 10nM GR or 3μM MS or both combined. Additional groups received 10 nM GR alone, 3 μM MS alone or no drugs (time-matched control). Aliquots of coronary effluent (1ml) were collected during the last min of drug perfusion for subsequent NO analysis by chemiluminescence (Menon *et al.*, 1991). NO was expressed as pmol $min^{-1}g^{-1}$ of ventricle. Values are expressed as mean ± s.e.mean (*$P < 0.05$ versus pre-drug, paired t-test).

ST produced a concentration-dependent decrease in CF e.g., ΔCF with 1μM ST was -3.6 ± 0.4 ml $min^{-1}g^{-1}$. This was not accompanied by any change in NO production. In the presence of GR, the vasoconstrictor response to ST was inhibited, revealing a concentration-dependent increase in CF e.g., at 0.01μM ST, ΔCF was 2.0 ± 0.1 ml $min^{-1}g^{-1}$. This was accompanied by a significant increase in NO release (Table 1). MS enhanced the maximum reduction in CF in response to ST ($P<0.05$) without changing the pEC_{50} (table 1). The combination of GR and MS inhibited the ΔNO and the ΔCF in response to ST. Alone, neither GR nor MS significantly altered CF or NO release compared to time-matched controls.

Table 1. ST-induced ΔNO, and ST pEC_{50} for ΔCF

Drug	ST (μM)	ΔNO pmol $min^{-1}g^{-1}$	ST pEC_{50}†
ST	0.1	no change	8.6 ± 0.14^a
ST+GR	0.01	+684 ± 124*	9.1 ± 0.15^b
ST+MS	0.1	no change	8.6 ± 0.1^a
ST+MS+GR	0.1	no change	ST inactive

†=pEC_{50} for ΔCF; [a]=CF decrease; [b]=CF increase

ST-induced coronary vasoconstriction may be mediated via 5-HT_{1D} receptor activation. Interestingly, when this is inhibited by the 5-HT_{1D} antagonist GR, there is activation of a MS-sensitive receptor which leads to an increase in CF and a release of NO. Thus it appears that ST activates at least one other process in addition to activation of the 5-HT_{1D} receptor subtype.

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265P EFFECTS OF NITRIC OXIDE SYNTHASE INHIBITION AND METHYLENE BLUE ON VASOCONSTRICTOR RESPONSES IN THE RAT

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Nitric oxide synthase inhibitors (NOSI) have been shown to increase vasoconstrictor responses, and this action has been assumed to be due to inhibition of release of nitric oxide (NO). However, methylene blue (MB), which prevents the action of NO to stimulate cGMP production, fails to increase blood pressure (Wang et al., 1995), and even effects of NOSI in vitro are not easily explained in terms of preventing release of NO (Cocks & Angus, 1991). In this study, we have compared the actions of the NOSI, L-NMMA (see Cawley et al., 1995), with those of MB in two preparations: the pithed rat and the rat isolated mesenteric artery.

Rats were pithed under ether anaesthesia, and respired with 100% O_2 (see Cawley et al., 1995). Pressor responses were obtained to noradrenaline (NA) (1 μg/kg), or to spinal stimulation of pressor nerves (1 Hz, 10 pulses). Small mesenteric arteries were contracted with KCl, NA or with prostaglandin F2α (PGF2α), and L-NMMA or MB was administered during the contraction.

In pithed rats, NA (1 μg/kg) produced a rise in diastolic blood pressure (DBP) of 26.7±2.0 mmHg (n=21) and pressor nerve stimulation at 1Hz for 10 pulses produced a rise of 28.4±2.8 mmHg (n=21). MB (1 or 5 mg/kg) failed to affect pressor responses, but L-NMMA (10 mg/kg), alone, or in combination with MB (1 mg/kg), significantly increased the rise in DBP produced by NA or nerve stimulation (e.g. L-NMMA increased the response to nerve stimulation to 146.4±7.7% of control, n=5, P<0.05 from effect of vehicle).

In rat isolated small mesenteric artery, MB (1 μM) was at least as effective as L-NMMA (100 μM) at reducing endothelium-dependent relaxations to acetylcholine (ACh) or bradykinin in vessels pre-contracted with NA or PGF2α. In tissues contracted with NA or PGF2α, MB (1 μM) caused a small but significant increase in the contraction (e.g. contraction to NA increased from 0.86±0.13g to 1.03±0.16g, n=8, P<0.05, paired t-test), and subsequent L-NMMA (100 μM) produced a further increase (to 1.15±0.19g). When the order of administration was reversed, MB produced no further increase in the contraction when adminstered after L-NMMA. Responses to NA or PGF2α were unchanged in vehicle experiments, and MB or L-NMMA had no effect in endothelium denuded vessels.

It is concluded that the actions of L-NMMA to increase vasoconstrictor responses cannot be explained simply in terms of block of the release of NO.

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266P PK 11195 MODIFIES RESPONSES OF ISOLATED HEARTS TO BOTH CENTRALLY AND PERIPHERALLY ACTING BENZODIAZEPINES

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Benzodiazepines interfere with inotropic properties of the perfused heart. The mechanism(s) underlying this interference - which is antagonized non-selectively in the presence of adrenergic blocking drugs (Zeegers et al. 1994) - is (are) as yet unknown. Calcium entry blockers both antagonize and potentiate the response to benzodiazepines (Leeuwin et al., 1994). This presentation describes inotropic responses of the isolated rat heart to benzodiazepines, diazepam and Ro-5-4864 [7-chloro-5-(chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4 benzodiazepin-2-one] in the absence or in the presence of the peripherally acting type of benzodiazepine antagonist PK 11195 [-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl-3-isoquinolinecarboxamide].
Female rats (160-180g) were anaesthetized with pentobarbitone, 50mg/kg ip, and heparinized. Hearts with cannulated aortae were excised and perfused with Tyrode's solution at 37° (pH 7; perfusion rate 8ml/min), gassed with CO_2+O_2. Contractile force was currently recorded isometrically, using a latex balloon filled with water, inserted in the left ventricle. After equilibration the heart was perfused with 2.10^{-5} to 6.10^{-4} M diazepam or 1.10^{-5} to 4.10^{-4} M Ro-5-4864 with or without PK 11195 in the perfusate (n=7 for each concentration). Inotropic response was expressed as percentage change of contraction force. Data collected before and after exposure to a benzodiazepine were analysed statistically using Student's t-test.
Perfusion with diazepam resulted in a biphasic inotropic response, i.e. a transient negative inotropic response followed by a positive inotropic response, proceeding in two steps. Both responses were related to the concentration. Ro-5-4864 only evoked a concentration-related positive inotropic response of the Langendorff heart, the curve flattening at higher concentrations. PK 11195 (10^{-7} and 5.10^{-5} M) - not affecting the response by itself - did not interfere with the shape of the curve, but the positive inotropic response to diazepam was blocked significantly (P<0.05) by 10^{-6} or 10^{-5}M (at 10^{-4}M diazepam was blocked from 120 ±15% to 61±9 and to 41±7% respectively), whereas in the presence of 5.10^{-5} M it was completely abolished. The negative inotropic response was not affected whatsoever. The positive inotropy induced by Ro-5-4864 was suppressed as well concentration-dependently by PK 11195 (10^{-7} to 10^{-6} M). At 5.10^{-5} M the response to diazepam was completely suppressed.

In conclusion, it is feasible that at least the positive inotropic response to diazepam or Ro-5-4864 is realized via binding to the peripheral-type of benzodiazepine receptor; since the negative response to diazepam was not altered, more complex mechanisms, e.g. involvement of calcium currents, must be considered as well.

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267P ROLE OF L-AROMATIC AMINO ACID DECARBOXYLASE IN THE REGULATION OF ALDOSTERONE SECRETION BY 5-HT

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Aldosterone secretion is regulated by a number of factors, including the neurotransmitter 5-hydroxytryptamine (5-HT). However the physiological source of 5-HT reaching the adrenal zona glomerulosa remains elusive.

The enzyme, L-aromatic amino acid decarboxylase (L-AAAD) converts 5-hydroxytryptophan (5-HTP) into 5-HT, and has been reported to be present in adrenal glands by immunostaining (Beltramo *et al.*, 1993).

The aims of this project were to (1) to localise L-AAAD within the adrenal gland using a specific monoclonal antibody (Nagatsu *et al.*,1988) against the purified enzyme, (2) to determine whether the rat zona glomerulosa can synthesise 5-HT from 5-HTP, and (3) to assess whether 5-HTP can stimulate aldosterone secretion in (a) rat whole adrenal capsules and (b) rat preparations of isolated zona glomerulosa cells.

Female Wistar rats (200-250g), maintained on a normal (1%) NaCl diet were killed by decapitation, blood samples taken and the adrenal glands dissected. The adrenal capsules and adhering zona glomerulosa were removed following the method of Haning *et al.*, 1970. Isolated zona glomerulosa cells were prepared using collagenase digestion (2mg/ml/rat) followed by three washes in Krebs-Ringer bicarbonate buffer (2%BSA), yielding at least 95% pure zona glomerulosa cells with minimal zona fasciculata cell contamination. Whole adrenal capsule or isolated zona glomerulosa cells were incubated for 1hr at 37°C, with increasing concentrations of 5-HTP (10^{-6}M-10^{-3}M), in the presence of 10^{-4}M pargyline in a total volume of 1ml Krebs buffer (0.1%BSA). The supernatant was removed and stored at -20°C for subsequent measurement of aldosterone by radioimmunoassay (RIA), and 5-HTP, 5-HT and 5-HIAA by HPLC. The tissue was retained for protein assay. The blood samples, treated either with EDTA (27mmol/l) inhibitor or with a cocktail containing thrombin (1100U/l), clomipramine (1.1μmol/l) and pargyline (11.1μmol/l), were measured for aldosterone via RIA and for 5-HT, 5-HTP and 5-hydroxyindoleacetic acid (5-HIAA) by HPLC.

Results are shown as mean±SEM (n=12). Statistical significance was assessed by ANOVA.

Plasma aldosterone and 5-HTP concentrations were 1.067nmoles/L±0.14 and 37.587μmoles/L±10.2 respectively. Basal aldosterone secretion, (pmoles/mg of protein), from whole adrenal capsules was 22.1±2.15, this increased dose dependently to a maximal of 41.658±1.87 at 10^{-4}M 5-HTP, which was significantly different from basal ($P<0.05$). Carbidopa (10^{-4}M), an inhibitor of L-AAAD, completely inhibited the aldosterone response to 10^{-4}M 5-HTP, but had no significant effect on basal aldosterone secretion. The basal aldosterone secretion from isolated zona glomerulosa cell preparations was 1.127±0.085, this increased dose dependently to a maximal response of 5.074±0.435 at 10^{-4}M 5-HTP, which was significantly different from basal ($P<0.05$). Carbidopa ($1J^{-4}$M) completely inhibited the aldosterone response to 10^{-4}M 5-HTP, but had no significant effect on basal aldosterone secretion. In whole adrenal capsules the production of 5-HT (pmoles/mg of protein) increased dose dependently when incubated with increasing concentrations of 5-HTP. Basal 5-HT was 42.033±26.41, increasing to a maximal level of 1219.117±150.259 at $5x10^{-4}$M 5-HTP, which was significantly different from basal secretion ($P<0.05$). In the presence of 10^{-4}M carbidopa production of 5-HT from 10^{-4}M 5-HTP was significantly attenuated (381.617±130.055;$P<0.05$).

For immunohistochemistry, adrenal glands were fixed in 10% neutral buffered formalin for approximately 24 hours, then paraffin processed and 4μm sections cut onto adhesive slides. Immunostaining was by a Streptavidin-biotin complex peroxidase method preceded by antigen retrieval using microwave according to the method of Gerdes *et al.*, 1992. The L-AAAD antibody was used at a dilution of 1:2000. L-AAAD immunoreactivity in the adrenal gland was located in the zona glomerulosa, zona fasciculata and medulla. The adrenal capsule and zona reticularis showed no visible L-AAAD immunoreactivity.

The results of this study demonstrate significant L-AAAD activity within the adrenal zona glomerulosa which may form part of a paracrine or autocrine mechanism for the modulation of aldosterone secretion by 5-HT. Furthermore these results strongly suggest that 5-HT in the adrenal zona glomerulosa most probably originates from circulating 5-HTP.

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268P STUDIES ON THE SYMPATHOLYTIC EFFECTS OF SOME 5-HT_{1D} RECEPTOR AGONISTS IN PITHED RATS AND GUINEA-PIGS

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5-HT_{1D} receptor selective agonists have direct vasoconstrictor effects by actions at post-junctional receptors on the smooth muscle of the vasculature but can also inhibit electrically-evoked contractions of blood vessels by acting at pre-junctional 5-HT receptors on sympathetic nerves to attenuate the release of the vasoconstrictor noradrenaline (Humphrey *et al*, 1983: 1988). The present studies have examined in pithed rats and guinea pigs, the peripheral sympathoinhibitory action of a number of 5-HT_{1D} agonists (sumatriptan, BW311C, MK-462, CP-122,288) currently of interest as anti-migraine agents.

Male Sprague-Dawley rats (350 g) or male Dunkin-Hartley guinea-pigs (300 g) were anaesthetised briefly with isoflurane or methohexitone (50 mg/kg i.p.) respectively. The trachea was cannulated before pithing by the transorbital route with a steel rod insulated to within 1cm of its tip and then artificially ventilating with room air. A femoral artery and vein (rats) and a carotid artery and jugular vein (guinea pigs) were cannulated to record blood pressure and intravenously dose test compounds, respectively. The tip of the pithing rod was adjusted to the T_2-T_6 vertebral level within the spinal column. After pre-treatment with gallamine (20 mg/kg i.v.) and atropine (1 mg/kg i.v.), increases in blood pressure were evoked by successive electrical stimulation (10 V, 1.0 ms duration for 30 s) of vasopressor pre-ganglionic sympathetic nerves via the pithing rod at frequencies of 0.2 - 1.0 Hz (rats) and 0.2-2.0 Hz (guinea pigs) at 3 min intervals. After completion of a control frequency-response curve the 5-HT_{1D} agonists were given (0.3 - 10 mg/kg) and the curve repeated 5 min later. The pressor response to sympathetic nerve stimulation in the presence of test compound was calculated as a percentage of the control response. The effects of test compounds on the post-junctional vasoconstrictor action of noradrenaline were assessed by examining their effects on the pressor response to a bolus dose of noradrenaline (0.1 μg/kg i.v.)

Sumatriptan, BW311C, MK-462 and CP-122,288 inhibited the pressor responses evoked by pre-ganglionic sympathetic stimulation in a dose and frequency dependent manner (Table 1) but the direct pressor response to exogenous noradrenaline was undiminished. The rank order of activity in rats was CP-122,288 ≥BW311C > MK-462 > sumatriptan and was the same for those compounds tested in guinea pigs. The results suggest that these 5-HT_{1D} agonists act to reduce the noradrenaline release from post-ganglionic sympathetic neurones rather than blocking its post-junctional activity on the vasculature. The dose and frequency dependence of this sympatho-inhibitory effect suggests that in intact animals, this effect will depend upon circulating drug plasma levels and the prevailing level of sympathetic vasomotor tone in a vascular bed. The overall haemodynamic profile of these 5-HT_{1D} agonists will be determined by the balance between this sympathoinhibitory, pro-dilator activity and their post-junctional vasoconstrictor activity.

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Table 1. Mean % inhibition of the increase in blood pressure produced by sympathetic stimulation in pithed animals.

	Rat (1 and 10 mg/kg, i.v., n=3-4)								Guinea pig (0.3 and 3 mg/kg, i.v., n=3-4)					
Stimulus frequency	sumatriptan		MK-462		BW311C		CP122,288		sumatriptan		MK-462		BW311C	
0.2 Hz	20	67	47	67	59	72	59	83	43	100	91	89	73	94
0.4 Hz	13	47	24	41	45	67	49	67	50	74	60	71	57	77
0.6 Hz	11	38	18	31	32	69	33	66	5	41	36	50	32	67
0.8 Hz	-2	37	17	27	20	66	26	64	10	42	-19	40	31	62
1.0 Hz	-2	26	14	22	7	63	22	58	25	26	-17	32	20	43
2.0 Hz	N.T.		N.T.		N.T.		N.T.		23	10	-11	4	7	23

269P CHARACTERISATION OF 5-HT RECEPTORS MEDIATING VASOCONSTRICTION OF ISOLATED EQUINE DIGITAL ARTERIES

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Equine laminitis is a common condition characterised by decreased perfusion of the hoof laminae and reperfusion injury. 5-HT is a very potent vasoconstrictor of both equine digital vessels and we have previously demonstrated the presence of 5-HT_1-like receptors and 5-HT_2 receptors mediating vasoconstriction in equine digital veins (Weller et al 1994). The aim of this study was to characterise the 5-HT receptors mediating vasoconstriction of equine digital arteries (EDA).

Rings of EDA were denuded of their endothelium and prepared for isometric tension recording as previously described (Bryant et al 1992). Rings were preincubated with pargyline (0.5 mM) and benextramine (0.1 mM) for 30 min before washing, after which the maximum contractile response to depolarising Krebs solution (DKS, 118 mM KCl) was obtained. Cumulative concentration response curves (CRCs) were constructed (0.1 nM to 0.1 mM; n=6) for 5-HT, 5-carboxamidotryptamine (5-CT), sumatriptan (SUM), 8-hydroxy-2-(N,N-dipropylamine) tetralin (8-OH-DPAT), α-methyl-5-HT (α-Me-5-HT), and 2-methyl-5-HT (2-Me-5-HT). In the case of 5-HT, CRC were also obtained 30 min after the addition of ketanserin (KET; 0.1 μM; n=6). Increases in tension were expressed as a percentage of the DKS response.

All the agonists examined produced dose-dependent contractions. 5-CT produced a biphasic CRC. The order of potency of the agonists their EC_{50} values and maximum contractile responses are shown in the table. The maximum response to SUM was the lowest of the agonists tested and was significantly less than the 1st phase of the response to 5-CT ($p<0.05$; Student's t-test). In the presence of KET, 5-HT gave a biphasic CRC with mean EC_{50} values (95% confidence limits) of 1.9 (0.4 - 8.4) x 10^{-8}M and 8.9 (2.1 - 38.8) x 10^{-6}M for the two phases and a plateau at 29.3 ± 8.3% of the DKS response.

AGONIST	EC_{50} (M) geometric mean (95% confidence limits)	% DKS response (mean ± sem.)
5-CT (1st phase)	9.0 (3.0 - 33.0) x 10^{-9}	21.2 ± 2.5
5-HT	3.7 (1.3 - 10.9) x 10^{-8}	157.1 ± 8.0
α-Me-5HT	8.0 (3.1 - 20.7) x 10^{-8}	137.2 ± 11.0
SUM	4.9 (1.8 - 25.4) x 10^{-7}	8.7 ± 1.4
5-CT (2nd phase)	1.9 (3.0 - 12.0) x 10^{-6}	100.8 ± 4.3
8-OH-DPAT	6.5 (3.6 - 11.9) x 10^{-6}	93.9 ± 1.4
2-Me-5-HT	2.4 (0.5 - 10.1) x 10^{-5}	71.4 ± 5.4

The high potency of 5-CT and the KET resistant component of the responses to 5-HT suggest that EDA possess 5-HT_1-like receptors in addition to 5-HT_2 receptors mediating vasoconstriction. The low potency of 8-OH-DPAT suggests that $5HT_{1A}$ receptors are unlikely to be present. If the 5-HT_1-like component alone is considered, both 5-CT and 5-HT gave similar maximum responses, indicating that these two agonists have similar efficacies at the 5-HT_1-like receptor. SUM, the 5-HT_{1D} selective agonist, proved to have a low efficacy and potency when compared with 5-HT and 5-CT in EDA. SUM has similar efficacy to 5-HT in mediating vasoconstriction of dog saphenous vein (Humphrey et al., 1988) and other vessels with 5-HT_1-like receptors. We suggest, either that EDA has a low 5-HT_1-like receptor reserve or a different type of 5-HT_1-like receptor to that found causing vasoconstriction in other vessels.

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

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270P UPREGULATION OF THE ENDOTHELIN ET_A RECEPTORS IN LEFT VENTRICLE FROM FAILING HUMAN HEARTS DEMONSTRATED USING COMPETITION BINDING STUDIES WITH FR139317

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The aim of our study was to identify changes in expression of endothelin receptor subtypes in human left ventricle (HLV) from heart transplant recipients suffering from dilated cardiomyopathy (DCM, n=8) and ischaemic heart disease (IHD, n=6), relative to non-failing hearts (NF, n=7). Further, we localised the endothelin receptor subtypes to different cell populations present in the heart muscle by using autoradiographical techniques.

For competition experiments, cross-sections of HLV were incubated with 0.1 nM [^{125}I]-ET-1 (2000 Ci mmol^{-1}; Amersham) for 2 h in the presence of increasing concentrations (10 pM - 10 μM) of the ET_A-selective antagonist, FR139317 (Aramori *et al.*, 1992). Binding parameters were calculated using the non-linear iterative curve fitting programme LIGAND. Autoradiography was carried out by labelling 10-20% of the total receptor population using the non-selective radioligand [^{125}I]-ET-1, ET_B-selective [^{125}I]-BQ3020 and ET_A-selective [^{125}I]-PD151242 (Davenport *et al.*, 1994). Non-specific binding was defined by the inclusion of 1μM of the unlabelled radioligand. Results are given as mean ± s.e.mean. All experiments were carried out at 23°C. Data were analysed by ANOVA followed by Student's *t*-tests.

Competition binding studies indicated the presence of two receptor populations and FR139317 was more than 1,000 fold selective for the higher affinity binding sites. The receptor densities (B_{max}) for NF hearts were 30.4±0.8 (ET_A) and 17.8±3.2 (ET_B) fmol mg^{-1} protein. For DCM there was no apparent change in ET_A receptor density (45.4±7.9 fmol mg^{-1} protein), whilst in IHD a significant increase ($p=0.0034$) in ET_A receptor density was observed: 46.0±2.9 fmol mg^{-1} protein. ET_B receptor densities were not altered in either DCM (13.9±2.1 fmol mg^{-1} protein) or IHD (11.7±1.1 fmol mg^{-1} protein) compared to NF hearts (Figure 1). In contrast to the absolute values, analysis of subtype ratios yielded significant increases in the relative proportion of ET_A receptors in both DCM (76.8%, $p=0.024$) and IHD (80.1%, $p=0.0073$), compared to NF hearts (67.0%). No changes were observed in the affinity for the binding of FR139317 to ET_A receptors in either disease ($K_D ET_A \approx$ 1nM).

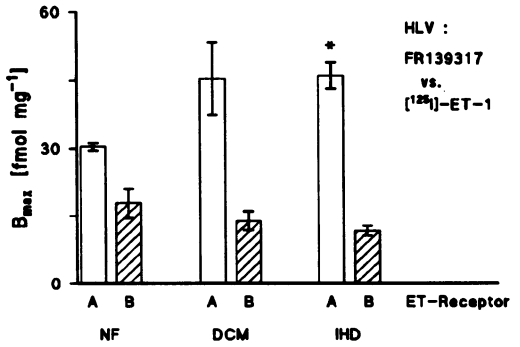

Figure 1 Density of ET_A and ET_B binding sites as determined by using the ET_A selective antagonist FR139317.

In NF hearts autoradiography showed binding of [^{125}I]-PD151242 to ET_A receptors located on intramyocardial vessels, with lower densities on cardiac muscle. Lower levels of [^{125}I]-BQ3020 binding to ET_B receptors were detected on the myocardium and binding to its vessels was below the level of detection.

Our data suggests that structural changes in human heart diseases may be regulated at the level of endothelin receptor subtype expression.

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271P DIRECT MEASUREMENT OF DURAL BLOOD VESSEL DIAMETER IN THE RAT USING INTRAVITAL MICROSCOPY

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Vasodilatation of the meningeal vasculature triggered by trigeminal activation has been suggested to cause migraine headache pain (Goadsby, 1994). Kurosawa et al. (1995) showed, using laser doppler fluxmetry, that electrical stimulation of trigeminal nerves produced an increase in meningeal blood flow mediated by calcitonin gene-related peptide (CGRP). However, such studies of meningeal blood vessels in anaesthetised animals often involve removal of the skull and this can lead to brain swelling and altered blood vessel reactivity. We have developed a novel technique using intravital microscopy to directly measure dural blood vessel diameter in a more physiological, closed cranial window preparation and studied the roles of substance P (SP) and CGRP in modulation of dural vessel calibre.

Male SD rats (300-400 g) were anaesthetised throughout with pentobarbitone sodium (initially 60 mg kg^{-1} i.p., then 6 mg kg^{-1} hr^{-1} i.v. infusion). A femoral artery and both femoral veins were cannulated for blood pressure recording, i.v. injection of drugs or infusion of anaesthetic. Rats were placed in a stereotaxic frame, the skull exposed and the right parietal bone thinned by drilling until the blood vessels of the dura mater were clearly visible through the intact skull. A branch of the middle meningeal artery (30 - 50 µm) was viewed using an intravital microscope (MV 2100, Finlay Microvision), the image displayed on a T.V. monitor and the diameter measured continuously using a video dimension analyser (V94, Living Systems Instrumentation). The change in dural vessel diameter to i.v. drugs or electrical stimulation was measured and expressed as % increase from baseline. Values are mean ± s.e.mean and statistical analysis was performed using ANOVA and matched t-test. Injection of SP (3 - 300 ng kg^{-1}, n = 6) produced transient (approx. 1 min) dose-related increases in dural vessel diameter reaching 84 ± 14% by the highest dose. A single dose of SP (100 ng kg^{-1}), evoked a 82 ± 1% increase in vessel diameter which was completely abolished ($p < 0.001$, n=3) by the NK_1 receptor antagonist RP 67580 (1 mg kg^{-1}). In comparison, the (-)-enantiomer, RP 68651 (1 mg kg^{-1}), which has lower affinity for NK_1 receptors, only reduced the SP response to 67 ± 1% ($p < 0.05$, n = 3). In another series of experiments, electrical stimulation of the surface of the cranial window (10-30 V, 5 Hz, 1 ms for 10 s) evoked an increase in vessel diameter (126 ± 26%, n = 6) which was longer lasting (approx. 3 min) than that seen with SP. Pretreatment with RP 67580 (1 mg kg^{-1}) did not reduce the magnitude of the stimulated response (118 ± 28%), whereas pretreatment with the CGRP receptor antagonist $CGRP_{8-37}$ (0.3 mg kg^{-1}) significantly attenuated this response (13 ± 4%, $p < 0.001$, n = 6). RP 67580 and $CGRP_{8-37}$ had no effects on vessel diameter *per se* suggesting that SP and CGRP are not involved in the maintenance of vascular tone in this preparation.

The results demonstrate that SP-evoked vasodilatation of dural blood vessels in rats is mediated through activation of NK_1 receptors. However, under the present experimental conditions, electrically stimulated vasodilatation appears to be mediated mainly via CGRP, presumably released from trigeminal nerves. This is consistent with the trigemino-vascular hypothesis of migraine and the observation that cranial venous CGRP levels are elevated during a migraine attack (see Goadsby, 1994). The present novel technique may thus be useful for investigating potential mechanisms and therapies for migraine.

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272P EFFECT OF NEOMYCIN OR STREPTOMYCIN ON ENZYME ACTIVITIES IN A HUMAN RENAL CELL LINE

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We have previously demonstrated (Kumar et al., 1995) that the human renal cell line, G401 is sensitive to the aminoglycoside antibiotic gentamicin. We have now determined enzyme activities in the G401; lactate dehydrogenase (LDH), alkaline phosphatase (AP) and leucine aminopeptidase (LAP) in response to neomycin & streptomycin. G401 cells were grown in Mcoy's 5α medium supplemented with 10% Foetal Clone. Once confluent, cells were incubated with neomycin or streptomycin (0-5mM) for 24, 48, or 72 hours prior to being washed, homogenised and protein content and enzyme activities determined.

Results for neomycin are given in Table 1. Streptomycin also showed a similar significant dose- and -time dependent decrease in enzyme activity i.e. after 24h incubation with streptomycin 0-5mM respectively: LDH was reduced from 0.092±0.008 to 0.057± 0.005*** iu/mg protein; AP from 0.38 ± 0.065 to 0.16± 0.025**mU/mg protein; LAP from 13.2±0.211 to 12.0±0.073** mU/mg protein, indicating that the G401 cell line is sensitive to aminoglycoside antibiotics. These data in conjunction with previous studies (Kumar et al 1995) confirm that the G401 is sensitive to neomycin < gentamicin < streptomycin which is consistent for rat primary cultures of proximal tubules (Parker *et al* 1982).

Table 1 The effect of varying concentrations (0-5mM) of neomycin on the specific activities of LDH, iu/mg protein; AP, mU/mg protein, LAP, mU/mg protein for either 24, 48 or 72h incubation. Results are expressed as mean ± s.e.mean, n=6. *p<0.05, **p<0.01 ***p<0.001 compared to media alone for each time, two tailed paired t-test.

	Neomycin concentration				
enzyme	control	10µM	100µM	1mM	5mM
time					
LDH	0.065	0.057*	0.048***	0.033***	0.013***
24h	±0.006	±0.005	±0.005	±0.007	±0.002
48h	0.058	0.050	0.043*	0.023***	0.013***
	±0.005	±0.005	±0.008	±0.004	±0.002
72h	0.052	0.045*	0.03***	0.013***	0.010***
	±0.002	±0.002	±0.002	±0.002	±0.001
AP	0.21	0.16*	0.14**	0.13**	0.11**
24h	±0.018	±0.016	±0.009	±0.009	±0.013
48h	0.29	0.23*	0.20*	0.17**	0.15**
	±0.025	±0.024	±0.025	±0.022	±0.023
72h	0.10	0.08*	0.06***	0.04***	0.03***
	±0.009	±0.007	±0.006	±0.007	±0.008
LAP	10.0	9.2	8.6**	7.4**	5.9***
24h	±0.40	±0.35	±0.36	±0.30	±0.29
48h	9.5	8.8*	8.1**	7.2***	5.6***
	±0.13	±0.19	±0.07	±0.22	±0.29
72h	9.4	8.5*	8.0**	7.1***	5.3***
	±0.13	±0.17	±0.14	±0.18	±0.22

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273P EVIDENCE THAT THE MITOGENIC RESPONSE TO ANGIOTENSIN II IN VASCULAR SMOOTH MUSCLE CELLS FROM THE HYPERTENSIVE RAT INVOLVES A TYROSINE KINASE DEPENDENT STIMULATION OF PHOSPHOLIPASE D ACTIVITY

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We have previously reported that angiotensin II (AII) stimulates thymidine incorporation in vascular smooth muscle (VSM) cells derived from spontaneously hypertensive rats (SHR), and that this response is in part due to a phospholipase D (PLD) dependent mechanism (Morton et al, 1995). Here we show that the response to AII is a true mitogenic response,and further investigate a role for PLD, and tyrosine kinases, in this response.

SHR derived cells were cultured from 12 week old animals and used at passages 8-14. To assess an increase in cell number cells were counted 48h after a 1h exposure to 1 μM AII, using a Coulter counter. DNA synthesis was estimated 19h after a 1h exposure to 100 nM AII, by incubation for a further 4h in the presence of 1μCi/ml [^{3}H]thymidine. [^{33}P]Phosphatidylbutanol accumulation in the presence of butanol was used as an index of PLD activity, as described (Morton et al, 1995). Butanol-1-ol (50mM) was also used to divert the products of PLD activity (Morton et al, 1995) and assess its role in the mitogenic response. Tertiary butanol, which does not divert the PLD reaction, was used as a control for other possible effects of butanol.

Treatment with AII gave an increase in cell number (basal, 3651 ± 98; AII stimulated, 4307 ± 136; x 10^3 cells/ml, mean ± s.e.mean, P<0.0002), indicating that cell division occurs with the stimulated thymidine incorporation reported previously. The tyrosine kinase inhibitor genistein substantially attenuated the stimulation of [^{3}H]thymidine incorporation by 100 nM AII: expressed as fold over basal, AII alone, 3.68 ± 0.26 fold; AII + genistein (30 μM), 1.13 ± 0.04 fold (mean ± s.e.mean, n = 3).The AII stimulation of PLD was inhibited by 3 different tyrosine kinase inhibitors (genistein, tyrphostin and methyl 2,5-dihydroxycinnamate) in a dose dependent manner. Butan-1-ol diverted the product of PLD from phosphatidic acid to phosphatidylbutanol, and also caused a 40.6 ± 6.0 % (n = 4, P<0.005) inhibition of the mitogenic response, reaching this maximal inhibition by 30 mM butan-1-ol. However tertiary butanol, at up to 50 mM, did not affect the product of the PLD reaction. It also failed to inhibit the mitogenic response.

These results show that AII stimulation of SHR derived VSM cells leads to a mitogenic response by a mechanism dependent in part on the sequential stimulation of tyrosine phosphorylation and phospholipase D.

Morton, C. et al, (1995) Br. J. Pharmacol. 115, 361-367.

274P COMPARISON OF THE CARDIOTOXICITY OF NEW ANTHRAQUINONE ANTITUMOUR AGENTS AND THEIR N-OXIDES PRODRUGS WITH DOXORUBICIN AND EPIRUBICIN USING ISOLATED RAT CARDIAC MYOCYTES

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The therapeutic use of anthracycline antitumour antibiotics is limited by dose-related chronic congestive cardiomyopathy (Doroshow 1991). Synthetic antitumour compounds such as mitoxantrone and ametantrone which contain the planar anthraquinone chromophore have also been shown to induce cardiotoxicity (Saletan 1987). This study examined the cardiotoxic effects of newly synthesised antitumour compounds (Patterson et al., 1994) structurally related to mitoxantrone, the bis-alkylaminoanthraquinones (AQ4 and AQ5) and their respective N-oxide prodrugs (AQ4N and AQ5N) for comparison with the cardiotoxicities of doxorubicin and epirubicin.

Cardiac myocytes were isolated (Tytgat 1994) from adult female Hooded Lister rats (200-250g) and cultured in medium M199 (200μl) containing 5% foetal bovine serum (FBS) using 96-well plates (10^4 cell/well). The cardiac myocytes were incubated with the drugs (1-500μM) under 5% CO_2 and 37°C for 4 or 24h after which the drug was removed. An MTT assay (Jabbar et al., 1989) was used to assess the survival of the cells after the periods of incubation. The response to the drug was expressed as the percentage cell survival compared with control (100% cell survival).

The estimated IC50 of the compounds after 4 and 24 h incubation with the isolated cardiac myocytes is shown in Table 1. Doxorubicin and epirubicin (concentration range used 10-500μM for 4h and 1-200μM for 24h) produced a dose-dependent decrease in cell survival with epirubicin being 2.5 times more toxic than doxorubicin. Similarly mitoxantrone, AQ4, and AQ5 (concentration range used 1-100μM for 4h and 1-50μM for 24h) produced a dose-dependent decrease in cell survival. However, AQ4N and AQ5N, the N-oxides of AQ4 and AQ5 respectively, produced no detectable decrease in cell survival in the same dose range.

These results show the order of toxicity of the antitumour agents as: mitoxantrone, AQ4 and AQ5> epirubicin > ametantrone > doxorubicin. These results also show that AQ4N and AQ5N have low toxicities using this system, consistent with their *in vitro* cytotoxicities against MCF7 and V79 tumour cell lines.

Table 1. Comparison of estimated IC50 values obtained using the MTT assay (n=3).

Compound	IC50(μM) ± SD 4h	IC50(μM) ± SD 24h
Doxorubicin	244 ± 17.4	88.0 ± 6.0
Epirubicin	99.7 ± 16.1	35.2 ± 6.6
Mitoxantrone	25.0 ± 6.0	10.7 ± 2.2
Ametantrone	>100	>50
AQ4	22.5 ± 5.7	10.8 ± 1.7
AQ5	28.5 ± 1.2	25.0 ± 4.6
AQ4N	>100	>50
AQ5N	>100	>50

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275P COMPARISON OF ANTAGONISM OF ADENOSINE RECEPTOR MEDIATED RESPONSES BY 8-(*P*-SULFOPHENYL) THEOPHYLLINE IN FOUR GUINEA-PIG ISOLATED CARDIAC PREPARATIONS

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It is now generally accepted that adenosine receptors can be divided into three types, A_1, A_2 and A_3 (Collis & Hourani, 1993). Although selective antagonists for adenosine A_1 and A_2 receptors have been described (Trevidi *et al.*, 1990), pharmacological classification of these receptors has usually depended on agonist potency orders. This study characterises the adenosine receptors mediating various cardiac responses in the guinea-pig from the pA_2 values of the antagonist 8-(*p*-sulfophenyl) theophylline.

Male Dunkin-Hartley guinea-pigs (250-300g) were killed and the left (LA) and right atria (RA) and a left papillary muscle (PM) removed. The tissues were bathed in Krebs bicarbonate solution at 37°C, gassed with 95% O_2:5% CO_2. Resting tensions of 1g were applied to LA and RA and 0.5g to the PM. LA and PM were paced at 2Hz with threshold voltage + 50% and tension recorded. Spontaneous rate of RA were recorded. After 30 minute equilibration, 8-SPT or vehicle was added. Cumulative concentration-response curves for the negative inotropic and chronotropic response to N^6-cyclopentyladenosine (CPA) were constructed either in naive LA and RA or in isoprenaline-stimulated LA and PM to measure antiadrenergic responses. The concentrations of isoprenaline (ISO) were 1 and 30nM respectively. stock drug solutions were prepared in 50:50 PEG 200: water and serially diluted with water. Decreases in tension or rate from basal or the ISO-stimulated plateau were recorded and EC_{50} determined. Geometric EC_{50} values were obtained in the absence of 8-SPT(control). Dose-ratios (DR) for antagonism by 8-SPT were calculated as the ratio of the individual EC_{50} in the presence of 8-SPT relative to the mean control EC_{50}. Geometric Mean (n=4) DR's are expressed as the $\log_{10}$ (DR-1) in Table 1.

At the highest concentration [A] of 8-SPT, the Schild plots plateaued, the DR values not increasing significantly ($P>0.05$) as concentration increased. This resulted in reduction of the Schild regression slope from unity. Omission of this point resulted in slopes closer to unity. pA_2 values were therefore calculated without this concentration from the equation, $pA_2=\log_{10}(DR-1) - \log_{10}[A]$ (Mackay,1978).Significant differences occurred between pA_2 values (ANOVA); in PM it was less than in other tissues ($P<0.05$, Student's t-test).

Table 1.

	Log_{10}(dose ratios - 1) ± S.E.M.			
	Left atria		Right atria	Papillary
8-SPT	Naive	ISO-stim.	Naive	ISO-stim.
$1x10^{-6}$ M	-0.34+/-0.39	-0.24+/-0.07	-0.04+/-0.08	-0.30+/-0.14
$3x10^{-6}$ M		0.18+/-0.12		
$1x10^{-5}$ M	0.84+/-0.31	0.16+/-0.35	1.01+/-0.10	0.55+/-0.19
$1x10^{-4}$M	1.71+/-0.07	1.43+/-0.03	2.02+/-0.17	1.16+/-0.46
$3x10^{-4}$ M	2.10+/-0.12	2.18+/-0.09	2.21+/-0.27	
$1x10^{-3}$ M	2.10+/-0.15	2.38+/-0.06	2.38+/-0.06	0.97+/-0.21
Slope-all points	0.85	0.87	0.83	0.44
Linear Points	0.98	0.95	0.94	0.73
pA2 +/- S.E.M	5.71+/-0.11	5.57+/-0.09 [a]	5.9+/-0.07	5.22+/-0.17[b]
conc. of SPT	(4)	(5)	(4)	(3)

[a] sig. different from right atria [b] sig. different from all others.

The plateauing of the Schild plots for the antagonism by 8-SPT suggests either an allosteric interaction at high 8-SPT concentrations or that the responses at high CPA concentrations are mediated via an alkylxanthine-resistant mechanism. The possibility that the balance of these heterogeneous mechanisms may vary between cardiac preparations could explain the different pA_2 values.

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276P ACCELERATING DRUG DISCOVERY: HIGH AFFINITY HUMAN MONOCLONAL ANTIBODIES ON FILAMENTOUS BACTERIOPHAGE

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Display of functional antibody fragments on the surface of filamentous bacteriophage is emerging as the method of choice for manipulating human antibodies (McCafferty *et al.*, 1990; Johnson & Chiswell, 1993). To avoid the need for immunisation, phage display repertoires have been constructed using antibody genes from non-immunised human donors, and these have proven to be a source of highly specific antibodies to a range of structurally diverse antigens (Marks *et al.*, 1991; Griffiths *et al.*, 1993). Recently, we have found that if these repertoires are large enough (Griffiths *et al.*, 1994), not only are they high specificity, the antibodies are also of high affinity. A small selection of the antibodies we have already isolated from a single repertoire of 10^{10} antibody genes are listed in Table 1. All have Kds <10nM, the best so far being 300pM, equivalent to the best that have been produced in rodents by immunisation. None of these antibodies took longer than two weeks to isolate. Amongst these examples are several that are difficult or impossible to produce using conventional approaches.

The high affinities of these monoclonal antibodies enables their direct use in immunoassays, *in vitro* biological assays and *in vivo* 'proof of principle' studies. We believe that these new techniques will have increasing applications in pharmacology, from basic research through to development of novel therapeutic products.

Table 1. Affinities of selected antibodies isolated directly from a human antibody repertoire of 10^{10} genes. Standard errors of the mean are ≤ 10%.

Antigen	K_d
Carcino Embryonic Antigen	7.7 nM
Doxorubicin	5.8 nM
DTPA	0.8 nM
Fluorescein	0.3 nM
Merozoite Surface Protein	8.0 nM
Oestradiol	3.7 nM
Very Low Density Lipoprotein Receptor	4.2 nM

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277P S 18149 IS A PARTIAL AGONIST AT α-ADRENOCEPTORS: STUDIES IN PITHED RATS AND ISOLATED CANINE AND HUMAN BLOOD VESSELS

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S 18149, (5S)-spiro [(1,3-diazacyclopent-1-ene)-5:2'-(7'-methyl - 1',2',3',4'-tetrahydronaphtalene)] fumarate, was selected in an evaluation program aimed at detecting compounds that possess partial agonist activity at α_1-adrenoceptors (Cordi et al., in press). The aim of the present studies was to examine the in vivo and in vitro α-adrenergic effects of S 18149.

The in vivo studies were performed in pithed rats (weight 250-400 g), previously anesthetized with ether. The animals were artificially ventilated and arterial blood pressure was recorded via a catheter inserted into the carotid artery. Mean resting blood pressure was 53 ± 2 mmHg (n=12).

The in vitro studies were performed on isolated canine (weight 15-25 kg) and human blood vessels, suspended in organ chambers filled with oxygenated (95 % O_2 / 5 % CO_2) Krebs Ringer solution kept at 37°C. In some experiments, the temperature of the organ bath solution was rapidly increased to 41°C (warming) or decreased to 24°C (cooling).

S 18149, at intravenous doses from 1 to 1000 µg/kg, induced dose-dependent pressor responses (ED_{50} : 26 ± 4 µg/kg) in the pithed rats (n=8) ; the maximal response (101 ± 4 mmHg) was lower than that to phenylephrine (148 ± 4 mmHg). The ED_{50} values of S 18149 in presence of the α_1-adrenoceptor antagonist prazosin (100 µg/kg i.v. ; n=5) and the α_2-adrenoceptor antagonist yohimbine (1 mg/kg i.v. ; n=5) averaged 230 ± 48 µg/kg and 135 ± 30 µg/kg, respectively.

In isolated dog saphenous veins (n=6), S 18149 (0.3 nM to 30 µM) evoked concentration-dependent contractions (EC_{50} : 0.22 ± 0.05 µM) ; the maximal responses averaged 78 ± 9 % of those to 100 mM KCl. In dog arteries, S 18149 only caused weak contractions at high concentrations, e.g. 12 ± 5 % and 18 ± 5 % at 100 µM (n=5) in femoral and coronary arteries, respectively. The EC_{50} values to S 18149 in the dog vein in presence of prazosin (0.1 µM ; n=6) and of the α_2-adrenoceptor antagonist rauwolscine (0.1 µM ; n=8) averaged 5.1 ± 1.9 µM and 2.2 ± 0.7 µM, respectively.

In the human saphenous vein, S 18149 (1 µM) evoked contractions that averaged 0.48 ± 0.20 g which represents 52 ± 17 % of those to KCl (n=6). These contractions were decreased by cooling (- 76 ± 20 %) and augmented by warming (+ 30 ± 5 %).

These data suggest that S 18149 is a partial agonist at α_1 and α_2-adrenoceptors, both in vivo and in vitro. In vitro, the compound produces more pronounced contractions of the saphenous vein than of arteries. S 18149-1 also contracts the human saphenous vein in a temperature sensitive manner.

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278P APAMIN-SENSITIVE RESPONSES TO ACETYLCHOLINE IN RABBIT ISOLATED MESENTERIC ARTERIES

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Cholinomimetic-evoked endothelium-dependent relaxation of small resistance arteries in some vascular beds has been shown to be resistant to nitric oxide synthase (NOS) inhibitors, and may be mediated by an endothelium-derived hyperpolarizing factor (EDHF), which is distinct from nitric oxide (NO: Garland *et al.*, 1995). Studies have indicated that the hyperpolarization and ensuing relaxation is mediated by potassium channels (K^+ channels: Chen & Suzuki 1989), although the type of channel involved is not clear. The involvement of ATP-sensitive K^+ channels (K_{ATP}) has been suggested (Standen *et al.*, 1989), whilst other studies suggest a role for apamin-sensitive K^+ channels (Adeagbo & Triggle, 1993). In this study, the relative contribution of NO and EDHF to acetylcholine-induced relaxation and the channels underlying hyperpolarization were investigated in third order branches of the rabbit mesenteric artery.

Female New Zealand White rabbits (2-2.5 kg) were injected with sodium pentobarbitone (60mg kg^{-1}) and killed by rapid exsanguination. The mesenteric bed was removed and the third branch of the mesenteric artery (D_{100} 349 ± 15 µm; n= 32) was isolated and mounted in a Mulvany-Halpern myograph for simultaneous recording of tension and membrane potential. The tissues were perfused with oxygenated Krebs buffer at 5ml min^{-1} and 37°C. Results are expressed as mean ± s.e.mean.

The cumulative addition of acetylcholine (3 nM-3 µM) stimulated concentration-dependent relaxation and repolarization in arteries contracted and depolarized by sub-maximal concentrations of phenylephrine (0.3-1 µM: mean contraction and depolarization 12.5 ± 1.4 mN and 31.1 ± 1.0 mV; n=6). The maximum relaxation and repolarization induced by acetylcholine (1 µM) was 97.1 ± 1.0 % and 33.0 ± 1.2 mV (n=4). The addition of the NOS inhibitor, N^G nitro-L-arginine (L-NOARG: 100 µM, 30mins) and the cyclo-oxygenase inhibitor indomethacin (3 µM) did not alter either the resting tension or the phenylephrine-induced contraction and depolarization (12.8 ± 0.8 mN and 32.3 ± 1.1 mV; n= 11; p>0.05). In the presence of these agents, the subsequent application of acetylcholine (3 nM-3 µM) produced a similar concentration-response curve with a maximum relaxation and repolarization of 93.6 ± 2.3 % and 35.8 ± 2.6 mV (n =5, p>0.05). These inhibitors were present in all further experiments.

In the presence of raised extracellular potassium ($[K^+]_o$: 30mM) and phenylephrine (0.3 - 1 µM: mean contraction and depolarization 12.4 ± 0.7 mN and 38.4 ± 2.1 mV; n=5) acetylcholine-induced relaxation and repolarization was abolished (n=4; p<0.05). In phenylephrine precontracted tissues, the K_{ATP} channel blocker, glibenclamide (3 µM: 30mins), did not affect the acetylcholine-induced relaxation or repolarization (n=4; p>0.05). However, apamin, a blocker of small conductance K^+ channels (10-20 nM: 35 mins), reduced the maximium acetylcholine-induced relaxation of pre-contracted vessels to only 3.6 ± 2.1 % and abolished the associated repolarization (n=4, p<0.05).

These data indicate that in the third branch of the rabbit mesenteric artery, acetylcholine-evoked relaxation is mediated mainly by a K^+-sensitive pathway and by a factor distinct from NO. Relaxation appears to be mediated predominantly via an apamin-sensitive pathway, and not through K_{ATP} channels, thus suggesting an important role for small conductance calcium-activated K^+ channels in the relaxation to acetylcholine.

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279P THE *IN VIVO* CARDIOVASCULAR PHARMACOLOGY OF THE SELECTIVE A_{2A} ADENOSINE ANTAGONIST SCH 58261 IN THE RAT AND RABBIT

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SCH 58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) is a new selective A2A adenosine receptor antagonist (Zocchi et al., 1996). The present studies describe the A2A antagonist properties of the compound "in vivo", as determined by studying cardiovascular parameters in conscious rats and anesthetized rabbits.
In spontaneously hypertensive rats (SHRs), weighing 350-400 g, and having a catheter implanted in the abdominal aorta for telemetry monitoring of blood pressure (BP) and heart rate (HR), antagonism to hypotensive responses induced by the A2A agonist 2-hexynyl-5'-N-ethylcarboxamidoadenosine (2HE-NECA) was assessed. Eight SHRs were given 2HE-NECA (0.01 mg/kg ip). Agonist injection was preceded by either SCH 58261 (10 mg/kg ip), or vehicle (5ml/kg, Tween 80 suspension ip), on two different days of experiment. Baseline diastolic BP and HR were 128±5 mmHg and 305±2 b/min, respectively. 2HE-NECA lowered diastolic BP (-58±7 mmHg) and increased HR (+198±11 b/min). SCH 58261 fully prevented 2HE-NECA induced hypotension (-1±11 mmHg, $p<0.01$), whereas it partially blocked the tachycardic response (+106±18 b/min, $p<0.01$). In anesthetized rabbits, weighing 2.5-3.5 kg, the antagonism to the hypotensive responses to the A2A agonist 2-[4-(2-carboxyethyl)-phenethylamino]-5'-N-ethyl-carboxamidoadenosine (CGS 21680), and the bradycardic responses to the A1 agonist N^6-cyclo-pentyladenosine (CPA), were determined. BP and HR were recorded continuously during the experiment. After a 30-min stabilization period, either CGS 21680 (3µg/kg iv bolus) or CPA (5µg/kg iv bolus) were injected and hemodynamic responses recorded. Following a recovery period of 1hr the rabbits were given infusions of SCH 58261 (3µg/kg iv x 10 min) or vehicle (DMSO 0.1 ml/min). They then received a second injection of either CPA or CGS 21680. This experimental period was repeated two more times, each separated by 1 hr of recovery. Baseline diastolic BP and HR values were 67±2 mmHg and 264±5 b/min, respectively. CGS 21680 reduced diastolic BP (-38±2 %), whereas HR was not influenced. CPA decreased HR by (-19±2 %), and had only modest hypotensive effects. Both agonists induced similar hemodynamic effects after vehicle infusions. SCH 58261 significantly inhibited the hypotensive effects of the three subsequent dosing with CGS 21680 (-7±3 %, -14±3 %, and -9±2 %; $p<0.01$), whereas it did not affect the bradycardic response to CPA. In conclusion, SCH 58261 was effective in blocking the hypotension induced by two A2A selective agonists in both SHRs and rabbits. The lack of effects on A1-mediated bradycardia in the rabbit indicates that SCH 58261 retains its A2A selectivity "in vivo".

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280P LACK OF EFFECT OF 1-(2-TRIFLUOROMETHYLPHENYL) IMIDAZOLE (TRIM) ON ENDOTHELIUM-DEPENDENT VASODILATATION IN RABBIT AORTA AND PERFUSED RAT MESENTERY

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1-(2-trifluoromethylphenyl) imidazole (TRIM) inhibits neuronal and inducible isoforms of nitric oxide synthase but is a poor inhibitor of endothelial NOS in vitro (Handy *et al.*, 1995). We have now compared the ability of TRIM and L-N^G nitro arginine methyl ester (L-NAME) to influence endothelium-dependent vasorelaxation in the isolated rabbit aorta and perfused rat mesenteric vascular bed preparations.

Rabbit (male, New Zealand White, 1.5-2.5kg) aortic rings were mounted under 2g tension in Krebs solution. After preconstriction with phenylephrine (PE: 0.75 µM) cumulative dose-response curves were constructed to carbachol (CCh: 0.01-20 µM) in the presence/absence of TRIM (1-100 µM) or L-NAME (100 µM). Rat (male, Sprague-Dawley, 300-350g) mesentery preparations were perfused at 5 ml min^{-1} with Krebs containing indomethacin (5 µM) and aliquots (5 ml) of perfusate collected for measurement of nitrite by chemiluminescence. After preconstriction with methoxamine (ME, 50 µM; perfusion pressure increased from 43.8±5.6 mm Hg to 123.6±13.5 mm Hg, n=42) the vasodilator response to CCh (0.1-20 µM added to the Krebs reservoir) was determined in the presence/absence of TRIM or L-NAME (both 50 µM).

CCh relaxed the phenylephrine-precontracted rabbit aorta with an EC_{50} of 0.4 µM. L-NAME (100 µM) greatly reduced the response to CCh (e.g. 27±2% maximum response at 10 µM CCh, n=8). In contrast, TRIM (1-100 µM) failed to influence CCh-mediated relaxation. CCh also produced dose related vasodilatation of the ME-preconstricted rat mesentery accompanied by increased release of nitrite into the perfusate (e.g. 10 µM; 133±8 pmol ml^{-1} perfusate c.f. 57.4±6 pmol ml^{-1} prior to CCh addition, n=42, $P<0.05$). L-NAME (50 µM) added to the Krebs reservoir caused a transient increase in perfusion pressure (10.1±1.8 mm Hg, n=12) accompanied by reduced nitrite release (51.8±7 pmol ml^{-1} perfusate c.f. 90±10 pmol ml^{-1}, n=12, $P<0.05$). L-NAME reduced both the vasodilator effect of CCh (e.g. 10µM CCh; 2.3±1.2 mm Hg c.f. 73.2±8.9 mm Hg, n=15, $P<0.05$) and the associated nitrite release (63.9±5 pmol ml^{-1} perfusate c.f. 154±9 pmol ml^{-1}, n=15, $P<0.05$). In contrast, addition of TRIM (50 µM) to the Krebs reservoir did not increase perfusion pressure or affect CCh-induced vasodilatation (e.g. 10 µM CCh; 58.6±6.3 mm Hg c.f. 61.2±7.4 mm Hg, n=15, $P>0.05$) or nitrite release (e.g. 10 µM CCh; 88.1±9 pmol ml^{-1} perfusate c.f 94.9±5 pmol ml^{-1}, n=15, $P>0.05$).

TRIM (unlike L-NAME) does not influence CCh-mediated endothelium-dependent relaxation in the rabbit aorta or rat mesentery preparations. Accordingly, the present results support the possibility that TRIM may prove to be a useful tool to study the biological effects of neuronally derived nitric oxide in the absence of cardiovascular side effects.

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281P EFFECT OF DOPAMINE D_1 RECEPTOR BLOCKADE ON NORADRENALINE-MEDIATED CONTRACTION OF THE RAT SMALL MESENTERIC ARTERY

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Recently, we showed that prazosin and other selective α_1-adrenoceptor antagonists do not produce the expected behaviour for simple competitive antagonism of noradrenaline (NA) in the rat isolated small mesenteric artery (s.m.a.) assay (Van der Graaf *et al.*, 1995a). Analysis of the steep Schild plots led us to hypothesise that NA also exhibits a receptor-mediated inhibitory action. Subsequently, we exposed a relaxant effect of NA in this assay (Van der Graaf *et al.*, 1995b) which appeared to be mediated by dopamine D_1 receptors (Van der Graaf *et al.*, 1995c). Consequently, we examined the effect of the presence of the D_1 receptor antagonist, SCH-23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride), on the antagonism of NA by prazosin.

S.m.a.'s (internal diameter 100-300μm) from male Wistar rats (225-350g) were mounted as 2mm ring segments in a myograph (37°C, gassed with 95%O_2/5%CO_2) as described before (Van der Graaf *et al.*, 1995a). The endothelium was removed, as confirmed by the lack of response to 10μM of the acetylcholine M-receptor agonist, 5-methylfurmethide, after precontraction with a single concentration of 10μM NA. After a 15min washout period, tissues were incubated for 90min with 30μM cocaine, 6μM timolol and antagonists or the appropriate vehicles. Single NA concentration-effect (E/[A]) curves (n=4) were obtained by cumulative dosing at half-log unit concentration increments. Effects were expressed as percentage of the response to the single concentration of 10μM NA.

NA produced concentration-dependent contraction of the s.m.a. and the individual E/[A] curves were fitted to the Hill equation to provide estimates (Table 1; data shown as mean±s.e.mean) of the midpoint slope (n_H), midpoint location ($p[A]_{50}$, that is $-\log[A]_{50}$) and upper asymptote (α). SCH-23390 (10nM) had no significant effect on the NA E/[A] curve, as judged by comparison between the Hill equation parameter estimates using Student's t-test (Table 1). Prazosin (0.1μM) produced rightward shift of the NA E/[A] curve with no significant effects on the upper asymptote or midpoint slope with an associated concentration ratio of 79 (Table 1). In the presence of 10nM SCH-23390 the apparent potency of prazosin at this concentration was significantly reduced by ~3.5-fold (concentration ratio = 22; Table 1). The $[A]_{50}$ values of the curves in the absence and presence of 0.1 μM prazosin alone were then fitted to the Schild equation with the pA_2 value constrained to the estimate (8.33 ± 0.14) obtained from the rightward shift produced by 0.1μM prazosin in the presence of 10nM SCH-23390. The apparent Schild slope parameter thus yielded (1.42 ± 0.10) was similar to values obtained in our previous study (Van der Graaf *et al.*, 1995a).

We conclude that these data indicate that the steep Schild plots obtained with NA in the s.m.a. were due to an action of NA at D_1 receptors mediating relaxation. Such a dual action of an agonist within one assay appears to be another way in which steep Schild plots can be obtained.

Table 1 Effects of SCH-23390 and prazosin on the Hill equation parameter estimates of noradrenaline E/[A] curves

	$p[A]_{50}$	α (%)	n_H
control	5.93±0.09	123±6	1.8±0.3
SCH-23390 (10nM)	5.76±0.18	123±6	1.7±0.2
prazosin (0.1μM)	4.03±0.19	107±10	2.1±0.1
SCH-23390 (10nM) + prazosin (0.1μM)	4.58±0.11	125±13	1.7±0.2

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282P INVERSE AGONISM OF THE NEUROLEPTIC DRUG (+)-BUTACLAMOL AT THE SHORT ISOFORM OF THE HUMAN D_2-DOPAMINE RECEPTOR HETEROLOGOUSLY EXPRESSED IN CHO CELLS

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Inverse agonism is a relatively poorly investigated phenomenon at G-protein-coupled receptors. This is probably largely due to a lack of experimental systems in which the receptors are present at a sufficiently high densities to cause a significant basal activation of the signal transduction systems for the inverse agonists to inhibit. However, there have been some reports on inverse agonism, although these have mostly studied adrenergic or opioid receptor subtypes in membrane preparations (e.g. Chidiac *et al.*, 1994; Tian *et al.*, 1994; Costa *et al.*, 1990). In at least one of these reports (Tian *et al.*, 1994), the observed inverse agonism was lost when the concentration of Na^+ in the assay buffer was increased to physiological levels. In a recent paper, however, Bond *et al.* (1995) have reported inverse agonist activity by a β_2-adrenoceptor ligand (ICI-118,551) *in vivo* in transgenic mice with myocardial over-expression of β_2-adrenoceptors, suggesting that inverse agonists (as opposed to neutral antagonists) may be of particular therapeutic importance in some pathological conditions. In this report we present data which suggest that the neuroleptic drug (+)-butaclamol behaves as an inverse agonist in Chinese hamster ovary (CHO) cells which heterologously express the short isoform of the human D_2-dopamine receptor ($D_{2S}R$).

Pre-incubation of cells for 40 min with (+)-butclamol resulted in a marked and concentration-dependent increase in the amount of cAMP that the cells produced in response to 10 μM forskolin (cells were pre-incubated for 40 min in the presence of 1 mM of the phosphodiesterase inhibitor isobutylmethylxanthine before addition of forskolin in all cases). The pD_2 value of (+)-butclamol was 8.25 ± 0.10 (sem, n=4) (EC_{50}=5.6 nM). The maximal stimulation above the forskolin control was 279.7 ± 42.2 % (sem, n=4). No such stimulation was observed in these cells with the inactive (-)-enantiomer of butaclamol at concentrations up to 10 μM. This strong stereoselectivity between butaclamol isomers is characteristic of D_2-like receptors. No such stimulatory effect of (+)-butclamol was seen in CHO cells which had not been transfected with the $D_{2S}R$ further suggesting that this effect is due to the presence of the transfected dopamine receptor rather than an effect of butaclamol at a non-dopaminergic site. Also, in the $D_{2S}R$ expressing cells, the effect of 1 μM (+)-butclamol was inhibited by incubation of the cells for 16 hours with 200 ng/ml pertussis toxin (PTX), an increase of 241.1 ± 69.1% (sem, n=4) above the forskolin control being reduced to -2.5 ± 1.4% (sem, n=3) in the PTX-treated cells showing that this effect is mediated by a G-protein of the Gi/Go family. The studies with PTX also confirmed that there is a large degree of basal inhibition of AC due to the presence of the $D_{2S}R$ in this cell line as in the PTX-treated cells the level of forskolin-stimulated cAMP accumulation was 2.7 ± 0.6 (sem, n=3) fold greater than that in untreated cells. In CHO cells lacking the $D_{2S}R$ PTX caused a slight inhibition of forskolin-stimulated cAMP accumulation the levels in the treated cells being 75.6 ± 4.4% (sem, n=3) of those in untreated cells. It would, therefore, appear that the potentiation of the effect of forskolin by (+)-butclamol in this cell line is true inverse agonism due to the binding of the (+)-butclamol to the $D_{2S}R$ and relieving the basal inhibition of adenylyl cyclase (AC) caused by it, rather than due to an interaction with AC or Gs or with a non-dopaminergic site which can stimulate AC via Gs.

Thus, we would like to report a functional assay system in which inverse agonism at D_2-dopamine receptors can be measured and that in this system the neuroleptic drug (+)-butclamol behaves as an inverse agonist.

The cells used in this study expressed the $D_{2S}R$ at 1.9 ± 0.4 pmole/mg protein (sem, n=3) when assayed using [^{3}H]-spiperone in membrane preparations.

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283P THE ROLE OF CONSERVED SERINE RESIDUES IN THE BINDING OF ANTAGONISTS TO THE D_2 DOPAMINE RECEPTOR

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Three serine residues are conserved in the fifth putative transmembrane spanning region of each of the dopamine receptors. Previously, using the cDNA for the long form of the rat D_2 receptor, these serines were mutated individually to alanines (Ala 193, Ala 194 and Ala 197) using site-directed mutagenesis and were shown to differentially affect the binding of selected antagonist drugs (Coley *et al.*, 1994; Woodward *et al.*, 1995a,b). The deductions based on these single serine mutations have been investigated further by constructing rat D_2 dopamine receptors in which two or three of these conserved serine residues have been mutated to alanines, expressing these mutant cDNA's in COS-7 cells and determining their properties using ligand binding assays.

In competition assays with [^{3}H]spiperone, there was no significant change in the affinities of a range of antagonist drugs for the mutant receptors compared with the native receptor (e. g. BRL 27320), indicating that these mutations had not altered the gross conformation of the receptor (Table 1). Drugs whose binding affinity was changed by these mutations (e. g. remoxipride, domperidone) were affected in different ways, suggesting that such drugs may bind in different modes to the receptor. However, in each case, the fold change in affinity observed with the multiple mutants was comparable to that observed with the single mutant receptors (Woodward *et al.*, 1995a,b) suggesting that these residues may act largely independently of each other. For example, all the mutants containing the Ala 193 mutation showed increases in affinity for certain substituted benzamides (e. g. remoxipride), while mutants in which the Ser 197 had been mutated to alanine, showed decreased affinity for certain compounds (e. g. domperidone), which may be consistent with the mutations changing hydrogen bonding interactions between these conserved serine residues and the ligands. From this data, it can be concluded that each of these conserved serine residues contributes in different ways to the binding of selected antagonist drugs to the D_2 dopamine receptor.

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Table 1. The binding of antagonists to native and mutant D_2 dopamine receptors. (Ki, nM, mean ±S.E.M., for three or more experiments).

	remoxipride	domperidone	BRL 27320
native	331.8±2.7	3.3±1.5	54.7±13.0
Ala 193/194	24.81±2.12	4.14±1.11	39.56±2.72
Ala 193/197	19.40±2.32	94.20±15.9	19.74±0.16
Ala 193/4/7	21.15±4.79	77.72±6.69	126±18.4

284P THE EFFECT OF AN ADENOSINE A_1 RECEPTOR AGONIST AND ANTAGONIST ON LONG-TERM DEPRESSION (LTD) IN THE RAT HIPPOCAMPUS

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A long-term reduction in synaptic strength (LTD) following low frequency stimulation (LFS) has been reported in the rat hippocampus (Dudek & Bear, 1992) and whereas it is known that long-term potentiation (LTP) is sensitive to adenosinergic agonists and antagonists it is not clear whether LTD is similarly sensitive. In this study we have examined the effect of the adenosine A_1 receptor agonist 2-Chloro-N^6-cyclopentyladenosine (CCPA) and the A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on LTD at Schaffer collateral/commissural synapses on CA1 hippocampal pyramidal neurones.

Hippocampal slices from Wistar rats were prepared conventionally and held submerged in the bathing solution at 31°C. The afferents were stimulated with a bipolar stainless steel electrode using stimulus intensities of 1.5-5V which produced responses 2/3 to 3/4 of maximum. Extracellular recordings of field excitatory post-synaptic potentials (fEPSPs) were made with glass microelectrodes (resistance 2-4 MΩ) filled with the extracellular fluid which had the following composition (mM): NaCl 126, KCl 2.75, $NaHCO_3$ 26, D-Glucose 10, $NaHPO_4$ 1.25, $MgSO_4$ 2, $CaCl_2$ 2.5. Test stimuli were applied every 30 s, LTD was induced by stimulating the afferents at 1 Hz for 10 minutes at the test intensity.

LFS reduced fEPSP slope by 21.5% ± 4.9 (s.e.m., n = 9) measured 30 minutes after the end of LFS in control. When slices were incubated for 2-4 hours in a solution which contained CCPA (20 nM) LTD was not significantly reduced (10.8% ± 3.2, n = 7; P > 0.1, Mann-Whitney U test). The magnitude of LTD in slices incubated for 2-4 hours in a solution containing DPCPX (30nM) was 9.1% ± 7.9 (n = 6) and although in 2 of 6 experiments LTD was blocked the mean reduction was not significantly different from control (P > 0.1).

These results suggest that adenosine A_1 receptors do not play a role in the induction of LTD or its maintenance for periods up to 30 minutes. Adenosine which reduces excitatory synaptic transmission and leaves GABAergic inhibition untouched appears only to modulate LTP with no significant action on long-term reductions in synaptic strength.

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285P ACCELERATED APOPTOSIS IN MESENCEPHALIC NEURONS: A ROLE IN PARKINSON'S DISEASE?

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Parkinson's Disease (PD) can be regarded as an abiotrophy (Lieberman, A., 1992), a premature, programmed cell death (apoptosis) of dopaminergic neurons in the substantia nigra leading to loss of dopaminergic control of the neostriatum. The premature death of neurons at the initial focal site of pathology must ultimately derive from biologic changes caused by disturbed genomic control of cell function or deleterious environmental factors or both. Current treatments are aimed at pharmacologically augmenting striatal dopamine (DA) levels but do not prevent continued neuronal degeneration. The present study investigates within cultured rat mesencephalic dopaminergic neurons, whether an alteration in ambient dopamine levels can induce neuronal apoptosis.

Primary cultures of mesencephalic cells were prepared from embryonic rats. Confirmation of dopaminergic neuron presence was achieved by immunoreactivity to the enzyme tyrosine hydroxylase. Cultured neurons were then exposed to varying concentrations of dopamine (0.1 to 100 μM) Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test. The mechanism of cell death was investigated by flow cytometry (Ormerod, M. G. *et al*, 1993). Apoptotic changes in neurons were confirmed by an *in situ* terminal deoxytransferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) technique which detects DNA strand breaks in cells undergoing apoptosis (Surh, C. D. , Sprent, J., 1994).

Exposure to dopamine led to a significant decline in mesencephalic neuronal cell viability in a dose responsive fashion with an observed LD_{50} of 28 μM (Figure 1). This decline was associated with an increase in the rate of neuronal apoptosis. The present study would suggest that dopamine induced activation of apoptosis may have a role in the premature death of neurons seen in PD.

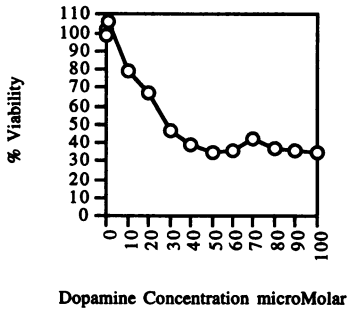

Fig. 1 : MTT test. Effect of dopamine on survival of mesencephalic neurons, measured by MTT conversion to formazan. All values are means (n = 4).

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286P CHARACTERISATION OF THE BINDING OF A NOVEL RADIOLIGAND TO CCK_B/GASTRIN RECEPTORS IN RAT CEREBRAL CORTICAL MEMBRANES

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Competition studies using JB93182 (5[[[(1S)-[[(3,5-Dicarboxyphenyl)amino]carbonyl]-2-phenylethyl]amino]-carbonyl]-6-[[(1-adamantylmethyl)amino]carbonyl]-indole) indicate that it is a high affinity CCK_B/gastrin-receptor antagonist with G_1-subtype selectivity (Harper *et al.*, 1996). Here we have characterised the binding of [^{3}H]-JB93182 to rat cortical membranes. Tissue was homogenised (Polytron PT10; setting 10 for 1min) in 10 vol (w/v) of ice-cold buffer (pH 7.4 at 21±3°C; composition, mM: 250 Sucrose, 5 EDTA, 0.1 PMSF, 25 imidazole) and centrifuged at 800 x g (12min at 4° C). Supernatants were pooled and stored at 4°C. Pellets were rehomogenised and recentrifuged (as above). This process was repeated, all supernatants were filtered through gauze, diluted to a final concentration of 50mM Tris HCl (pH 7.4 at 4°C) and centrifuged at 39,800 x g (20min at 4°C). The pellet was resuspended in 50mM Tris HCl buffer (pH 6.9 at 21±3°C containing 0.089mM bacitracin).

[^{3}H]-JB93182 (>97% purity by RP-HPLC) did not bind to GF/B filter discs (<0.5%) or bind specifically to heat-denatured tissue. There was no significant difference in the non-specific binding of [^{3}H]-JB93182 when defined with a 1μM concentration of the selective CCK_B/gastrin receptor antagonists, t-butyl-N-(8-quinolinyl)-N-(3-methylphenyl aminocarbonylmethylenecarbonyl)glycinate and YM022 (Nishida *et al.*, 1994), although it was higher ($p<0.005$) when defined with 1μM L-365,260 (n=6). The tissue concentration curve was linear between 1 and 30mg ml^{-1} (original wet weight). At a 20mg ml^{-1} tissue concentration, 12.03±1.14% (n=4±s.e.mean) of the radioligand was bound and specific binding was 46.4±1.8% (n=4). Under these assay conditions, [^{3}H]-JB93182 was stable for >3h. [^{3}H]-JB93182 specific binding was saturable and Scatchard plots and Hill plots were linear (pK_D=9.48±0.08, Bmax=3.61±0.65pmol g^{-1}; n_H=0.97± 0.02; n=5). [^{3}H]- JB93182 binding reached equilibrium after a 50min incubation (21±3°C) and remained constant for >3.5h. Association and dissociation rate constant values of 16.9± 4.5nM min^{-1} and 0.023±0.001 min^{-1}, respectively (n=10) were estimated by kinetic analysis. The corresponding pK_D estimate (9.73±0.11) was not significantly different from that determined by saturation analysis.

For competition studies tissue (400μl; 20mg ml^{-1} original wet weight), [^{3}H]-JB93182 (50μl; 3nM) and competing ligand (50 μl; 0.01nM - 300μM) were incubated (21°C) for 150 min.

Table 1 pIC_{50} and n_H values for competing ligands

Compound	pIC_{50}	n_H	n
L-365,260	7.63 ± 0.07	1.17 ± 0.13	8
PD-134,308	7.93 ± 0.07	0.99± 0.09	8
L-364,718	6.75 ± 0.13	1.20 ± 0.08	8
YM022	10.27 ± 0.20	0.79 ± 0.11	7

Competition curves for all ligands were not significantly different from rectangular hyperbolae (table 1). The low affinity of L-364,718 indicated that no CCK_A receptors were labelled. The low pIC_{50} values obtained for L-365,260 indicated that [^{3}H]-JB93182 predominantly labels the G_1-subtype, which we previously defined in this assay (Harper *et al.*, 1995). This is expected from the behaviour of JB93182 when [^{125}I]-BH-CCK8S was used to label CCK_B/gastrin receptors (Harper *et al.*, 1996). Overall, [^{3}H]-JB93182 appears to selectively label a homogeneous population of non-interacting CCK_B/gastrin-G_1 subtype receptors in rat cortical membranes.

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287P CHARACTERISATION OF THE BINDING OF A NOVEL CCK_B/GASTRIN RECEPTOR ANTAGONIST (JB93182) IN MOUSE AND RAT CEREBRAL CORTEX AND GUINEA-PIG PANCREAS

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Recently we presented evidence that L-365,260 can distinguish between two CCK_B/gastrin receptor subtypes (G_1/G_2) present in several peripheral and central nervous tissues (Harper *et al.*, 1995; Roberts *et al.*, 1995). Here we present evidence from radioligand binding assays that a novel antagonist, JB93182, (5-[[[(1S-[[(3,5-Dicarboxyphenyl)amino]carbonyl]-2-phenyl ethyl] amino]-carbonyl) 6-[[(1-adamantylmethyl)amino] carbonyl] -indole) is selective for the G_1-subtype. Guinea-pig pancreatic, mouse and rat cerebral cortex tissue were prepared as described previously (Harper *et al.*, 1995). In the mouse assays competition curves were obtained by incubating (21°C for 150min) competing ligands with either [^{125}I]-BH-CCK8S (20pM) or [^{3}H]-PD140,376 (0.2nM) and tissue concentrations of 2 and 8mg ml^{-1}, respectively. In the rat cortex and guinea-pig pancreas competing ligand and [^{125}I]-BH-CCK8S (20pM) were incubated (21°C for 150min) with 5 and 1mg ml^{-1} of tissue, respectively.

JB93182 (pK_I=5.29±0.12 at CCK_A-receptors in guinea-pig pancreas, n_H=1.55±0.21, n=5) competition curves were not significantly different from rectangular hyperbolae when [^{125}I]-BH-CCK8S or [^{3}H]-PD140,376 was used to label the receptors in mouse cortex (table 1).

The behaviour of several reference ligands in each assay was similar to those previously reported in these tissues (table 1; Lotti & Chang, 1989; Hughes *et al.*, 1990; Nishida *et al.*, 1994). In mouse cortex, no significant differences in pK_I values were obtained for the CCK_B/gastrin receptor selective ligands when [^{3}H]-PD140,376 or [^{125}I]-BH-CCK8S were used. However, the pK_I value for L-364,718 was significantly higher when [^{125}I]-BH-CCK8S was used. In the rat cortex n_H values for L-365,260 and PD134,308 were significantly less than unity. The rat cortex data could be simulated by assuming the presence of a mixed population of G_1 and G_2-subtypes. The application of a two-site model indicates that JB93182 is ~6-fold more potent at the G_1-subtype in contrast to L-365,260 which exhibits the reverse selectivity. In conclusion, JB93182 behaves as a G_1-subtype selective receptor antagonist.

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Table 1 pK_I and n_H parameters for JB93182 and reference compounds in mouse and rat cortex (±.s.e.mean)

	mouse cortex						rat cortex		
ligand	[^{3}H]-PD140,376			[^{125}I]-BH-CCK8S			[^{125}I]-BH-CCK8S		
	pK_I	n_H	n	pK_I	n_H		pIC_{50}	n_H	n
L-365,260	8.28 ± 0.08	1.00 ± 0.08	9	8.40 ± 0.01	0.96 ± 0.02	47	7.96 ± 0.02	0.75 ± 0.03*	50
JB93182	8.89 ± 0.10	0.85 ± 0.08	3	8.95 ± 0.24	0.99 ± 0.09	5	9.25 ± 0.18	0.81 ± 0.14	6
PD134,308	8.66 ± 0.18	1.14 ± 0.13	5	8.78 ± 0.08	0.87 ± 0.11	7	8.46 ± 0.08	0.75 ± 0.05*	5
PD140,376	9.28 ± 0.13	0.87 ± 0.09	6	9.23 ± 0.12	1.07 ± 0.05	6	8.75 ± 0.12	0.73 ± 0.11	3
L-364,718	7.26 ± 0.15	0.84 ± 0.11	5	7.78 ± 0.05	0.93 ± 0.04	17	6.77 ± 0.15	1.22 ± 0.17	5

288P DEXAMETHASONE MODULATION OF SECOND MESSENGER RESPONSES IN A LOCUS COERULEUS-LIKE CELL LINE

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Glucocorticoids and other components of the adrenal/pituitary axis, such as corticotrophin releasing factor (CRF), are involved in the CNS response to stress. A recent study has shown that CRF receptor mRNA is markedly reduced by corticosteroids (Iredale & Duman, 1995) in a noradrenergic cell line (CATH.a) derived from the locus coeruleus (Suri *et al.*, 1993). We have described cAMP responses in this cell line in a recent communication to the society (Bundey et al., 1995). We have therefore examined the effects of corticosteroid treatment on a number of second messenger responses in the CATH.a cell line.

Cyclic AMP responses were measured in intact cells using a [^{3}H]-adenine prelabelling assay (Johnson et al., 1994). Phosphoinositide hydrolysis was assessed by measuring the accumulation of [^{3}H]-inositol phosphates ([^{3}H]-IP) in subconfluent monolayers of CATH.a cells prelabelled with [^{3}H]-*myo*-inositol. Cells were incubated with agonists in the presence of lithium chloride (20mM) for 40 mins at 37°C. [^{3}H]-inositol phosphates were separated from other [^{3}H]-products using dowexCl$^-$ columns. The data are expressed as [^{3}H]-IP accumulation fold basal (means±s.e.mean).

The cholinergic agonist carbachol increased [^{3}H]-IP accumulation to a maximum of 3.01±0.79 fold basal in a concentration dependent manner (EC_{50}=7.94±0.14μM, n=3). Bradykinin produced a maximum 1.81±0.05 fold stimulation of basal [^{3}H]-IP accumulation (EC_{50}=9.12±0.16nM, n=3). The receptor agonists (1S,3R)-1-amino-1,3-cyclopentane dicarboxylic acid (300μM), 5-hydroxytryptamine (300μM), adenosine trisphosphate (1mM), L-glutamate (100μM), histamine (1mM) and noradrenaline (300μM) were unable to alter significantly [^{3}H]-IP accumulation compared with basal levels (p>0.05, n=3, Student's t test).

Incubation for 24 hours with 1μM of the glucocorticoid receptor agonist, dexamethasone, significantly potentiated the VIP (1μM) stimulated enhancement in cAMP production (28.7±8.8%; increase over control±s.e.mean) (p<0.05, n=3). However, the action of CRF (1μM) and forskolin (10μM) upon cAMP production was unaffected by dexamethasone treatment. The response to forskolin was reduced by the α_2-adrenoceptor agonist UK14304 (1μM) and this inhibition was not altered by the dexamethasone treatment. Incubation for 24 hours with 1μM dexamethasone caused a significant increase in the [^{3}H]-IP accumulation stimulated by carbachol (100μM) (46.0±6.9%, p<0.05, n=3) but not by bradykinin (1μM) (61.9±31.7%, p>0.05, n=3). Following dexamethasone treatment there was also a significant increase (67.8±6.7%, p<0.05, n=3) in [^{3}H]-IP accumulation in the presence of histamine (1mM).

The results presented demonstrate that glucocorticoid treatment can modulate the activity of two important signal transduction pathways in the CATH.a cell line. It appears however that the reductions previously observed in CRF receptor mRNA are not translated into functional changes under these conditions.

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