192P NITRIC OXIDE (NO) MODULATION OF VASOPRESSIN-INDUCED VASOCONSTRICTION IN THE ISOLATED RAT KIDNEY DEPENDS ON THE VISCOSITY OF THE PERFUSATE

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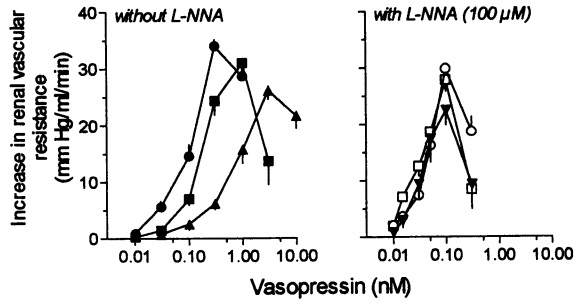
Previously we reported that vasopressin is a potent vasoconstrictor in the Tyrode's perfused rat kidney (Barthelmebs *et al.*, 1996). In vivo however vasopressin elicited only minor effects on renal blood flow (Hofbauer *et al.*, 1984). We hypothetized that differences in shear stress, in particular as related to differences in viscosity, could be involved. The aim of this study was to investigate the role of perfusate viscosity in the NO modulation of vasopressin-induced renal vasoconstriction.

Experiments were performed in kidneys isolated from male Sprague Dawley rats (250g). Kidneys were perfused at a constant flow (8 ml/min), using a recirculating system. Tyrode's solution was supplemented with 6% bovin serum albumin (BSA) or 4.7% Ficoll 400. The respective viscosities relative to water were 1.03 (Tyrode), 1.33 (BSA) and 2.32 (Ficoll 400). Concentration-response curves to vasopressin were constructed for separate kidneys, in the absence or presence of an inhibitor of NO synthesis, N°-nitro-L-arginine (L-NNA, 100 µM) and compared to those obtained with Tyrode's solution (open circuit perfusion). Results are presented as means ± s.e.m.

Vasopressin elicited a concentration-dependent renal vasoconstriction (Figure 1). With increasing viscosity, the curves were progressively shifted to the right with an increase in $logEC_{50}$ (-9.9 ± 0.1, -9.7 ± 0.1, -9.0 ± 0.1; Anova, $p<0.001$) and a decrease in E_{max} (34 ± 1, 31 ± 2, 26 ± 3, Anova, $p<0.001$).

Figure 1: Vasopressin-induced vasoconstriction in isolated kidneys perfused with solutions of different viscosities (● or ○ Tyrode, n = 14 and 11; ■ or □ BSA 6%, n = 5; ▲ or ▼ Ficoll 400 4.7%, n = 5 and 6).



L-NNA abolished the differences in $logEC_{50}$ but not in E_{max} (Anova, $p<0.001$) for vasopressin between kidneys perfused with solutions of different viscosities. L-NNA increased basal renal vascular resistance differently according to the viscosity of the perfusate (15 ± 4%, 35 ± 9%, 49 ± 10%; Anova, $p<0.001$).

Conclusions: 1) the modulation of vasopressin-induced renal vasoconstriction is more pronounced with the perfusate (Ficoll 400 4.7%) having a viscosity comparable to plasma *2)* the most widely used solution for kidney perfusion (6% BSA) has a low viscosity; 3) NO is involved in the modulation elicited by viscosity. Viscosity, a major determinant of shear stress, needs to be considered for studies using isolated kidneys.

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193P EFFECT OF SUPEROXIDE ANION GENERATION ON VASODILATOR AND VASOCONSTRICTOR RESPONSES IN PERINATAL RABBIT PULMONARY RESISTANCE ARTERIES

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We have previously shown that superoxide anion ($O_2^{\cdot-}$) accumulation may compromise the activity of nitric oxide (NO) in newborn rabbit pulmonary conduit arteries (Morecroft & MacLean, 1996). $O_2^{\cdot-}$ have been shown to produce pulmonary vasoconstriction in young pigs (Sanderud *et al.*, 1991). Here, we studied the effect of $O_2^{\cdot-}$ on ACh-induced vasorelaxation and 5-HT-induced vasoconstriction in pulmonary resistance arteries (PRAs) from foetal and neonatal NZW rabbits. The rabbits were killed with sodium pentobarbitone (200mg kg^{-1}). The PRAs (~250µm internal diameter) were dissected out and set up on a wire myograph under tension (in Krebs at 37°C). They were bubbled with 16% O_2/6%CO_2 balance N_2 (3% O_2/6%CO_2 balance N_2 for foetal tissue). The PRAs were pre-constricted with endothelin-1 (ET-1) (1.0-10.0nM) and subsequently, responses to ACh (1.0nM-10µM) were obtained in the presence/absence of the $O_2^{\cdot-}$ generating system hypoxanthine/xanthine oxidase (HX/XO) or pre-incubation (45 minutes) with the superoxide dismutase (SOD) inhibitor triethylenetetramine (TETA). Results are shown in Table 1. The results show that ACh-induced relaxation in perinatal rabbit PRAs was significantly attenuated both by the generation of $O_2^{\cdot-}$ and by inhibition of endogenous SOD. The effect of HX/XO was also obtained in 5-HT (0.1-1µM) contracted PRAs. Data are expressed as mean ± s.e. mean of >6 paired observations. In foetal PRAs, $O_2^{\cdot-}$ generation significantly reduced 5-HT induced vasoconstriction from 46.7 ± 7.0% (% 50mM KCl) to 11.3 ± 4.0% whereas in 4 day PRAs, 5-HT-induced vasoconstriction was significantly increased from 10.3 ± 2.6% (% 50mM KCl) to 79.3 ± 8.4% suggesting inactivation of basal NO by $O_2^{\cdot-}$. Together, these observations suggest that the activity of NO in perinatal PRAs could be profoundly affected by endogenous SOD activity and by the interaction between $O_2^{\cdot-}$ and NO. This interaction inactivates basal NO in newborn PRAs whilst in foetal PRAs, the interaction between basal NO and $O_2^{\cdot-}$ may result in the generation of a vasodilator.

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Sanderud, J., Norstein, J., Sagstad, O.D. (1991). *Ped. Res.*, 29, 543-547.

Table 1. Effect of superoxide anion generation and endogenous SOD inhibition on ACh induced relaxation in ET-1 pre-contracted 2 day preterm foetal and 4 day old rabbit PRAs (n/n≥5/4).

Treatment		Foetus		4 day	
		pEC50	Emax	pEC50	Emax
ACh	-control	7.1 ± 0.1	92.4 ± 3.6	7.4 ± 0.1	85.7 ± 2.4
	+ HX(0.1mM)/XO(15mu ml^{-1})	5.7 ± 0.1**	74.4 ± 3.2*	6.5 ± 0.2**	64.8 ± 3.8**
	+ TETA (1mM)	6.6 ± 0.2**	71.4 ± 3.4*	6.5 ± 0.1**	63.3 ± 5.0*
	+ 150 u ml^{-1}SOD + HX/XO	7.0 ± 0.1	88.6 ± 4.0	7.1 ± 0.2	80.2 ± 5.8

n/n = number of vessels/number of animals. Emax: maximum relaxation expressed as % of ET-1 pre-contraction. Statistical comparisons using unpaired Student's t test: compared with appropriate control *P<0.01 **P<0.001.

194P *IN VITRO* ASSESSMENT OF SUPEROXIDE DISMUTASE MIMETICS

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Interest is growing in the potential use of low molecular weight superoxide dismutase (SOD) mimetic compounds as therapeutic agents. A number of assays of SOD and SOD mimetic activity have been developed to screen for lead compounds and these generally involve inhibition of a colourimetric reaction catalysed by superoxide anion. For example, assays have been based on inhibition of the ability of superoxide anion to catalyse the oxidation of pyrogallol (Marklund & Marklund, 1974), reduction of ferricytochrome c (cyt c; Laight *et al.*, 1997) and reduction of nitro blue tetrazolium (NBT; Ewing & Janero, 1995). We wished to compare the utility of each of these assays in determining the activities of a number of putative SOD mimetics.

The oxidation of pyrogallol (0.2 mM) was assessed at 420 nm following a 30 min incubation in 50 mM Tris-HCl buffer (pH 7.7) containing diethylenetriaminepentaacetic acid (DETPA, 1 mM). The reduction of cyt c (30 μM) was assessed at 550 nm following a 30 min incubation in 50 mM Tris-HCl (pH 7.7) containing hypoxanthine (0.1 mM)/xanthine oxidase (3 mU ml^{-1}) and catalase (100 U ml^{-1}). The reduction of NBT (50 μM) was assessed at 550 nm following a 5 min incubation in 50 mM phosphate buffer (pH 7.4) containing NADH (78 μM), phenazine methosulphate (3.3 μM) and EDTA (0.1 mM). All assays were conducted at room temperature in 96 well plates and monitored using a plate reader. Assays were conducted on authentic SOD and the SOD mimetics 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron), Cu(II)-(diisopropylsalicylate)$_2$ (CuDIPS), 4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), 4-phenyl-2,2,5,5-tetramethyl-3-imidazolin-1-yloxy-3-oxide (PTIYO). Log IC_{50} values for each agent were calculated using analytical software (Graph Pad Prism).

The results (Table 1) show that authentic SOD was active in each of the assays. In the pyrogallol assay only tiron exhibited SOD-like activity and this was weak. Tempol and PTIYO enhanced the oxidation of pyrogallol and could not be used. The NBT assay was more sensitive than the cyt c assay, but the rank-order of potency was similar in each case (tempol = tiron > PTIYO). CuDIPS was the most potent agent, but was active only in the cyt c assay, probably because of removal of Cu by the chelators, DETPA and EDTA, which are vital components in the pyrogallol and NBT assays, respectively. Consequently, it would seem prudent to screen for SOD mimetics using a variety of *in vitro* assays.

Ewing, J.F. & Janero, D.R. (1995) *Anal. Biochem.* 232, 243-248.
Laight, D.W. *et al.* (1997) *Environmental Toxicol. Pharmacol.* 3, 65-68.
Marklund, S. & Marklund, G. (1974) *Eur. J. Biochem.* 47, 469-474.

Table 1. Log IC_{50} concentrations (mean ± s.e. mean, n=6-8) of SOD mimetics in the pyrogallol, cyt c and NBT assays.

	SOD (U ml^{-1})	Tiron (M)	CuDIPS (M)	Tempol (M)	PTIYO (M)
Pyrogallol assay	0.40 ± 0.05	-2.25 ± 0.21	---	---	---
Cyt c assay	0.49 ± 0.12	-3.19 ± 0.18	-6.27 ± 0.28	-3.36 ± 0.11	-2.59 ± 0.30
NBT assay	0.07 ± 0.04	-4.28 ± 0.19	---	-4.27 ± 0.03	-3.88 ± 0.05

195P ASSESSMENT OF SUPEROXIDE DISMUTASE MIMETIC COMPOUNDS IN THE RABBIT AORTA

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Nitric oxide (NO) is rapidly inactivated by superoxide anion (O_2^-). We have previously demonstrated in the rabbit aorta that endothelium-derived NO is protected against destruction from O_2^- by endogenous Cu-Zn superoxide dismutase (SOD; MacKenzie & Martin, 1997). The aim of this study was to determine if, following inhibition of endogenous Cu-Zn SOD and exogenous generation of O_2^-, endothelium-dependent relaxation could be restored by exogenously applied SOD and by putative SOD mimetic compounds.

Rabbits were killed with an i.v. injection of sodium pentobarbitone (200 mg Kg^{-1}). The thoracic aorta was removed, cleaned of fat and connective tissue, and cut into rings (2.5 mm wide). Aortic rings were then suspended in tissue baths containing oxygenated Krebs solution at 37°C and contracted with phenylephrine (30 nM - 0.3 μM). Relaxation was induced with acetylcholine (ACh; 10 nM - 3 μM). Endogenous Cu-Zn SOD was inactivated by incubating aortic rings for 60 min with diethyldithiocarbamate (DETCA; 0.3 mM). Hypoxanthine (HX; 0.1 mM)/xanthine oxidase (XO; 4.8 mU ml^{-1}) was used to generate O_2^-. The SOD mimetic compounds investigated were 4-hydoxy 2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), 4-phenyl-2,2,5,5-tetramethyl-3-imidazolin-1-yloxy-3-oxide (PTIYO; Ewing & Janero, 1995) and Cu(II)-(diisopropylsalicylate)2 (CuDIPS; Burdon *et al.*, 1995). Data are expressed as mean ± s.e. mean of ≥ 5 observations and differences determined by ANOVA followed by Bonferroni's post-test.

The relaxation induced by the maximal concentration of ACh (3 μM) was reduced from 93.8 ± 1.6 % to 55.3 ± 2.7 % ($P<0.001$) following exposure to HX/XO in DETCA-treated tissues. This blockade was completely prevented following treatment with exogenous SOD (250 U ml^{-1}; maximum ACh-induced relaxation was 92.1 ± 1.3 %). The blockade was unaffected by pretreatment with PTIYO at 10 and 100 μM (maximum ACh-induced relaxation was 60.3 ± 2.7 and 50.6 ± 2.9 %, respectively). Tempol was ineffective at 1 mM but partially prevented ($P<0.05$) the blockade at a concentration of 0.1 mM (maximum ACh-induced relaxation was 64.4 ± 5.7 and 69.4 ± 4.6 %, respectively). The blockade was unaffected following treatment with CuDIPS at 0.1 μM but prevented ($P<0.001$) at a concentration of 1 μM (maximum ACh-induced relaxation was 66.5 ± 7.4 and 84.3 ± 2.9 %, respectively).

In conclusion, exogenous SOD fully restored ACh-induced relaxation following inactivation of endogenous SOD and generation of O_2^-. The stable nitroxides, PTIYO and tempol, showed poor SOD-like activity, probably because they bind and inactivate NO as well as O_2^- (Akaike *et al.*, 1993). In contrast, the metal centred compound, CuDIPS, did exhibit powerful SOD-like activity and may provide a lead in the development of SOD mimetics as therapeutic agents.

Akaike, T., Yoshida, M., Miyamoto, Y. *et al.* (1993) *Biochemistry* 32, 827-832.
Burdon, R.H., Allianagana, D. & Gill, V. (1995) *Free Rad. Res.* 23, 471-486.
Ewing, J.F. & Janero, D.R. (1995) *Anal. Biochem.* 232, 243-248.
MacKenzie, A. & Martin, W. (1997) *Br. J. Pharmacol.* 120, 159P.

196P EVIDENCE OF OXIDATIVE STRESS-INDUCED ENDOTHELIAL DYSFUNCTION IN RATS WITH CHRONIC HEART FAILURE

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We have shown previously that chronic heart failure (CHF) impairs the flow-dependent, NO mediated dilatation of small muscular arteries in rats, and that this impairment is partly prevented by chronic exercise (Varin et al, 1997). The present study was designed to assess whether oxygen-derived free radicals, which inactivate NO, contribute to this impairment.

Heart failure was induced by left coronary artery ligation in methohexital-anaesthetised rats. Exercise consisted of 2x30 min swimming per day. After 2 months, rats were anaesthetised with pentobarbital, and small segments of gracilis muscle arteries were dissected out and placed in an arteriograph. Arterial segments were pressurized (30 mm Hg) and preconstricted using phenylephrine 10^{-6}-10^{-5}M (preconstricted internal diameter 100±5mm). Responses to stepwise increases in intraluminal flow were assessed using a video camera connected to an image analyser. The role of free radicals was assessed using the free radical scavenger N-2-mercaptopropionyl glycine (MPG 10^{-5}M).

In arteries isolated from sham rats, flow induced gradual dilatation (figure 1). CHF abolished flow-dependent vasodilatation and converted it to a vasoconstriction, especially at low values of flow. Chronic exercise partially restored the impaired flow-dependent dilatation.

MPG did not affect the responses obtained in sham rats. In CHF rats, the flow-induced vasoconstriction was abolished by MPG, although MPG did not restore an active vasodilatation. MPG also significantly improved the flow-dependent dilatation in arteries taken from exercised CHF rats.

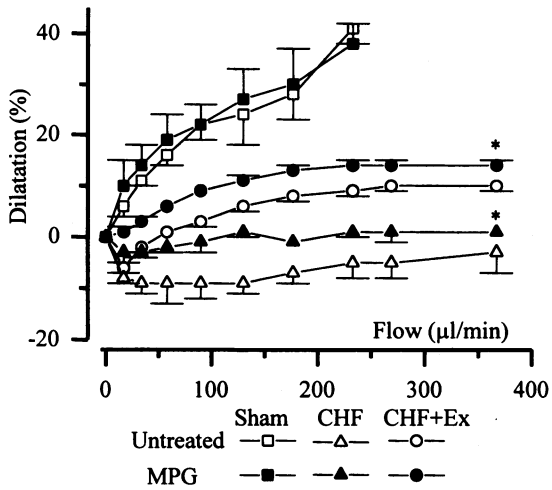

Figure 1. Flow-dependent responses in arteries from sham rats or rats with CHF, either sedentary or exercised (n= 6 in each group), in the absence or the presence of MPG. *: $p<0.05$ vs corresponding basal value (ANOVA).

Thus, these results demonstrate that oxygen-derived free radicals contribute to the impaired flow-dependent vasodilatation of peripheral muscular arteries in CHF. However, the exercise-induced improvement of this endothelium-mediated response does not appear to involve a reduced oxidative stress.

Varin, R., et al., (1997). *Fund. Clin. Pharmacol.*, **11**, 198.

197P PRIOR HEAT STRESS INCREASES ENDOTHELIUM-DEPENDENT RELAXATIONS OF RAT ISOLATED ARTERIES

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Heat Stress (HS) has been used to stimulate the expression of stress proteins (including heat shock proteins), and is associated with various cardiovascular changes, such as delayed myocardial protection against ischemic injury (Richard *et al.*, 1996). However, the effect of HS on endothelial cells is unknown. Thus, the present study was designed to assess whether HS affects endothelium-dependent relaxations in rat arteries (aorta, mesenteric and coronary arteries).

HS was induced in methohexital-anaesthetised rats (n=8 per group) by increasing body temperature to 42°C for 15 min. Twenty four hours later, segments of rat aorta were isolated and mounted in organ chambers, while segments of mesenteric and coronary arteries (normalised internal diameter 250-300 µm) were mounted in small vessel myographs. Relaxing responses were studied after precontraction by phenylephrine (aorta and mesenteric arteries) or serotonin (coronary arteries).

In all 3 vessels studied, HS markedly increased the endothelium-dependent relaxations to acetylcholine (figure 1), without affecting the responses to the NO donor SIN-1.

In the aorta, the NO synthase inhibitor N^G-nitro L-arginine (L-NA) abolished the response to acetylcholine both in control and HS rings (maximal responses: sham + L-NA 4±2%; HS + L-NA 6±2%), whereas the inhibitor of cyclooxygenase diclofenac did not affect the response to acetylcholine in control or HS rings. HS also increased the NO-dependent relaxing responses of isolated aorta to histamine (responses to 10^{-5}M histamine: sham: 24±3; HS 60±9, n=8, $p<0.01$) or to the calcium ionophore A23187 (sham: 57±8; HS 76±7%, n=8, $p<0.05$).

In the aorta, HS did not affect the contractions to phenylephrine, either in the absence or presence of endothelium, or in the presence of the NO synthase inhibitor nitro L-arginine (L-NA), suggesting that it did not affect the basal release of NO.

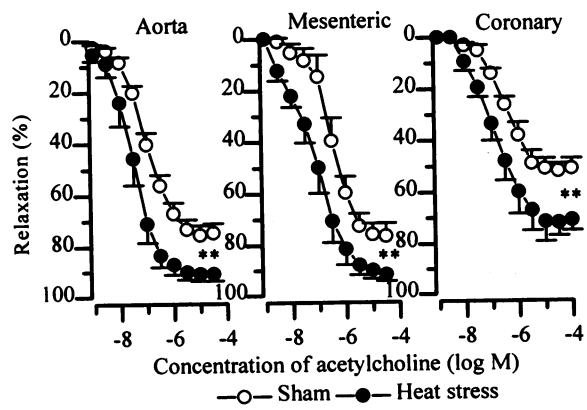

Figure 1. Relaxing responses to acetylcholine in arteries from sham or heat – stressed rats (n=8). **:$p<0.01$ (ANOVA)

Thus, HS increases the stimulated release of NO in different rat artery preparations. This may contribute to some of the previously described effects of HS, such as the anti-ischemic properties.

Richard, V., Kaeffer, N. & Thuillez, C. (1996) *Fundam. Clin. Pharmacol.*, 10, 409-415

198P DIFFERENCES IN AORTA ENDOTHELIUM-DEPENDENT FUNCTION IN SHR AND LH HYPERTENSIVE RATS

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Aim: This work was done to analyse the aorta vasoactive properties in two hypertensive rat strains in relation with their smooth muscle and endothelium functions and structure.

Material and methods:

12 week-old male spontaneously hypertensive (SHR) and Lyon hypertensive (LH) rats (n=5-23) were anaesthetised by injection of sodium pentobarbital (50mg/Kg; ip). Thoracic aortas were rapidly excised and placed in a Krebs-Ringer solution of the following composition (mM): NaCl: 115.0; KCl: 4.6; $CaCl_2$, $2H_2O$: 2.5; $MgSO_4$: 1.2; KH_2PO_4: 1.2; Glucose: 11.1 and $NaHCO_3$: 25.0, (pH 7.2-7.4; 4°C). Blood vessels were carefully dissected under a microscope, cut into rings of 3-4 mm length. In some vessels, the endothelium was removed by rubbing the intimal surface. Then, rings were mounted on two stainless wires in a 20 ml organ bath and isometric tension was recorded. Bath solution was aerated by a gas mixture 95%O_2-5% CO_2 at 37±1°C. α-adrenergic response was evaluated in presence of 10^{-7}M Noradrenaline (NA). Functional endothelium, was studied in presence of 10^{-5}M carbamylcholine (CARB) on aorta precontracted with 10^{-7}M NA. NO dependent vasodilation in both NA stimulated and non stimulated preparations was evaluated in presence of Nω-Nitro-L-arginine (LNNA) at 10^{-4}M (10 min).Medial cross sectional area (MCSA), media thickness (MT), total collagen and elastin contents were evaluated by histomorphometry. Values are expressed as mean ± sem, relaxation values were calculated as percentage of the preceding contraction. One or two factors ANOVA were used for statistical analysis. Differences were considered significant when p<.05; (*p<.05; **p<.01; ***p<.005).

Results:

Our results show, that a) SHR and LH aortas are characterised by distinct biological parameters such as weight, mean arterial pressure (MAP) and collagen content (table 1), whereas no difference can be seen in MCSA, MT as for the elastin content (40.34±1.82 and 38.99±2.74% respectively)

Table 1: Biological and histomorphometric characteristics:

	Weight (g)	MAP (mmHg)	MCSA (mm^2)	MT (mm)	Collagen (%)
SHR	311±1	161±4	0.636±0.038	0.105±0.002	15.04±0.41
LH	374±8 ***	146±5 **	0.689±0.035 ns	0.105±0.003 ns	11.33±0.59***

b) SHR and LH aorta functions, in the absence of endothelium (E-), are similar in regard to their developed tension in presence of NA (table 2), c) in the presence of endothelium (E+) SHR and LH aortas display a much lower developed tension than that found in the absence of endothelium (p<.005). Furthermore, under the same conditions, LH aorta presents a significant lower tension than SHR aorta (table 2).

Table 2: Endothelium dependent and independent developed tension to NA:

	NA (E-) (mg)	NA (E+) (mg)	NA+LNNA (E+) (mg)	CARB (%)
SHR	2869±300	1774±249	3296±361	71±9
LH	2947±422 ns	1124±149 **	2931±406 ns	93±1 ***

d) addition of LNNA on resting tension (Fig. 1) induced a larger NO dependent developed tension in SHR than in LH aorta (p<.005). In opposition, maximal developed tension in presence of NA is not significantly different in SHR and LH aorta when vessels are incubated with LNNA (table 2),

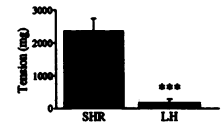

Figure 1:Developed tension in presence of LNNA.

and e) the amplitude of the endothelium dependent relaxation to CARB is smaller in SHR than in LH aortas (table 2) with an EC_{50} value of 140±31nM and 50±9 nM respectively (p<.05).

Conclusion:

Taken together our results show that the differences observed between the two hypertensive strains are associated to the endothelium dependent NO pathway, since they are abolished both in the presence of LNNA and in the absence of endothelium. Furthermore, they show that endothelium dependent NO production is differently regulated in SHR than in LH aortas. The endothelium characteristics observed for the two hypertensive strains reinforce the observed differences in MAP and total collagen.

199P VASCULAR RESPONSE OF PORCINE ARTERIAL VASA VASORUM TO VASODILATOR PEPTIDES

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The walls of certain large blood vessels are nourished by the vasa vasorum, a network of microvessels which penetrate the adventitia and media of the vessel wall. Recently we have demonstrated that isolated vasa vasorum of the bovine and porcine aorta react to vasoactive agents (Ahluwalia & Vallance, 1996). We have now investigated the mechanisms of relaxation in isolated vasa vasorum of porcine aorta.

Porcine aortas were collected from an abattoir and arterial vasa vasorum isolated from the adventitial-medial border of the thoracic aorta and cleaned of extraneous tissue. 1-2 mm lengths were cut and mounted in a myograph for measurement of isometric tension bathed in Krebs solution at 37°C gassed with 5 % CO_2 in O_2. Following equilibration the internal diameter was determined (Mulvany & Halpern,1977). The diameter of the vessels studied was 179 ± 6 μm (n=85). Vessels were repeatedly contracted with 125mM KCl until the contraction was constant and then precontracted with a submaximal concentration of endothelin-1 (ET-1, 3-30 nM, approximate EC_{70}). Relaxation concentration-response curves to bradykinin (BK, 0.01-300 nM) and substance P (SP, 0.1-100 nM) were constructed. The results were expressed as a percentage reversal of the ET-1 contractile response.

To further characterise the responses, concentration-response curves to BK and SP were constructed in the absence or presence of a nitric oxide synthase inhibitor L-NAME (300 μM, 30 min.), the NO scavenger oxy-haemoglobin (oxyHb, 20μM, 15 min.), soluble guanylyl cyclase inhibitor ODQ (1 μM, 30 min.) or the cyclooxygenase inhibitor indomethacin(5μM, 30 min.). To determine the relative importance of hyperpolarisation in mediation of endothelium-dependent relaxation vessels were precontracted with 40 mM KCl followed by application of the dilator.

SP and BK caused concentration-dependent relaxations (EC_{50} = 3.5± 0.7 nM, n=17) (EC_{50} = 3.8 ± 0.5 nM, n=16) respectively. The SP response was significantly inhibited by L-NAME (n=6, p<0.05) but not indomethacin (n=9, p<0.05). The response to BK was abolished by endothelial denudation (n=3) but was not significantly shifted to the right by indomethacin (n=5, p<0.05), L-NAME (n=5, p<0.05), ODQ(n=8, p<0.05) or oxyHb (n=5, p<0.05). The BK response was significantly attenuated in the presence of 40mM KCl (n=5, p<0.05).

These results show that porcine arterial vasa vasorum are sensitive to vasodilators. Moreover, it seems that the response to SP is mediated by nitric oxide whereas the response to BK appears to be predominantly mediated by an endothelium-dependent hyperpolarisation of vascular smooth muscle.

Ahluwalia,A. & Vallance,P.J.(1996).*Circulation (Supp)* **94** I-588.

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

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200P PROCYANIDIN OLIGOMERS FROM GRAPE SEED INDUCE NO-DEPENDENT RELAXATION OF RAT AORTIC RINGS VIA $P2Y_1$ RECEPTOR ACTIVATION

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It has been reported that extracts from wine, grape juice and grape skin (Fitzpatrick *et al.*, 1993) and polyphenol compounds from red wine (Andriambeloson *et al*, 1997) could induce a NO-dependent relaxation in rat isolated aorta. The aim of the present study was two fold : first to investigate if such a relaxant effect could also be evidenced with procyanidin oligomers (PCO) extracted from grape seeds, second to try to determine the mechanisms of action of these extracts in *in vitro* relaxation of rat aortic rings, since it has been reported that neither muscarinic, β-adrenergic, histamine nor 5-HT receptor activation was involved in this effect (Fitzpatrick *et al.*, 1995). For this purpose, male Wistar rats (250-300 g) were anaesthetized with sodium pentobarbitone (50 mg/kg) and bled. Aortae were removed, cleared from connective tissue and 3-4 mm rings were mounted in organ bath. Preparations were either intact (+E) or endothelium-denuded (by rubbing, -E). They were bathed in a physiological salt solution (mM) : NaCl 119, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO4 1.2, $CaCl_2$ 2.5, glucose 5.5, bubbled with 95% O_2 / 5% CO_2 mixture and maintained at 37°C. Rings were initially contracted with an hyperpotassic solution (K-PSS, 60 mM) and then challenged with PCO (10^{-7} g/l to 10^{-1} g/l) added in the bath.These polyphenolic compounds were prepared by classical methods (mixture of water and acetone as solvent on non ground seeds).

In +E rings, PCO induced relaxations which were concentration-dependent (maximal effect : 41 ± 6%, expressed as percent of the initial contraction in K-PSS, EC_{50} = $1.3.10^{-3}$ g/l, n=21). These relaxations could be fully reversed by L-NAME ($1.5.10^{-5}$ M, 94,2 ± 2,4%, n=5).A slight relaxation was observed in the preparations without endothelium (11,6 ± 1%, n=8).

To determine the mechanism of action of PCO, reactive blue 2 (RB2, 10^{-6} and 3.10^{-5} M, an antagonist of the $P2Y_1$ receptors, Burnstock & Warland, 1987) and apyrase (0.8 U/ml, Grade V, Sigma, which selectively hydrolyses ATP) were used. The vasorelaxant effects of PCO (3.10^{-3} g/l) were tested after incubation with RB2 or apyrase. Results are displayed in Table I.

	PCO control	+ RB2 (10^{-6}M)	+ RB2 3.10^{-5}M)
% relaxation	23 ± 4 % n=10	11,5 ± 6,1% n=6	8 ± 2 % * n=10
	PCO control	+ apyrase (0.4 UI/ml)	+ apyrase (0.8 UI/ml)
% relaxation	22,1 ± 2,3% n=19	11,5 ± 3% n=4	5,6 ± 1,1%*** n=19

Values (mean ± SEM) different from control : * : $p < 0,05$
*** : $p < 0.001$ vs control (Student's t test for paired groups)

Table1 : Maximal relaxation (expressed as percent of initial constriction) of aortic rings induced by PCO (3.10^{-5} g/l) in control conditions and after incubation with RB2 or apyrase

These results show that grape seed extract contains a mixture of substances - procyanidin oligomers- which can induce an endothelium-dependent arterial relaxation through the NO pathway. The concentration-dependent inhibitory effects of RB2 and apyrase strongly suggests that this endothelium-dependent relaxation is mediated by a release of ATP which activates a $P2Y_1$ purinoceptor and subsequently leads to a NO release.

Andriambeloson E. *et al.* (1997) *Br. J. Pharmacol.*,120:1053-8.
Fitzpatrick D.F. *et al.* (1993) *Am. J. Physiol.*, 265:H774-8.
Fitzpatrick D.F.*et al.* (1995) *J. Cardiovasc. Pharmacol.*,26:90-5.
Burnstock & Warland, (1987) *Br. J. Pharmacol.*, 90:383-391.

201P ERGONOVINE AND THE CORONARY VASCULAR BED IN CONSCIOUS DOGS: ROLE OF VASCULAR ENDOTHELIUM, NITRIC OXIDE AND 5-HT RECEPTORS

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The ergonovine (Erg) test remains one of the most currently used for the diagnosis of coronary artery spasms in patients with resting angina pectoris. To date, it is well known that Erg mainly acts through activation of 5-HT1 subtype receptors located on coronary vascular smooth muscle but the role of the vascular endothelium in the modulation of this response in vivo remains presently unclear.

To answer this question, 15 dogs were instrumented under general anaesthesia and artificial ventilation to measure aortic pressure (AP), left circumflex coronary artery diameter (CD, ultrasonic crystals) and blood flow (CBF, doppler flow probe). All experiments were conducted in the conscious state. Data are expressed as mean values ± s.e.mean.

In 7 dogs with an intact endothelium, Erg administered at increasing doses (30 to 1000 µg, iv bolus) did not alter cardiac and systemic haemodynamics up to 100 µg but increased heart rate and AP at higher doses, e.g., +7±2 beats/min from 88±5 beats/min ($p<0.05$) and +6±1 mmHg from 98±4 mmHg ($p<0.01$) at 1000 µg. Erg also induced a biphasic response on epicardial coronary artery with an early and transient increase in CD (up to +2.9±0.5% from 3.31±0.16 mm, $p<0.01$) followed by a sustained decrease (up to -4.9±0.5%, $p<0.001$) which occurred simultaneously with a progressive and sustained increase in CBF (up to +100±26% from 28±4 ml/min, $p<0.001$). Using a modified angioplasty technique (Berdeaux *et al*, 1994), the endothelium of epicardial coronary artery was then removed at the level of crystal attachment. Three days after this procedure, the adequacy of endothelium removal (E-) was verified by administrations of acetylcholine (Ach, 0.3 µg/kg, iv bolus) and nitroglycerin (Nitro, 1 µg/kg, iv bolus). Ach-induced increase in CD was severely depressed after E- (+0.8±0.2% after vs +4.6±0.7% before E-) whereas Nitro-induced increase in CD was not altered (+5.2±1.2% after vs +6.9±1.0% before E-). By that time, Erg-induced early increase in CD before E- was abolished whereas the delayed and sustained epicardial vasoconstriction was potentiated (CD: -6.4±0.9% after vs -4.9±0.5% before E-, $p<0.01$).

Erg (300 µg, iv bolus) was administered to 4 other dogs before and after sustained blockade of nitric oxide (NO) synthesis by N^{ω}-nitro-L-arginine (L-NNA, 30 mg/kg/day/7 days, iv infusion over 30 min). The early increase in CD induced by Erg was abolished by L-NNA but the delayed vasoconstrictor component of the response as well as the increase in CBF remained unchanged.

Finally, Erg (300 µg, iv bolus) was administered to 4 other dogs before and after 5-HT1 receptor blockade by methysergide (M, 200 mg/kg, iv infusion over 10 min). M, per se, did not alter systemic haemodynamics but decreased CD (-3.8±1.4% from 2.80±0.10 mm, $p<0.05$) and increased CBF (+32±6% from 34±8 ml/min, $p<0.05$). Whereas M exhibited almost no effect on Erg-induced early epicardial vasodilation (+2.5±0.6% after vs +3.3±0.3% before M, NS), Erg-induced delayed decrease in CD and increase in CBF were reduced after M (CD : -2.0±0.6% after vs -6.0±0.7% before M, CBF: +16±2% after vs +38±6% before M, all $p<0.01$).

We conclude that in conscious dogs, Erg induces a biphasic effect on large epicardial coronary arteries: an early but transient endothelium- and NO-dependent coronary vasodilation followed by a sustained vasoconstriction mainly mediated through the activation of 5-HT1 receptors. The latter effect of Erg is increased after endothelium removal but not affected by NO blockade. Finally, Erg also dilates small coronary arteries, an effect which is partly mediated through the activation of 5-HT1 receptors but is independent of NO release.

Berdeaux, A. *et al.* (1994). Circulation 89, 2799-2808.

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During sepsis, bacterial products can induce the inducible nitric oxide synthase isoform (NOS-2) activity in arteries from animals (Stoclet et al., 1993) and from patients (Tsuneyoshi et al., 1996). This may result in generalized production of large amounts of vasodilating nitric oxide (NO), but the role of NO in « unrelenting » hypotension refractory to catecholamines that occurs in septic shock has not yet been established. NOS-2 expression and activity and reactivity to noradrenaline (NA) were investigated here in resistance arteries from septic and non septic patients.

Small omental arteries (internal diameter: 200-400 µm) were harvested from patients undergoing abdominal surgery for cancer (large bowel resection for cancer) (36-61 years, non septic) or peritonitis (caused by intestinal perforation, and associated to systemic inflammatory response syndrome with high cardiac output and low peripheral resistance haemodynamic profile) (28-71 years, septic). The presence of NOS-2 was detected using the anti-murine macrophage NOS-2 antibody and was visualised by confocal microscopy. NO assay was performed using electron paramagnetic resonance (e.p.r.) spectroscopy with Fe^{2+}-diethyldithiocarbamate (Fe-DETC) as spin trap. The arteries were incubated with Fe-DETC for 60 min, in the presence of L-arginine (300 µM). The accumulation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) was determined by radioimmunoassay in tissues incubated with isobutylmethylxantine for 30 min, in the presence or in the absence of L-arginine (300 µM). Cyclic GMP content is expressed as fmol μg^{-1} DNA. For contraction experiments, segments of small omental arteries were mounted on a myograph filled with physiological salt solution 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 continuously kept at 37°C and gassed with a mixture of 95% O_2, 5% CO_2 (pH 7.4). All results are given as mean ± s.e.mean. The sensitivity to agonists is expressed as the pD_2 value, where pD_2 represents -log of the half maximally effective molar concentrations. Student's *t* test was used for statistical analysis regarding the cGMP assay. Analysis of variance was used for the difference between pD_2 of the agonist. A *P* level of 0.05 or less was considered significant.

NOS-2 staining was observed in small omental arteries from septic patients ($n = 3$) but not in 2 out of 3 arteries from non septic patients. NOS-2 was present in endothelium and sub-endothelium layers. An e.p.r. spectrum characteristic of NO-Fe(DETC)$_2$ was observed in 2 out of 3 arteries from septic patients but only in 1 out of 6 arteries from non septic patients. However the cyclic GMP level was not significantly different in arteries from septic patients ($n = 4$) and in those from non septic patients ($n = 5$) in the absence of L-arginine (310 ± 126 and 402 ± 85 fmol μg^{-1} DNA, respectively) and in its presence (659 ± 236 and 693 ± 200 fmol μg^{-1} DNA, respectively). The concentration-effect curves of NA (from 10 nM to 30 µM) and the thromboxane A_2 analogue, U 46619, (from 1 nM to 1 µM) were not significantly different in arteries from septic ($n = 7$) and non septic ($n = 7$) patients. The pD_2 values for NA and U 46619 were 5.76 ± 0.11 and 7.00 ± 0.11 respectively in arteries from non septic patients and 5.93 ± 0.21 and 6.74 ± 0.11 in arteries from septic patients. However, the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 300 µM), produced a significant leftward shift of the concentration-effect curves of the two agonists in arteries from patients with sepsis, the pD_2 values being 6.82 ± 0.10 ($P<0.05$) and 7.54 ± 0.08 ($P<0.05$) for NA and U 46619 respectively. However, L-NAME had no effect in arteries from non septic patients (the pD_2 values being 6.12 ± 0.20 and 7.00 ± 0.38 for NA and U 46619 respectively).

These results show that NOS-2 is present in small omental arteries of patients with sepsis, resulting in enhanced NO production but not in a significant increase of cyclic GMP level nor in hyporesponsiveness to agonists. This dissociation and the potentiating effect of L-NAME in arteries from septic patients suggest that antagonistic mechanisms blunting NO biological activity may be induced in arteries during sepsis, in addition to NOS-2 activity.

Stoclet et al., Circulation, **87 (suppl.V)**: V77-V80, 1993.
Tsuneyoshi et al., Crit. Care Med., **24**: 1083-1085, 1996.

203P BLOOD PRESSURE AND RENAL EFFECTS OF SR 49059, A SELECTIVE VASOPRESSIN V_{1A} RECEPTOR ANTAGONIST, IN HYPERTENSION INDUCED BY CHRONIC INHIBITION OF NITRIC OXIDE SYNTHESIS

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Chronic inhibition of nitric oxide (NO) synthesis induces hypertension with alteration of renal hemodynamics (Baylis *et al.*, 1992). Because NO inhibits peptide secretion from the neuronal lobe of pituitary gland (Lutz-Bucher & Koch, 1994) and partially masks renal vasoconstriction linked to the activation of vasopressin V_{1A} receptors (Barthelmebs *et al.*, 1996), we decided to determine the role of vasopressin in NO-deficient hypertension. Therefore a selective, non peptide, orally active vasopressin V_{1A} receptor antagonist, SR 49059 (Serradeil-Le Gal *et al.*, 1993) was used.

Male Sprague Dawley rats (200g) were divided in four groups, controls or rats treated for 6 weeks with N^{ω}-nitro-L-arginine (L-NNA, 15 mg/kg/d) and/or SR 49059 (30 mg/kg/d). Both compounds were mixed with the food. After the 6 week treatment, systolic blood pressure (SBP) was measured (tail-cuff method) and 24h urine collected for determination of water, sodium (UVNa) and albumin (UValb) excretions. Rats were thereafter anaesthetised with inactin (100 mg/kg i.p.) for glomerular filtration rate (GFR) and renal plasma flow (RPF) measurements by polyfructosan and para-amino-hippuric acid clearances. Data (means ± s.e.m.) were analysed by a two-way Anova.

L-NNA-induced hypertension was associated with unchanged diuresis (not shown) and UVNa (Table 1). UValb was increased while RPF and GFR were decreased.

Table 1: Effects of a 6 week treatment with SR 49059 and/or L-NNA on SBP and renal functions

Treatment	n	SBP mmHg	UVNa mmol/d	UValb mg/d	RPF ml/min/g	GFR ml/min/g
Control	9	134 (4)	1.8 (0.2)	4.1 (0.6)	5.65 (0.87)	1.35 (0.13)
L-NNA	12	173 (4)	1.9 (0.1)	13.1 (3.5)	3.95 (0.77)	0.90 (0.14)
SR 49059	9	129 (3)	2.0 (0.1)	3.8 (0.4)	6.86 (1.02)	1.37 (0.17)
L-NNA + SR 49059	12	198 (3)	2.0 (0.1)	13.2 (3.3)	3.41 (1.31)	0.86 (0.19)
L-NNA factor		*< 0.01*	*ns*	*< 0.01*	*< 0.02*	*< 0.01*
SR 49059 factor		*< 0.05*	*ns*	*ns*	*ns*	*ns*
interaction		*< 0.01*	*ns*	*ns*	*ns*	*ns*

SR 49059 per se had no effect on any parameter and also failed to affect the alterations in renal functions induced by L-NNA. SBP was even higher in L-NNA+SR 49059 treated rats. The pressor response to vasopressin (100 ng/kg i.v.) was inhibited in SR 49059 treated groups ($p < 0.05$).

Conclusion: The results suggest no role for activation of vasopressin V_{1A} receptor and subsequent vasoconstriction in hypertension and alteration of renal hemodynamics induced by chronic inhibition of NO synthesis.

Barthelmebs M. *et al.* (1996) *Eur. J. Pharmacol.* 314, 325-332.
Baylis C., Miruka B. & Deng A. (1992) *J. Clin. Invest.* 90, 278-281.
Lutz-Bucher B. & Koch B. (1994) *Neurosc. Lett.* 165, 48-50.
Serradeil-Le Gal C. *et al.* (1993) *J. Clin. Invest.* 92, 224-231.

204P EFFECTS OF NO SYNTHASE INHIBITION ON ACETYLCHOLINE-INDUCED RELAXATION IN THE ARTERIAL NETWORK OF THE RABBIT ISOLATED EAR

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In many blood vessels, the relaxation induced by ACh is not fully abolished in the presence of NO synthase inhibitors suggesting that other relaxing or hyperpolarising factors such as EDHF may also be released from the endothelium. Using an X-ray microangiographic technique (Griffith *et al.*, 1987), we have investigated ACh-induced relaxation in the rabbit isolated ear preconstricted with 5-HT (300 nM) and histamine (300 nM). The effects of ACh under control conditions were compared with those obtained in the presence of L-NAME (300 μM) which was administered in combination with indomethacin (10 μM) to additionally inhibit any possible effects of prostacyclin.

Under control conditions, ACh (10 nM–30 μM) induced a concentration-dependent relaxation of tone reaching 63.0± 13.3% at 30 μM (n=6). In the presence of L-NAME and indomethacin, a concentration-dependent relaxation was still observed although its magnitude was significantly reduced reaching only 29.8±5.7% at 30 μM ($P<0.05$; n=6).

Direct visualisation with X-ray microangiography revealed that under control conditions the relaxation induced by ACh was associated with a large increase in diameter of the central ear artery (G_0), a small increase in diameter of the first generation daughter branch artery (G_1) but no significant effect on the diameter of the second generation daughter branch artery (G_2). In contrast, in the presence of L-NAME and indomethacin, the relaxation induced by ACh was associated with no significant effect on vessel diameter of either G_0 or G_1 vessels but a significant increase in vessel diameter of G_2 vessels.

The relaxation induced by ACh in the presence of L-NAME and indomethacin was inhibited, but not abolished, by two K_{Ca} channel blockers, charybdotoxin (ChTX; 10 nM) and Penitrem A (100 nM). The relaxation induced by 30 μM ACh was significantly reduced to 15.6±5.4% by ChTX ($P<0.01$; n=6) and 16.0±3.8% ($P<0.01$; n=6) by Penitrem A. Microangiographic analysis revealed that both compounds had no significant effect on the lack of response to ACh in either G_0 or G_1 vessels in the presence of L-NAME and indomethacin but that both significantly inhibited the increase in diameter induced by ACh in G_2 vessels.

The data indicate that the presence of L-NAME and indomethacin causes a change in the pattern of relaxation induced by ACh within the network of the rabbit ear. This may be because under control conditions NO is the dominant relaxing factor released and is mainly effective on larger vessels. In the presence of L-NAME and indomethacin, the dominant mediator of relaxation may be EDHF which, in turn, may be more effective on smaller vessels. The ability of K_{Ca} channel blockers to inhibit this relaxation confirms that hyperpolarisation via K_{Ca} channels is involved in mediating the response.

Griffith, T.M., Edwards, D.H., Davies, R.L., Harrison, T.J. & Evans, K.T. (1987). *Nature*, **329**, 442-445.

205P ENDOTHELIUM-DEPENDENT HYPERPOLARIZATIONS AFTER CHRONIC *IN VIVO* TREATMENT WITH NITRIC OXIDE SYNTHASE INHIBITORS

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Membrane potential and changes in tension were recorded from isolated canine coronary arteries and guinea-pig carotid arteries and aortas to study the effects of chronic *in vivo* NO-synthase inhibition on endothelium-dependent hyperpolarization.
In coronary arteries taken from control dogs contracted with U 46619 (3×10^{-8} M), acetylcholine (10^{-9} - 10^{-4} M) and bradykinin (10^{-10} - 10^{-5} M) induced endothelium-dependent relaxations (-log ED_{50}: 7.4 ± 0.1, n = 6 and 8.5 ± 0.1, n= 8, respectively) which were unaffected by acute *in vitro* exposure to indomethacin (5×10^{-6} M), but partially inhibited by L-nitro-arginine (L-NA: 10^{-4} M) (-log ED_{50}: 6.6 ± 0.1 and 7.8 ± 0.1 for acetylcholine and bradykinin respectively, $p < 0.05$). The combination of L-NA plus indomethacin did not produce any further inhibition than L-NA alone. In coronary arteries taken from animals chronically treated *in vivo* with L-NA (30 $mg.kg^{-1}$ on the first day and 20 $mg.kg^{-1}$ the 7 following days, intra-arterially, Puybasset *et al* 1996), the response to the two agonists were significantly inhibited when compared to control dogs (-log ED_{50}: 6.7 ± 0.1 and 7.7 ± 0.1 for acetylcholine and bradykinin respectively, $p < 0.05$). However, in these coronary arteries taken from chronically treated dogs, acute *in vitro* exposure to L-NA, and/or indomethacin no longer influenced the effects of either agonist. In the isolated canine coronary artery, the hyperpolarizing response to acetylcholine (10^{-6} M), observed in the presence of L-NA and indomethacin, was enhanced in dogs chronically treated *in vivo* with L-NA (6 ± 1 and 13 ± 2 mV respectively in control and treated animals, $p < 0.05$ unpaired t test). Conversely the hyperpolarization to bradykinin (10^{-7} M) was partially but significantly inhibited in isolated coronary arteries taken from treated animals (14 ± 2 and 8 ± 2 mV in arteries from control and treated animals respectively, $p < 0.05$ unpaired t test).
In the guinea-pig isolated aorta, the relaxation to bradykinin (10^{-7} M) was abolished by chronic *in vivo* treatment with L-nitro-arginine-methyl-ester (L-NAME: 1.5 $mg.ml^{-1}$ in the drinking water for at least 4 days; relaxation in % of the contraction induced by U 46619: - 46 ± 8 and + 3 ± 5 in control and L-NAME treated animals respectively). In the isolated guinea-pig carotid artery, (in the presence of L-NA and indomethacin) acetylcholine (10^{-6} M) induced a hyperpolarization which was not significantly affected by chronic *in vivo* L-NAME treatment (18 ± 2 and 16 ± 1 mV in control and treated animals respectively).
This study shows that in canine coronary artery, under our experimental conditions, acute *in vitro* and chronic *in vivo* L-NA treatment produce a similar inhibition of the endothelium-dependent relaxations. These inhibitory effects were not additive. In the guinea-pig aorta the chronic *in vivo* L-NAME treatment abolished the endothelium-dependent relaxation to bradykinin. These results suggest that either treatment induced a complete inhibition of the NO-synthase. Endothelium-dependent hyperpolarizations are maintained during chronic NO-synthase inhibition. The proposed negative regulatory role of NO on endothelium-derived hyperpolarizing factor production and/or release is not observed consistently

Puybasset L. Bea ML, Ghaleh B et al (1996) Cir. Res. 79, 343-357.

206P MODULATION BY THE ENDOTHELIUM OF CONTRACTILE RESPONSES EVOKED BY 5-HT$_{1B/D}$ RECEPTOR AGONISTS IN THE RABBIT ISOLATED SAPHENOUS VEIN

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We recently reported that a component of the contractile response evoked by the 5-HT$_{1B/D}$ receptor agonist, sumatriptan, in the rabbit isolated saphenous vein was dependent upon an intact endothelium (E) (Valentin *et al.*, 1996). In the present study, we compared the contractile responses induced by the recently described 5-HT$_{1B/D}$ receptor agonists, naratriptan (NAR), rizatriptan (RIZ) and zolmitriptan (ZOL) with those of 5-hydroxytryptamine (5-HT) in the rabbit isolated saphenous vein.

Saphenous vein rings were set up for isometric tension recording, as previously described (Valentin *et al.*, 1996), in tissue baths containing oxygenated Krebs solution which contained (μM): idazoxan (1), indomethacin (10), ketanserin (0.1), prazosin (10), and Nω Nitro-L-Arginine Methyl Ester (L-NAME; 0 or 10), a nitric oxide synthase inhibitor (NOSI). In some experiments, the E was removed mechanically. Following tension readjustments for stress relaxation, 2g tension was applied to each ring and cumulative concentration-isometric tension response curves to NAR, RIZ, ZOL and 5-HT (n=7-38 rings *per* group) were constructed.

Potencies of NAR (pD_2 range: 5.0-5.9), RIZ (5.2-5.9) and ZOL (5.8-6.2) were lower than those of 5-HT (6.5-7.2) but were not significantly affected by either the E or NOSI. In E-intact rings, efficacy, as assessed by the maximal contractile response (Emax), was significantly greater for RIZ and ZOL, but not for NAR, compared to that of 5-HT irrespective of the absence (19±1, 54±7, 10±3 and 9±2mN respectively; $P<0.05$ for RIZ and ZOL *vs.* 5-HT) or presence (86±7, 89±5, 65±6 and 61±3mN respectively; all. $P<0.05$ *vs.* absence of NOSI and $P<0.05$ for RIZ and ZOL *vs.* 5-HT) of NOSI. Emax values of each agonist were potentiated by NOSI, suggesting an inhibitory influence of either spontaneously and/or agonist-stimulated release of NO (Valentin *et al.*, 1996). In E-denuded rings, the Emax values did not differ between the 4 agonists (30±3, 24±5, 25±3 and 32±8mN for 5-HT, NAR, RIZ and ZOL respectively; NS between agonists) and were statistically significantly lower than those obtained in E-intact rings in the presence of NOSI ($P<0.05$ in each case); suggesting that part of the contractile responses evoked by the 5-HT$_{1B/D}$ receptor agonists studied are E-dependent. Moreover, the contractile responses elicited by the agonists were concentration-dependently (from 1nM) antagonized by the 5-HT$_{1B/D}$ receptor antagonist, GR 127935, being abolished by 1μM, supporting the involvement of 5-HT$_{1B/D}$ receptors.

The data suggest that a component of the contractile responses evoked by 5-HT and NAR, and moreso for ZOL and RIZ is dependent upon an intact E.

Valentin, JP; Bonnafous, R and John, GW. (1996), Br. J. Pharmacol. 119, 35-42.

207P COMPARISON OF 5-HT AUTORECEPTOR CONTROL IN GUINEA-PIG DORSAL AND MEDIAN RAPHE INNERVATED BRAIN REGIONS

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We previously reported that neither selective h5-HT$_{1B}$ nor non-selective h5-HT$_{1B/1D}$ receptor antagonists increase extracellular 5-HT in the frontal cortex of the freely moving guinea-pig (Roberts *et al.*, 1994,1997a,b). This failure to demonstrate an increase may be attributable to a lack of endogenous tone at the terminal or multiple modulatory inputs in this brain region. Therefore, the aim of this work was to study multiple brain regions to assess effects of: (i) the selective 5-HT$_{1B}$ receptor antagonist, SB-224289, and the non-selective 5-HT$_{1B/1D}$ receptor antagonist, GR 127935; and (ii) systemic paroxetine, to compare the control on 5-HT release exerted by 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor antagonism in different brain regions. Frontal cortex and striatum were used as example areas receiving 5-HT projections from the dorsal raphe nucleus (DRN) and dentate gyrus as an example of median raphe nucleus (MRN) projections.

Guinea-pigs (300-400g) were anaesthetised with isoflurane and dialysis probes implanted either into the frontal cortex, dentate gyrus or striatum. (Co-ordinates: AP +4.5mm, ML ±2mm, V -3mm; AP -4.1mm, ML ±2.1mm, V -4.5mm or AP +3.0mm; ML ±3.3mm, V -7mm respectively from Bregma). Animals were allowed to recover overnight before probes were perfused with a.CSF at 2µl/min. After 2 hours perfusion, samples were collected every 20 min into 10µl of a.CSF. 5-HT was separated by HPLC and detected by ECD (electrode potential of 0.65V).

In the frontal cortex and striatum, GR 127935 (0.3mg/kg i.p.) evoked a significant decrease in extracellular 5-HT, reaching minima of 41±12% (n=6) and 32±6% (n=3) of basal respectively ($p<0.05$). In contrast, SB-224289 (4mg/kg p.o.) had no effect on 5-HT levels in either region (n=4). Paroxetine (10mg/kg p.o.) produced a small but non-significant increase in extracellular 5-HT in the frontal cortex (n=5). In the dentate gyrus, GR 127935 and SB-224289 significantly increased extracellular 5-HT, reaching maxima of 146±11% (n=4) and 151±19% (n=4) of basal respectively ($p<0.05$). Paroxetine (10mg/kg p.o.) produced a small decrease in extracellular 5-HT ($p=0.08$), reaching a minimum of 59±16% (n=3) of basal.

Differential effects of non-selective versus selective ligands on 5-HT levels were observed in the DRN innervated brain regions. The selective 5-HT$_{1B}$ receptor antagonist had no effect on 5-HT while the mixed 5-HT$_{1B/1D}$ receptor antagonist decreased 5-HT levels. The decrease may be explained by antagonism of inhibitory 5-HT$_{1D}$ receptors on raphe cell bodies, leading to a local increase in 5-HT, which in turn stimulated 5-HT$_{1A}$ receptors to decrease cell firing and hence 5-HT release from terminals.

In the MRN innervated region both compounds had a similar effect. The ability of both compounds to increase 5-HT levels in the dentate gyrus implies, that in this region the above mechanism is not present, which in turn suggests a lack of 5-HT$_{1D}$ receptors in the MRN. The lack of effect of paroxetine in all regions may be due to the limiting effects of cell body 5-HT$_{1A}$ receptors. However, the tendency to decrease 5-HT in the median innervated area suggest that the density of 5-HT$_{1A}$ and/or 5-HT re-uptake sites may be higher in this region.

Roberts C. *et al.* (1994) Br. J. Pharmacol., 112, 489P.
Roberts C., Price G.W. & Jones B.J. (1997a) Eur. J. Pharmacol., 326, 23-30.
Roberts C. *et al.* (1997b) Neuropharmacol., 36, 549-557.

208P SB-224289, A SELECTIVE h5-HT$_{1B}$ RECEPTOR ANTAGONIST WITH NEGATIVE INTRINSIC ACTIVITY

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Despite the availability of numerous 5-HT$_{1B/1D}$ receptor agonists and the selective antagonist GR127935, few compounds pharmacologically discriminate these receptor subtypes. SB-216641 is 30-fold selective for 5-HT$_{1B}$ over 5-HT$_{1D}$ receptors and BRL-15572, almost 100-fold selective for 5-HT$_{1D}$ over 5-HT$_{1B}$ receptors (Price et al., 1997). However, as with GR127935, these compounds are low efficacy partial agonists. We now report on SB-224289 (2,3,6,7-tetrahydro-1'-methyl-5-{2'-methyl-4'-[(5-methyl-1,2,4-oxadiazole-3-yl)biphenyl-4-yl]carbonyl}furo[2,3-f]indole-3-spiro-4'-piperidine oxalate), a selective h5-HT$_{1B}$ receptor antagonist with negative intrinsic activity.

Recombinant h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors were stably expressed in CHO cells, yielding receptor levels of 3.2 and 8.7 pmoles/mg protein, respectively. SB-224289 was characterised in membranes from these cell lines using [^{3}H]5-HT radioligand binding and [^{35}S]GTPγS binding (for methods, see Watson *et al.*, 1996), to measure its affinity and functional efficacy, respectively, at h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors. SB-224289 was also tested for its effects on electrically evoked (1Hz and 3Hz frequencies) [^{3}H]5-HT release in guinea-pig cerebral cortical slices.

In receptor binding studies, SB-224289 showed nearly 100-fold higher affinty for h5-HT$_{1B}$ receptors over all other 5-HT receptors tested, including h5-HT$_{1D}$ receptors. In functional studies at h5-HT$_{1B}$ receptors, 5-HT stimulated [^{35}S]GTPγS binding by 194% (± 16%, pEC50 = 7.8 ± 0.1) above basal levels, whereas SB-224289 inhibited basal binding (Table 1). This was attributed to negative intrinsic activity at a constitutively coupled receptor. In the presence of SB-224289, the 5-HT concentration response curve was shifted to the right in a parallel manner with no suppression of the maximal response. Affinity estimates again correlated with receptor binding affinity (Table 1). SB-224289(1μM) demonstrated 5-HT terminal autoreceptor antagonism by blocking 5-HT inhibition of release and by potentiating release to 182% (± 12%, n=4) at higher frequencies. In conclusion, SB-224289 has high affinity and selectivity for h5-HT$_{1B}$ receptors and this is the first selective 5-HT$_{1B}$ receptor antagonist devoid of any partial agonism.

Price, G.W., Burton M.J., Roberts C., et al., *Br. J. Pharmacol.* **119**, 301P
Watson J., Burton M.J., Price G.W., et al., *Eur. J. Pharmacol.* 314, 365-372

Table 1. Receptor binding affinity and functional potency of SB-224289 at h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors

	Receptor Binding (pKi)		[^{35}S]GTPγS - h5-HT$_{1B}$ Receptors			[^{35}S]GTPγS - h5-HT$_{1D}$ Receptors		
	h5-HT$_{1B}$	h5-HT$_{1D}$	EC$_{50}$	E$_{max}$	pA$_2$	EC$_{50}$	E$_{max}$	app. pA$_2$
SB-224289	8.0 (± 0.1)	6.0 (± 0.1)	7.9 (±0.1)	-65% (± 2%)	8.4 (± 0.2)	6.8 (± 0.1)	-25% (± 2%)	6.9 (± 0.1)

All data are at least n = 3, mean , (± SEM), Emax = % inhibition of basal binding

209P PHARMACOLOGICAL CHARACTERISATION OF THE 5-HT TRANSPORTER IN RAT ADRENAL MEDULLA

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5-hydroxytryptamine (5-HT) is a specific stimulus for steroid secretion in the adrenal zona glomerulosa of several species including man and rat. It has been hypothesised that 5-HT synthesised in the adrenal medulla could regulate steroidogenesis via medullary-cortical interactions. 5-HT transporter mRNA is expressed in rat adrenal medulla (Blakely *et al.*, 1994) and [^{3}H]paroxetine binding autoradiography has shown a high density of sites in rat adrenal medulla (220 fmoles/mg of tissue) with no sites in adrenal cortex (Burns *et al.*, 1996). The aim of this study was to characterise the rat adrenal 5-HT transporter by comparing its pharmacology with that of the brain 5-HT transporter.

Rat adrenal glands from adult male Wistar rats (250 - 300g) were dissected into capsular (including zona glomerulosa) and medullary (including inner cortex) portions. Membranes from adrenal tissue and rat cerebral cortex were prepared as described previously (Lawrence *et al.*, 1993). The affinities of a number of drugs were determined by incubating membranes with 0.25nM [^{3}H]citalopram (NEN; 85Ci/mmol.) and test drug for 60min at 25°C in 1ml 50mM Tris-HCl (containing 120mM NaCl and 5mM KCl, pH 7.4). Assays were terminated by rapid filtration using a Brandell Cell Harvester. Non-specific binding was defined using 10μM citalopram. Statistical comparisons were performed by ANOVA.

The K$_D$ value for [^{3}H]citalopram binding in adrenal medulla (1.43 ± 0.06nM) was not significantly different from that determined in cerebral cortex (1.62 ± 0.06). Corresponding B$_{max}$ values were 657 ± 28 and 1663 ± 29 fmoles/mg protein respectively (means ± s.e.mean; n = 3). Specific binding of [^{3}H]citalopram was not detected in adrenal capsule. The rank order of affinity for the drugs tested in adrenal medulla was paroxetine > citalopram > fluoxetine > DMI (desmethylimipramine) > 5-HT >> noradrenaline. K$_i$ values determined for the drugs in adrenal medulla were not significantly different from those in cerebral cortex (Table 1). K$_i$ values for noradrenaline were greater than 1mM.

The membrane [^{3}H]citalopram binding data together with the [^{3}H]paroxetine autoradiography data are consistent with the specific location of a high density of 5-HT transporters in the rat adrenal medulla. The pharmacological profile of this transporter is indistinguishable from the membrane 5-HT transporter found in serotonergic neurones and blood platelets and is distinct from the amine transporters located in catecholaminergic neurones and adrenal chromaffin cells.

Blakely, R.D *et al.* (1994) *J. Exp. Biol.* 196, 263-281.
Burns, N.J. *et al.* (1996) *Br. J. Pharmacol.* 117, 91P
Lawrence, J.A. *et al.* (1993) *Brain. Res.* 612, 326-329.
P.B.W. & N.J.B. are MRC Research Scholars.

Table 1: Inhibition of [^{3}H]Citalopram binding to rat adrenal medullary and cerebral cortical membranes

	K$_i$ (nM) (mean ± s.e.mean: n=3)	
Drug	Adrenal Medulla	Cerebral Cortex
paroxetine	0.37 ± 0.06	0.31 ± 0.11
fluoxetine	9.15 ± 1.34	6.85 ± 0.69
DMI	254 ± 74	156 ± 12
5-HT	510 ± 24	343 ± 20

210P *IN VIVO* AND *IN VITRO* CHARACTERISATION OF RELEASE-REGULATING 5-HT AUTORECEPTORS IN THE GUINEA-PIG RAPHE NUCLEUS AND HYPOTHALAMUS

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The release of 5-HT from nerve terminals is under the control of inhibitory 5-HT1B autoreceptors in human brain (Fink et al., 1995) and in animals (guinea pig cerebral cortex, Bühlen et al., 1996). Autoreceptors have also been described in the guinea pig raphe nucleus (El Mansari and Blier, 1996). The aim of the present study was to determine the subtype of the 5-HT autoreceptor implicated in the regulation of 5-HT release in the guinea pig dorsal raphe nucleus in comparison to the 5-HT terminal field in the hypothalamus.

For in vitro release studies, raphe nucleus or hypothalamic slices from male Hartley guinea pigs (300-350 g) were incubated with [^{3}H]5-HT, superfused with Krebs solution containing 10 μM paroxetine and stimulated electrically at 3 Hz for 2 min. In vivo release from the dorsal raphe nucleus or hypothalamus of freely moving guinea pigs was measured by microdialysis at a flow rate of 0.32 μl/min. Dialysate samples were collected every 30 min and analyzed with HPLC-ECD.

In vitro, stimulation-induced [^{3}H]5-HT release represented 3.93 ± 0.33 % (n = 18) and 4.72 ± 0.56 % (n = 11) of the total tissue radioactivity in the raphe and hypothalamus, respectively. The non-selective 5-HT receptor agonist, 5-carboxamidotryptamine (5-CT) decreased (-76 % at 10 nM in raphe, -64 % at 100 nM in hypothalamus), while the antagonist, methiothepin, increased (+168 % and +194 % in raphe and hypothalamus at 1 μM, respectively) the evoked release of [^{3}H]5-HT. At 1 μM, the selective 5-HT1B/D receptor antagonist, GR127935, and the 5-HT1D receptor antagonist, ketanserin, had no effect. Both methiothepin and GR127935 (100-1000 nM) attenuated the inhibition by 5-CT in both regions. At 300 nM, ketanserin shifted to the right the concentration-effect curve of 5-CT in the raphe but not in the hypothalamus.

In vivo, baseline levels of 5-HT were (fmol/15 μl) 10.0 ± 0.6 (n = 28) and 9.7 ± 1.0 (n = 23) in the raphe and hypothalamus, respectively. When perfused through the dialysis probe at 10 μM, ketanserin increased the extracellular 5-HT levels in the raphe to a maximum of 209 % of baseline but not in hypothalamus. The selective 5-HT1B/D receptor agonist, naratriptan, decreased extracellular 5-HT levels (area under curve for 330 min post-infusion period: 73.1 ± 3.3 and 65.0 ± 7.3 % of controls, at 10 and 0.1 μM in the raphe and hypothalamus, respectively). Ketanserin (1 μM) by itself was inactive and did not modify the decrease of 5-HT levels by naratriptan (10 μM in raphe and 0.1 μM in hypothalamus).

These results suggest that, under the conditions used in this study, the release of 5-HT at the terminal level in the hypothalamus is regulated by 5-HT1B receptors. In the dorsal raphe nucleus, 5-HT release is modulated by 5-HT1D receptors but possibly also by 5-HT receptors the subtype of which remains to be determined.

Bühlen M. et al. (1996) *Naunyn-Schmied. Arch. Pharmacol.* 353, 281-289.
El Mansari M. and Blier P. (1996) *Br. J. Pharmacol.* 118, 681-689.
Fink K. et al. (1995) *Naunyn-Schmied. Arch. Pharmacol.* 352, 451-454.

211P DOES 5HT-MODULINE INTERACT WITH THE 5HT$_{1B}$ AGONIST CP 93,129-INDUCED RELEASE OF STRIATAL DOPAMINE IN RATS?

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5HT-moduline is an endogenous tetrapeptide (leu-ser-ala-leu), which has recently been isolated and characterized (Rousselle *et al*, 1996). This specific and allosteric modulator has a high affinity for the 5HT$_{1B/1D}$ receptor playing an important role in the pathophysiologic modulation of the serotonergic transmission ; through heteroreceptors it also regulates other neurotransmission systems (Massot *et al* 1996). The aim of the present study is two-fold : (i) to check the possibility, while using the *in vivo* microdialysis method in awake rats, whether basal extracellular concentrations of dopamine (DA) and metabolites (DOPAC, HVA, 3MT) are modified ; (ii) to research the modulation properties through a preliminary induced- release of DA with a 5HT$_{1B}$ agonist, the CP 93, 129 (CP) (Galloway *et al* 1993).

This study was conducted with Sprague-Dawley (Charles River) male adult rats with a mean weight of 300-320 g. The animals were anesthetized with 350 mg. kg^{-1} chloral hydrate injected intraperitoneally. A probe guide canula was implanted above the right striatum (AP + 1.5, L - 2, V - 3.4 relative to the bregma and the dura surface). The day after a 4 mm long microdialysis probe was positioned into the probe guide, the animal being awake. The dialysis probe was perfused with Ringer solution (NaCl 147 mM, KCl 4 mM $CaCl_2$ 1.2 mM) at a flow rate of 1 µl. min^{-1} without or - with 5HT-moduline (500 µg. ml^{-1}), - with CP (0.05, 0.1 or 0.5 mM) or - with CP (same three dosages) + 5HT-moduline administered 1 h after. The Carnegie Medicine system was used. Assay of DA and its metabolites were performed on an automated HPLC processor (Spectra-Physics) with electrochemical detector (ESA). Concentrations were compared using variance analysis for repeated measures (ANOVA).

Local infusion of 5HT-moduline increased the striatal level of DA by 104 % ($p = 0.0015$), of 3MT by 293 % ($p = 0.0001$) without any modification of the terminal metabolites concentrations, DOPAC and HVA (n = 6 for the control group and 10 for the treated group). The intrastriatal administration of CP induced a statistically significant increase of DA ($p < 0.0001$), which is dose-dependant : CP 0.05 (n = 6) *versus* CP 0.1 (n = 7) $p = 0.0004$, CP 0.1 *versus* CP 0.5 (n = 7) $p = 0.0006$), without modifying terminal metabolites levels. 5HT-moduline administration after CP only modified DA concentrations for the lowest CP dosage ($p = 0.0321$).

5HT-moduline, as well as the 5HT$_{1B}$ selective agonist, increases striatal DA concentrations ; these results do not show a direct inhibitory effect of 5HT-moduline on the dopamine release induced by a 5HT$_{1B}$ agonist, however they indicate the existence of an interactive mechanism between 5HT-moduline and the dopaminergic system.

Galloway, M.P., Suchowski, C.S., Keegan, M.J. *et* al., (1993) *Synapse*, 15, 90-92.
Massot, O., Rousselle, J.C., Fillion, M.P. *et al.*, (1996) *Mol. Pharmacol.*, 50, 752-762.
Rousselle, J.C., Massot, O. Delepierre, M. *et al.*, (1996) *J. Biol. Chem.*, 271, 726-735.

212P A MICRODIALYSIS STUDY OF THE EFFECTS OF FLUOXETINE INFUSION ON NORADRENALINE (NA) EFFLUX IN THE FRONTAL CORTEX OF SALINE OR DSP-4-TREATED RATS

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Experiments using microdialysis *in vivo* have shown that local infusion of the selective serotonin reuptake inhibitor (SSRI) fluoxetine into rat frontal cortex causes an increase in noradrenaline (NA) efflux. Inhibition of NA reuptake could contribute to this increase (Hughes & Stanford, 1996). However, a 5,7-dihydroxytryptamine lesion of central 5-HT neurones did not affect the inhibition of synaptosomal uptake of [^{3}H]NA by fluoxetine or citalopram. This suggests that these two SSRIs, at least, act at a site which is not on 5-HT neurones (Hughes & Stanford, 1997). The present experiments used the selective neurotoxin, *N*-(2-chloroethyl)-*N*-2-bromobenzylamine (DSP-4), to investigate whether a noradrenergic lesion would diminish the effects of fluoxetine on the extracellular concentration of NA in the frontal cortex.

Male SD rats (260-320 g) were used. A partial noradrenergic lesion was induced by injection of DSP-4 (40 mg kg^{-1}, i.p.). Control rats were injected with saline vehicle (2 ml kg^{-1}, i.p.). 4 days later, microdialysis probes were implanted stereotaxically under halothane anaesthesia. The following day, probes were perfused with modified Ringer's at 1 µl min^{-1}. Dialysis samples were collected every 20 min and their NA content measured using HPLC-ECD. Once a stable basal efflux of NA was obtained, the perfusion medium was changed to one containing fluoxetine. Fluoxetine was infused by retrodialysis at a concentration of either 5 or 50 µM for 3 h. After this, rats were killed, their brains removed, and a sample of cortex taken for measurement of NA content using HPLC-ECD. Statistical analysis used split-plot ANOVA or Mann-Whitney U-Test where appropriate.

The NA content of cortical tissue from DSP-4 treated animals was 66% lower than in saline-injected controls (saline: 534.0 ± 47.9 ng g^{-1}(n=15); DSP-4: 178.8 ± 17.0 ng g^{-1}(n=5); P=0.001). Despite this, basal NA efflux in DSP-4 treated rats wasnearly 2-fold greater than that in saline-injected controls (saline: 28.1 ± 2.2 fmol 20 min^{-1}(n=13); DSP-4: 46.7 ± 5.4 fmol 20 min^{-1}(n=15); $F_{1,19}$=5.03; P=0.037). In saline-injected controls, fluoxetine infusion caused an increase in extracellular NA which was statistically significant at 5 and 50 µM (*cf* basal samples: 5 µM: $F_{2,13}$=9.25;P=0.003 ; 50 µM: $F_{2,14}$=10.9; P=0.001). However, after a DSP-4 lesion, infusion of neither concentration of fluoxetine affected NA efflux (5 µM: $F_{2,24}$=0.16; P=0.78; 50 µM: $F_{3,45}$=0.85; P=0.46).

There are several possible explanations for the lack of effect of fluoxetine in DSP-4 lesioned rats. One is that the target site for fluoxetine predominates on noradrenergic neurones which are targeted by DSP-4. However, our experiments looking at synaptosomal [^{3}H]NA uptake *in vitro* indicate that inhibition of uptake by fluoxetine was not reduced by a DSP-4 lesion. Another possibility is that the lesion leaves a population of noradrenergic neurones which have 2 distinct NA uptake sites, only one of which is fluoxetine-sensitive. It remains to be seen whether either of these, or some other, factor explains the abolition of the effects of fluoxetine in DSP-4 lesioned rats. Whatever the explanation, we are currently investigating whether this action of fluoxetine on the noradrenergic system is a general feature of SSRIs.

Z. Hughes is an MRC scholar.

Hughes, Z.A.& Stanford, S.C. (1996) *Eur. J. Pharmacol.* 317, 83-90
Hughes, Z.A.& Stanford, S.C. (1997) *Br. J. Pharmacol.* (abstract in press)

213P NOCICEPTIN STIMULATES FOOD INTAKE AND HYPERPOLARISES NEURONS IN THE RAT VENTROMEDIAL HYPOTHALAMUS

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The ventromedial hypothalamus (VMH) is a brain region thought to be involved in the control of food intake and *in situ* hybridisation studies have shown the presence of a high density of nociceptin-receptor mRNA in this area (Bunzow *et al.*, 1994). We have designed a feeding paradigm to investigate the effect of intracerebroventricular (icv) nociceptin on food intake in satiated rats and have investigated the effects of nociceptin electrophysiologically on VMH neurones contained in acutely isolated rat brain slices.

Male Hooded Lister rats (~300g) were implanted with cannulas into the right lateral ventricle under isoflurane anaesthesia (coordinates from Bregma: ventral 0.8, lateral 1.5 and caudal 3.5mm from the surface of the skull). Seven days following surgery the animals were starved overnight. At 10am on the day of the experiment animals were allowed free access to food for 30 minutes after which a 2µl injection of either aCSF or nociceptin (5 nmoles) was administered icv over 30 seconds. Animals were placed back into their home cages with food available *ad libitum*. At 30 and 60 minutes the remaining food was weighed. Data are presented as mean±s.e.mean. Statistical analysis was performed using the student's t-test.

Brains were removed from 14-28 day-old male Sprague Dawley rats and 300µm coronal slices containing the VMH were prepared in physiological saline. All experiments were performed at 32-35^{o}C and neurones were identified using infrared differential interference contrast videomicroscopy. The physiological saline contained (mM) 125.0 NaCl, 25.0 $NaHCO_3$, 10.0 glucose, 2.5 KCl,1.25 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$ and was bubbled with a 95%, 5% O_2/CO_2 gas mixture. This solution was also used as the extracellular solution whilst the intracellular (pipette) solution comprised (mM) 120.0 Kgluconate, 10.0 NaCl, 2.0 $MgCl_2$, 0.5 K_2EGTA, 10.0 HEPES, 1.0 Na_2ATP, 0.1 Na_2GTP, pH 7.2.

In the first 30 minutes following icv administration of nociceptin, satiated rats consumed significantly more food compared with vehicle treated controls ($p<0.01$) (2.20±0.33g, n=6 and 0.98±0.22g, n=5 respectively). For the time period 30-60 minutes post-icv injection, neither treatment group consumed any food.

In whole-cell current clamp recordings, bath application of nociceptin hyperpolarised 25 out of 32 neurones tested in a concentration-dependent manner; 10nM had little effect (n=3), 50nM produced a 3.2 ± 0.6mV hyperpolarisation (n=3), 100nM produced a 7.0 ± 1.1mV hyperpolarisation 7(n=5) and 200nM produced 9.2 ± 1.1mV hyperpolarisation (n=7). These effects were associated with a decrease in the apparent input resistance of the neurone and were maintained in the presence of either 1µM TTX (n=4) or 10µM naloxone (n=4).

In whole-cell voltage clamp recordings, 200nM nociceptin activated an outward current (16.3 ± 3.5pA (n=7) in amplitude) that had a reversal potential of 100.2 ± 4.6 mV. The magnitude of this current was unaffected by bath application of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxalone (NBQX, 1µM) and 50µM D-2-amino-5-phosphovalerate (D-APV; 13.1 ± 1.4pA (n=3)) but was abolished by the addition of 2mM $BaCl_2$ to the bath solution (n=3).

Thus we have shown that icv nociceptin stimulates feeding in satiated rats and consistent with this, we found that *in vitro*, nociceptin causes a hyperpolarisation in most neurones of the VMH.

Bunzow, J.R., Saez, C. , Mortrud, M. *et al.*, (1994) FEBS Lett., 347 284-288.

214P DIFFERENTIATION OF NEUROLEPTICS BASED ON MOTOR AND COGNITIVE PERFORMANCE

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The cognitive disturbance associated with schizophrenia, and treatment, is a matter of significant interest. Pharmacological intervention is most effective in attenuating the positive symptoms of this disease, however, cognitive dysfunction is associated with the negative symptoms (Addington et al. 1991). Ideally, neuroleptics which attenuate positive symptoms, should not introduce further deleterious cognitive sequelae. Thus, in this preliminary study three neuroleptics were assessed on the performance of a delayed non-match to place (DNMTP) procedure using a latin square design.

Male Lister Hooded rats (280-310 g) maintained on a restricted diet in light (12/12 light/dark cycle) and temperature (21°C) controlled facility were divided into three groups of six. The animals had previously been trained to perform the DNMTP task to a criterion of 85% accuracy and 120 trials per 40 minute test session. Six operant boxes each fitted with two retractable levers and a central reinforcement magazine were used. One of two levers (the 'cue' lever), chosen at random, was advanced into the box then retracted once a response had been made. Following a delay, chosen at random from one of seven predetermined periods (0.3 to 30 seconds) during which a repetitive non-reinforced response was performed in the central magazine, both levers were advanced. A 45mg food pellet was earned if a response was made on the lever opposite to the cue lever. Drugs were administered 30 minutes prior to testing. Haloperidol (0.1 to 3.0 $mgkg^{-1}$) and clozapine (0.01 to 1.0 $mgkg^{-1}$) were prepared in acidified saline and risperidone (0.1 to 1.0 $mgkg^{-1}$) in cyclodextrin. Performance of the task was assessed with respect to percent accuracy by delay (arcsine transformed) and total trials completed. Both parameters were analysed by ANOVA followed by appropriate *post hoc* procedures.

At higher doses haloperidol induced a delay independent impairment of accuracy ($F_{(4,174)}=27.72$, $p<0.001$) that was accompanied by a commensurate reduction in trials completed ($F_{(4,25)}=12.12$, $p<0.001$). Clozapine impaired accuracy at longer delays in a dose dependent manner ($F_{(1,174)}=10.08$. $p<0.001$) without affecting trials completed ($F_{(3,20)}=2.61$, $p=0.1$). Risperidone at the highest dose administered reduced the number of trials completed ($F_{(4,25)}=14.13$, $p<0.001$) but had no significant effect upon accuracy ($F_{(4,174)}=1.4$, $p=0.21$). In a parallel study the highest dose of each neuroleptic was shown to significantly attenuate amphetamine stimulated locomotor activity (Marston, unpublished data).

These results extend the earlier work of Didriksen (1995) by using a more taxing paradigm. With the improved dissociation of the pattern of effects, found in this study, it is clearly possible to classify anti-psychotic agents by their cognitive and motoric sequelae in the rat. From these patterns further conclusions as to the pharmaco-anatomical substrates of anti-psychotic activity, and possibly a clearer rational in the choice of neuroleptics in clinical practice, can be made.

Addington J.D., *et al.* (1991), Schizophr. Res. 5,123.
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215P A SELECTIVE INVERSE AGONIST FOR CENTRAL CANNABINOID RECEPTOR INHIBITS MAPK ACTIVATION STIMULATED BY INSULIN OR IGF1

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In the present study, we showed that CHO cells transfected with human central cannabinoid receptor (CB1) exhibit high constitutive activity of both levels of mitogen-activated protein kinase (MAPK) and adenylyl cyclase. These activities could be blocked by the CB1 selective ligand, SR 141716A, that functions as an inverse agonist. Moreover, binding studies showed that guanine nucleotides decreased the binding of the agonist CP-55,940, an effect usually observed with agonists, whereas it enhanced the binding of SR 141716A, a property of inverse agonists.

Unexpectedly, we found that CB1-mediated effects of SR 141716A included inhibition of MAPK activation by pertussis toxin-sensitive receptor-tyrosine kinase such as insulin or insulin-like growth factor 1 receptors but not by pertussis toxin-insensitive receptor-tyrosine kinase such as the fibroblast growth factor receptor. These results are shown in the figure 1. We also observed similar results when cells were stimulated with Mas-7, a mastoparan analog, which directly activates the Gi protein. Furthermore, SR 141716A inhibited GTPγS uptake induced by CP-55,940 or Mas-7 in CHO-CB1 cell membranes.

This indicates that, in addition to the inhibition of autoactivated CB1, SR 141716A can deliver a biological signal which blocks the Gi protein and consequently abrogates most of the Gi-mediated responses. SR 141716A was without effect in untransfected CHO cells eliminating a direct interaction of SR 141716A with Gi protein.This supports the notion that the Gi protein may act as a negative intracellular signaling cross-talk molecule.

From these original results, which considerably enlarge the biological properties of the inverse agonist, we propose a novel model for receptor/ligand interactions.

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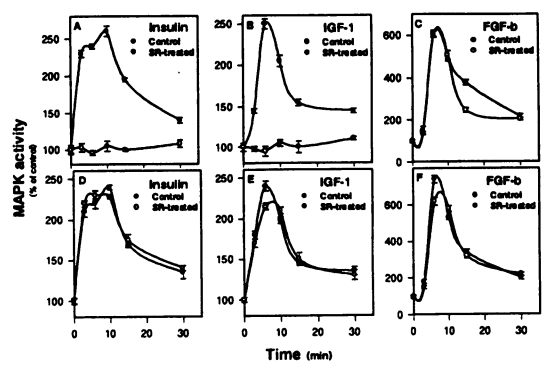

Figure 1

216P TGF-β1 AND BFGF MODULATE THE ANGIOTENSIN II-INDUCED CALCIUM RELEASE IN VASCULAR SMOOTH MUSCLE CELLS FROM NORMOTENSIVE AND HYPERTENSIVE RATS

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Angiotensin (AII) and growth factors play an important role in the development of hypertrophy of the arterial wall in hypertension, characterized by an increase in collagen and elastin content.

As Ca^{++} is implicated in the regulation of cell cycle, and in the synthesis of proteins, we wished to assess the effect of TGF-ß1 and bFGF on the mobilisation of intracellular Ca^{++} (Ca_i^{++}) induced by AII in smooth muscle cells (SMC) from rat aorta of spontaneous hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY).

The effect of AII (10^{-6} M) on Ca_i^{++} release was studied in cultured SMC isolated from the aorta of 5 wks old WKY and SHR (male, mean weight: 150g). Ca_i^{++} was assessed in Fura-2 loaded cells (Molecular Probe, USA), using fluorescent imaging microscopy (Newcastle Photometric System, UK). Several protocol were developped to assess the effect of growth factors, on the AII-induced Ca_i^{++} release: a) within two minutes after addition of TGF-ß1 (0.1 $ng.ml^{-1}$) or bFGF (0.5 $ng.ml^{-1}$); b) after 1 hour pretreatment or c) after 24 hrs pretreatment with TGF-ß1 or bFGF. Experiments were performed in the presence (Ca^{++}: 1mM) and in the absence of external Ca^{++}. Results are expressed as % of control values. Statistical significance was assessed with ANOVA followed by Student's *t*-test for unpaired data ($p<0.05$).

The results showed that AII-induced Ca_i^{++} release was not modified by either immediate addition or pretreatment for one hour with TGF-ß1 or bFGF. However 24 hrs pretreatment of SMC with TGF-ß1, increased markedly the AII-induced Ca_i^{++} release in SHR (+65%, n=54, $p<0.001$). This was consequent to a significant increase in Ca^{++} release from the internal stores (+66%, n=58, $p<0.001$), while Ca^{++} influx from external media was not significantly modified (-3%, n=144, n.s.). Similarly, in WKY rats , pretreatment for 24 hrs with TGF-ß1 significantly increased the AII-induced Ca_i^{++} release from internal stores (+50%, n=80, $p<0.001$).
Pretreatment of SMC for 24 hrs with bFGF was without significant effect on AII-induced Ca_i^{++} release from SHR (-7%, n=59, n.s.). In contrast, in SMC from WKY, a significant increase in Ca^{++} release from the internal stores (+46%, n=86, $p<0.001$) and in Ca^{++} influx from external media (+88%, n=189, $p<0.001$) was observed.
On the other hand, Ca_i^{++} steady state in SMC from both SHR and WKY was not significantly lodified by either TGB-ß1 or bFGF.

In conclusion, these results show that TGF-ß1 modulates the activity of Ca^{++} transport mechanisms elicited by AII in SMC from bothSHR and WKY. On the other hand, the regulation of these mechanisms by bFGF is different in WKY and SHR, suggesting differences in the signaling pathways for this growth factor between normotensive and hypertensive rats. These effects of TGB-ß1 and bFGF could be of relevance in the development of hypertrophy of the aortic arterial wall observed in this model of hypertension .

217P DIRECT EVIDENCE THAT LIGAND ACTIVATION OF THE HUMAN 5-HT_{5A} RECEPTOR RESULTS IN COUPLING TO G-PROTEINS IN HEK-293 CELLS

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The neurotransmitter, 5-HT (serotonin) mediates its physiological effects through a diverse family of receptors which regulate a variety of second messenger pathways (Hoyer *et al*, 1994). The human 5-HT_{5A} receptor is a member of this family, however its function is unknown. In the past, hydrophobicity plots have shown the 5-HT_{5A} receptor to possess characteristics of a G-protein coupled receptor (Rees *et al*, 1994). The aim of this study was to clone, express and characterise this receptor subtype.

A complete cDNA, (ORF 357-amino acids) encoding the human 5-HT_{5A} receptor was obtained by PCR, using the high fidelity DNA polymerase, *Pwo*, and stably expressed in HEK-293 cells using the mammalian expression vector, pCI-neo. Radioligand binding assays using [^{3}H]5-carboxyamidotryptamine([^{3}H]5-CT) (0.25-20 nM) were carried out on membrane preparations expressing the receptor. Non-specific binding was defined by the inclusion of 10 µM methiothepin. Competition experiments were performed using 2 nM [^{3}H]5-CT and varying concentrations of competing ligand. The possibility that the human 5-HT_{5A} receptor may couple to G-proteins in HEK-293 cells was investigated using a [^{35}S]GTPγS {guanosine-5'-O-(3-[^{35}S]thio)triphosphate} binding assay. Membranes were incubated with [^{35}S]GTPγS (50,000 cpm) (0.3-0.5 nM) and agonist drug (5-CT), in the presence of GDP to supress basal binding. Non specific binding was defined using 10 µM unlabelled GTPγS.

Saturation binding data for the receptor gave a Kd =7.5 nM and a Bmax =2-3 pmol/mg membrane protein. In competition studies (n=3), methiothepin and 5-CT showed high affinity with Ki =1.6±0.1 nM and 4.6±0.9 nM respectively. The pharmacological profile obtained for the receptor was methiothepin≥5-CT>5-HT≥methysergide>(-)propranolol. Since it is well established that the rat 5-HT_{1B} receptor is negatively coupled to adenylyl cyclase through G-protein recruitment, membranes expressing recombinant rat 5-HT_{1B} receptors were used as a positive control in this study. Dose-dependent increases in [^{35}S]GTPγS binding in response to 5-CT (10^{-8}-10^{-4} M) were obtained for both the 5-HT receptors tested (n=5). The rat 5-HT_{1B} expressing membranes showed a maximal 26.2±3.1% increase in [^{35}S]GTPγS binding over basal. The human 5-HT_{5A} gave a maximal response of 18.8±3.1%. The concentration of 5-CT required to cause half maximal stimulation (EC_{50}) was 8.51×10^{-7} M for the human 5-HT_{5A} receptor and 2.95×10^{-7} M for the rat 5-HT_{1B}. Membranes prepared from untransfected HEK-293 cells showed no such dose-dependent increase in binding of the GTP analogue.

In conclusion, the human 5-HT_{5A} receptor cDNA was amplified by PCR as a 1071 bp open reading frame and stably expressed in HEK-293 cells. The ligand binding profile obtained for the receptor was in agreement to that previously obtained for the receptor when transiently expressed in CosM6 cells (Rees *et al*, 1994). This is the first report describing a G-protein activation for the human 5-HT_{5A} receptor in any cell.

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218P DELAYED BUT LONG-LASTING PRODUCTION OF CYCLIC GMP BY CULTURED SMOOTH MUSCLE CELLS FROM ATHEROSCLEROTIC AORTA IN RESPONSE TO NO

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In response to NO, soluble guanylate cyclases extracted from rabbit atherosclerotic aortas have been described to possess a lower activity than control aortas (Schmidt et al., 1993). However, high concentrations of cGMP have been found in rabbit atherosclerotic aortae due to the induction of NO synthase type II in this tissue (Rupin et al., 1996). The present investigation was designed to evaluate the cGMP production by viable cultured smooth muscle cells from rabbit atherosclerotic aortas in response to NO.

The studies were performed on cultured smooth muscle cells isolated from medial explants from aortas of White New Zealand rabbits fed either a control or a cholesterol rich (0.3%) diet for 45 weeks. Experiments were performed with confluent cells (second or third passage) in a culture medium (M199) without fetal calf serum. Intracellular and extracellular cGMP produced by subcultured smooth muscle cells were measured in cellular extracts and culture supernatants respectively, 30 min after the addition of a phosphodiesterase inhibitor (IBMX, 100 μM). A competitive immunoenzymatic assay was used to quantify cGMP and results were expressed in function of the cellular protein content of each culture determined by the Lowry procedure.

After 48 h of culture under basal conditions, the intracellular and extracellular cGMP produced by subcultured smooth muscle cells from atherosclerotic and control aortas were found to be similar (3.6 ± 0.7 versus 4.3 ± 1.5 pmol/mg of protein in intracellular extracts, n=5, NS, unpaired t-test and 9.1 ± 3.4 versus 4.1 ± 1.6 pmol/mg of protein in the supernatants, n=5, NS, unpaired t-test). After 24 h incubation in the presence of 30 iu/ml interleukin-1β, the NO synthase type II was induced in both cell types as shown by the inhibitory effects of cycloheximide and L-NOARG on cGMP supernatants levels. Under these conditions, the maximal concentration of cGMP in the culture medium was found with 10 iu/ml interleukin-1β for control cells (n=4) and 100 iu/ml interleukin-1β with atherosclerotic cells (n=4). No intracellular increase was found at this time with both cell types. The time course of the supernatant cGMP content after 3, 6, 24 and 48 h of incubation with 100 iu/ml interleukin-1β was also evaluated and cGMP liberation and/or degradation in the supernatant expressed in pmol/mg/hour. Between 3 and 6 h, a cGMP production was found with both cell types. The cGMP production strongly decreased between 6 and 24 h with control cells but remained elevated with atherosclerotic cells (0.1 ± 0.1 pmol/mg/hour versus 3.2 ± 1.3 pmol/mg/hour, n=5, P<0.05, unpaired t-test). In response to sodium nitroprusside (100 μM), a maximal cGMP production was found between 0 and 3 hours with control cells (n=5) and between 3 and 6 h with atherosclerotic cells (n=5). Between 6 and 24 h, control cells no longer produced cGMP while the production persisted with atherosclerotic cells (0.0 ± 0.3 pmol/mg/hour versus 3.7 ± 1.5 pmol/mg/hour, n=5, P<0.05, unpaired t-test).

Our data demonstrate that NO synthase type II is inducible in cultured smooth muscle cells from control and atherosclerotic rabbit aortas in response to interleukin-1β. However, atherosclerotic cells appear less sensitive to the cytokine than control cells suggesting a down-regulation of this pathway in these cells. Moreover, the cGMP production in reponse to NO appears delayed and long-lasting in the atherosclerotic smooth muscle cells suggesting a modulation of the soluble guanylate cyclase activity in these cells even if they are cultured out of the atherosclerotic plaque.

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219P SOMATOSTATIN-INDUCED INCREASES IN EXTRACELLULAR ACIDIFICATION RATES IN CHO-K1 CELLS EXPRESSING HUMAN RECOMBINANT sst_2 AND sst_4 RECEPTORS

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The microphysiometer measures rates of extracellular acidification (EAR) as a means of monitoring cellular metabolism (McConnell et al., 1992). In the present study we have compared the effects of somatostatin (SRIF) and a number of selective SRIF receptor ligands (Raynor et al., 1993) on basal EAR in CHO-K1 cells expressing human recombinant sst_2 and sst_4 receptors which are respectively, members of the distinct $SRIF_1$ and $SRIF_2$ group of receptors. Additional studies to determine the mechanism responsible for SRIF-induced increases in EAR in these cells were also determined.

Approximately 500,000 CHO-K1 cells expressing either human sst_2 ($CHOsst_2$) or sst_4 ($CHOsst_4$) receptors were plated into microphysiometer cups approximately 18h before experimentation. Either a bicarbonate free DMEM (pH7.4) solution or a balanced salt solution (BSS) containing NaCl (138mM), Na_2HPO_4 (0.81mM), KCl (5mM), NaH_2PO_4 (0.11mM), $CaCl_2$ (1.3mM) and glucose (10.0mM) was perfused over the cells at a rate of 120 μL/min. In some studies NaCl was replaced with choline chloride (138mM) and all other sodium salts replaced with potassium equivalents. EARs were measured over a 10s period every 43s and drugs remained in contact with the cells for 3min 40secs. The basal rates of acidification were 100-300μV/s (0.1-0.3 pH unit/min). After an equilibration period of 1h, UTP (3μM) was initially added as a standard. Increasing concentrations of SRIF or other SRIF ligands were added every 30min. All values are mean ± se mean from at least 4 experiments.

In $CHOsst_2$ and $CHOsst_4$ cells perfused with DMEM, UTP caused an increase in basal EAR of 211.1±21.1 and 210.3 ± 23 μV/s, respectively. SRIF caused a concentration-dependent increase in basal EAR in both $CHOsst_2$ and $CHOsst_4$ cells. The potency of SRIF was identical in both cell types (Table 1), although the respective maxima differed ($p<0.05$). Whilst BIM-23027 and MK-678 were the most potent agonists in $CHOsst_2$ cells, they were the least potent at increasing basal EAR in $CHOsst_4$ cells being at least 500 times weaker than SRIF. It is also noteworthy that the maximal response to L-362855 in $CHOsst_4$ cells was approximately 200% of the SRIF maximum. SRIF and UTP also caused increases in EAR when cells were perfused with BSS which were abolished by removal of extracellular sodium or by amiloride (1mM) (Table 2).

Table 1: Potencies of SRIF analogues at increasing EAR in CHO-K1 cells expressing human recombinant sst_2 and sst_4 receptors.

	$CHOsst_2$ pEC_{50}	max %UTP	$CHOsst_4$ pEC_{50}	max % UTP
SRIF	9.6±0.34	43.0±14.5	9.6±0.3	20.5±2.5
MK-678	9.9±0.24	43.3±5.5	<7.0	
BIM-23027	9.9±0.29	40.2±6.2	<7.0	
L-362,855	9.2±0.40	53.9±7.1	8.0±0.1	43.0±4.8
BIM-23056	7.9±0.44	29.7±6.3	8.1±0.2	22.0±5.8

Table 2: SRIF and UTP induced increases in EAR (μV/s) in absence of sodium or presence of amiloride (1mM).

	$CHOsst_2$ 3μM UTP	$CHOsst_2$ 3nM SRIF	$CHOsst_4$ 3μM UTP	$CHOsst_4$ 3nM SRIF
BSS	106.5±14.4	62.0±14.0	129.5±16.9	29.3±10.0
-Na+	-66.8±12.9	-3.9±3.5	-49.5±30.5	-0.3±1.1
+ amiloride	-32.0±20.5	2.7±1.6	-49.8±9.3	-0.3±0.1

The results from the present study demonstrate that there are marked differences in the operational characteristics of human sst_2 and sst_4 receptors expressed in CHO-K1 cells. The increases in EAR following SRIF receptor activation in these cells appears to be entirely attributable to the activity of the Na+/H+ antiporter.

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220P SOMATOSTATIN INCREASES VASCULAR SMOOTH MUSCLE CELL PROLIFERATION IN THE PRESENCE OF TYROSINE KINASE AND PHOSPHATASE INHIBITORS

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Somatostatin-14 (SRIF) has potent inhibitory effects on the growth of vascular smooth muscle cells (Foegh et al., 1989). The mechanism of this antiproliferative effect is at least in part believed to involve activation of tyrosine phosphatase (Buscail et al., 1994). We have therefore investigated the effect of SRIF on the proliferation of rat aortic vascular smooth muscle cells (RAVSMC) in the presence of the tyrosine kinase inhibitors (genistein and lavendustin A) and the tyrosine phosphatase inhibitor, sodium orthovanadate.

Parallel lesions of a monolayer of RAVSMC grown to confluence on Thermanox™ coverslips in DMEM:F12 media supplemented with 10% foetal calf serum (FCS) were made as described by Fan & Frost, 1990. Regeneration of cells onto the denuded area (in the absence of FCS) was measured with a Seescan ™ image analyser and by direct cell counting of cell suspensions after washing the coverslips with 0.05% trypsin/0.02% EDTA for 2-5 min. Unless otherwise stated, all values quoted are mean ± se mean from at least 3 experiments with 4 replicates per test group. SRIF receptor types in RAVSMC were detected by Western analysis using specific anti-sst receptor antibodies.

The basal recovery of the cells into the denuded area 18h after lesioning was 6.0±0.3%. Genistein (50μM), lavendustin A (11nM) and sodium orthovanadate (5μM) had no effect on this recovery but abolished the increase in regeneration (18.9±0.7%) induced by bFGF (10ng/ml). SRIF (100nM) had no effect on basal recovery, but increased regrowth to 17.3±0.9%, 16.8±0.5% and 17.0±1.5% in the presence of genistein, lavendustin and sodium orthovanadate, respectively. In the presence of genistein, SRIF (100nM) increased the number of RAVSMC from 57045±3000 to 89295±2337. The increase in recovery-induced by SRIF in the presence of genistein was unaffected by taxol (18.0±1.4%) but reduced to basal levels by actinomycin D (6.1±1.7%) as well as pretreatment with pertussis toxin (100ng/ml) (6.4±0.7%).

The SRIF-induced increase in recovery was concentration-dependent (pEC_{50} 8.0±0.1) and the effect mimicked by a number of SRIF-receptor selective ligands (Raynor et al., 1993). The sst_5-receptor selective ligand, L-362855 (pEC_{50} 10.4±0.2) was the most potent. SRIF-28 and BIM-23027 were approximately 3 times weaker than SRIF. The sst_5 receptor blocking drug (BIM-23056) had no effect on regrowth in concentrations up to 10μM. In the presence of BIM-23056 (100nM), concentration-effect curves to SRIF were displaced to the right [concentration ratio 43(12-74), geometric mean (95% confidence limits)] with an estimated pK_B of 8.6±0.1.

Western analysis of membrane proteins of RAVSMC with specific anti-SRIF receptor antisera detected the presence of all five (sst_1-sst_5) SRIF receptor types.

The results from the present study demonstrate that in the presence of tyrosine kinase or a phosphatase inhibitor, SRIF promotes rather than inhibits the growth of RAVSMC. The transduction pathway mediating the stimulant effect of SRIF involves activation of pertussis toxin-sensitive G-proteins and is distinct from that activated by bFGF. Although all five different SRIF receptor types were detected in RAVSMC, the high potency of L-362855 and antagonism of SRIF-induced increases in cell regrowth by BIM-23056 (Wilkinson et al., 1996) is consistent with this proliferative effect being mediated by sst_5 receptors.

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221P FUNCTIONAL EFFECTS OF SOMATOSTATIN-14 (SRIF) ON PROLIFERATION OF HUMAN PARIETAL HGT-1 CELLS

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We have previously demonstrated in rat isolated mucosa that SRIF receptors resembling the recombinant sst_2 receptor mediate inhibition of acid secretion directly at the level of the parietal cell (Wyatt *et al.*, 1996). SRIF has been reported to have inhibitory effects on cellular proliferation (Yamada *et al.*, 1994), and we were interested to determine the effect of SRIF on the growth of the human parietal HGT-1 cell line (Reyl-Desmars *et al.*, 1986).

Parallel lesions of a monolayer of HGT-1 cells grown to confluence on Thermanox™ coverslips in DMEM media supplemented with 10% heat inactivated foetal calf serum (FCS) were made as described by (Fan & Frost, 1990). Regeneration of cells into the denuded area, after 24hr in the absence of FCS, was measured with a Seescan™ image analyser and by direct cell counting of cell suspensions after washing the coverslips with 0.05% trypsin/0.02% EDTA for 2-5 min. All values quoted are mean ± se mean from at least 3 experiments with 4 replicates per test group. SRIF receptor subtypes in HGT-1 cells were detected by Western analysis using specific anti-sst receptor antibodies.

Basal rate of recovery after partial denudation was 11.7±0.3% (n=12). PDGF (5ngml^{-1}) and SRIF (100nM) increased recovery to 21.6±1.1% (n=12) and 20.1±0.7% (n=6), respectively. This increase in recovery was unaffected by taxol (1ngml^{-1}) and was associated with an increase in cell number from 51747±6704 to 93304±5609 and 91119±3024, respectively. Interestingly, SRIF (100nM) also inhibited PDGF (5ngml^{-1}) stimulated increases back to control values (50957±3759).

In other experiments a range of SRIF receptor selective ligands(Raynor *et al.*, 1993) were tested on basal and PDGF (5ngml^{-1}) stimulated regrowth. SRIF (pEC_{50} 8.3±0.3) and BIM23027 (pEC_{50} 8.2±0.1) concentration-dependently increased regrowth with maxima of 19.6±1.7% and 25.9±2.5%, respectively. L-362855 and BIM23056 had no effect on basal regrowth at concentrations up to 1μM. SRIF and L-362855 were able to inhibit PDGF stimulated regrowth back to basal values with pIC_{50} values of 8.3±0.3 and 8.1±0.3, respectively. BIM23027 and BIM23056 did not cause inhibitions of PDGF stimulated regrowth at concentrations up to 1μM. Both the stimulatory response to BIM23027 and the inhibitory response to L-362855 were abolished by 100 ngml^{-1} pertussis toxin.

Western blot analysis of whole cell samples of HGT-1 cells with specific anti-sst receptor antisera detected the presence of sst_1, sst_2, sst_4 and sst_5 but not sst_3 receptor types.

The results from this study demonstrate that SRIF can both stimulate and inhibit HGT-1 cell proliferation depending on the growth factor stimulus present. The marked difference in responsiveness to BIM23027 and L-362855 on basal or stimulated regrowth strongly suggests that the proliferative effects are mediated by sst_2 receptors and the inhibitory effects by sst_5 receptors (see Wilkinson *et al.*, 1997). Western analysis demonstrated the presence of both sst_2 and sst_5 receptors in HGT-1 cells, however the possibility that sst_1 or sst_4 receptors are also involved cannot be excluded. Both the mitogenic and antiproliferative effects are mediated via pertussis toxin-sensitive mechanisms.

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222P ANGIOPEPTIN ANTAGONISES THE ANTI-PROLIFERATIVE EFFECTS OF SRIF IN CHO-K1 CELLS EXPRESSING HUMAN RECOMBINANT sst_5 RECEPTORS

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The somatostatin (SRIF) analogue, angiopeptin (BIM23014), has been shown to inhibit vascular smooth muscle cell (VSMC) proliferation associated with restenosis after balloon angioplasty in animal models (Lundergan *et al.*, 1989) and SRIF may modulate the growth of rat VSMC through activation of the sst_5 receptor (Lauder *et al.*, 1997). However, in clinical trials angiopeptin has proven less effective than anticipated at preventing restenosis (Emanuelsson *et al.*, 1995). The present study has functionally examined the action of angiopeptin at inhibiting proliferation of CHO-K1 cells expressing human recombinant sst_5 receptors ($CHOsst_5$).

$CHOsst_5$ cells were seeded at a density of 2×10^5 on to 13mm Thermanox™ coverslips in 24-well plates and grown to confluence. Using the mechanical "wounder" described by Fan & Frost (1990), eleven parallel areas (400μm wide) of the confluent monolayer were denuded of cells. Coverslips were washed three times in PBS, and placed in a fresh well containing drug or vehicle in appropriate media. Experiments were terminated after 24h by washing coverslips three times in PBS and either fixed in absolute ethanol for 5 min, and allowed to air dry before image analysis, or harvested by adding 0.05% trypsin/0.02% EDTA solution for 2-5 min. The digestion process was terminated by adding complete media and single cell suspension counted using a Coulter Counter™. Image analysis was carried out with a Seescan™ semi-automated, image analysis machine. For each coverslip 5 fields of view selected at random were analysed and data expressed as the mean percentage of the area re-covered (%R) following the period of re-growth, using the expression:

$$\%R = [1-(\text{denuded areas at } T_t/\text{denuded areas at } T_0)] \times 100$$

where T_t = period of re-growth and T_0 is the time immediately post-denudation. All values are mean±s.e.mean from 3 experiments with 4 replicates per test group.

SRIF (0.1-1000 nM) had no effect on basal re-growth (7.4±0.1%) after denudation but caused a concentration-dependent (pIC_{50}=8.56± 0.12) inhibition of bFGF-induced (10 ng ml^{-1}) re-growth (20.7±1.2%). At 1μM, SRIF abolished the effect of bFGF. In contrast, angiopeptin (0.1 nM-10 μM) did not affect basal or bFGF-induced (10 ng ml^{-1}) re-growth as seen measuring either % re-growth into the denuded area or direct cell counts (Table 1).

Table 1 Effect of angiopeptin (AP; 100 nM) upon basal and bFGF-induced (10 ng ml^{-1}) re-growth and proliferation after denudation. *P<0.05 to basal re-growth and cell counts.

	% re-growth	Cell counts
Basal	6.4±0.3	232149±19554
AP	5.8±0.2	245102±15099
bFGF	19.6±0.6*	358137±13936*
bFGF+AP	19.0±0.7*	353731±19768*

Angiopeptin was further examined as a potential antagonist at the human recombinant sst_5 receptor and was found to potently inhibit SRIF-induced inhibition of bFGF-induced proliferation (pK_B=10.4±0.3). 5-HT (0.1 nM-10 μM) also inhibited bFGF-induced re-growth (pIC_{50}= 8.36± 0.11). Angiopeptin had no effect on this response (pK_B< 7).

These findings may explain the lack of efficacy of angiopeptin in clinical trials for restenosis, which contrasts with encouraging data found in equivalent studies in the rat.

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223P THE ANTI-PROLIFERATIVE EFFECT OF THE SELECTIVE PHOSPHODIESTERASE (PDE) 4 INHIBITOR DENBUFYLLINE, BUT NOT THEOPHYLLINE, IS REDUCED BY A SELECTIVE PROTEIN KINASE A INHIBITOR

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The proliferation of lymphocytes is involved in orchestrating the inflammatory response in bronchial asthma and other allergic diseases (Azzawi *et al.*, 1992). Lymphocyte proliferation can be inhibited by phosphodiesterase (PDE) 4 as well as isozyme non-selective PDE inhibitors (Torphy & Undem, 1991). Interestingly, the anti-proliferative effects of the PDE4 inhibitors, rolipram and R-(+)-4-[2-(3-cyclopentoxy-4-methoxyphenyl)-2-phenylethyl] pyridine (CDP840) but not theophylline are reduced by indomethacin (Banner & Page, 1997). Whilst the actions of indomethacin are usually attributed to prostaglandin synthesis inhibition, indomethacin is known to have other effects including inhibition of cAMP-dependent protein kinase (Kantor & Hampton, 1978). The present study compared the effects of indomethacin with those of the protein kinase A (PKA) inhibitor, 8-bromoadenosine 3',5'-cyclic monophosphothioate Rp-Isomer (Rp-8-Br-cAMPS) on the anti-proliferative actions of another PDE4 inhibitor, denbufylline. The effect of the PKA inhibitor was also examined on the anti-proliferative actions of theophylline. Peripheral venous blood (25ml) was drawn from healthy volunteers and mononuclear cells (10^5 per well) stimulated to proliferate with phytohaemagglutinin (PHA) (2μg ml^{-1}) in the absence or presence of the PDE 4 inhibitor, denbufylline (0.1-10μM), or the non-selective PDE inhibitor, theophylline (10-1000μM) for 24h at 37°C in a 95% air, 5% CO_2 atmosphere. [^{3}H]-thymidine (0.1μCi per well) was then added and cells incubated for a further 24h before being harvested onto glass fibre filters for scintillation counting of β emission.

Denbufylline was tested alone and in the presence of indomethacin (3μM) or Rp-8-Br-cAMPS (3μM and 10μM). Theophylline was tested alone or with Rp-8-Br-cAMPS (3μM and 10μM). Each drug was examined on 5 blood samples (each from a separate individual) and each concentration of PDE inhibitor was examined in triplicate. Data were analysed by ANCOVA and unpaired t test and results are expressed as % PHA control. Indomethacin alone had no effect on basal or PHA stimulated proliferation. Denbufylline and theophylline produced concentration related inhibition of proliferation (P<0.05). Indomethacin reduced the anti-proliferative effect of denbufylline (1μM) from 47 ± 2% to 104 ± 11% (P<0.05). At a concentration of 3μM, Rp-8-Br-cAMPS had no effect on the anti-proliferative actions of denbufylline whereas Rp-8-Br-cAMPS (10μM) reduced the effect of denbufylline (1μM) from 55 ± 2.5% to 103 ± 18% (P<0.05). In contrast, Rp-8-Br-cAMPS had no effect on the anti-proliferative effect of theophylline (300μM) which was 65 ± 10% in the absence and 64 ± 7% in the presence of Rp-8-Br-cAMPS. These data suggest that denbufylline inhibits proliferation by a PKA dependent mechanism whereas theophylline does not. They also indicate prevention of anti-proliferative effects by indomethacin may occur through inhibition of cAMP-dependent PKA.

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224P TRANSCRIPTIONAL UP-REGULATION OF cAMP PHOSPHODIESTERASES 4A5, 4D1, 4D2 AND 4D3 IN JURKAT T-CELLS: ROLE IN β_2-ADRENOCEPTOR DESENSITISATION

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Inhaled β_2-adrenoceptor agonists are used widely in the treatment of asthma but their excessive use might mask disease progression and thereby increase mortality. One explanation for this paradox is the development of tolerance by leukocytes within the lung to the anti-inflammatory effects of endogenous agents that act through Gs-coupled receptors. Traditionally, desensitisation has been attributed to the phosphorylation of the β_2-adrenoceptor by G-protein receptor-coupled kinases and cAMP-dependent protein kinase. In this study, we have explored an additional mechanism that could produce a state of tolerance that is based upon the enhanced degradation of cAMP by phosphodiesterase (PDE). We have specifically determined whether chronic treatment of Jurkat T-cells with cAMP-elevating agents up-regulates PDE4, the main isoenzyme in immune cells, and the extent to which this compromises β_2-adrenoceptor-mediated cAMP accumulation.

Jurkat T-cells were cultured for 0.5 to 24 h in the absence and presence of 8-Br-cAMP, prostaglandin E_2 (PGE_2) and fenoterol. At various time-points, cells were harvested and cAMP PDE activity was measured together with the expression of PDE4 proteins (western analysis) and mRNA transcripts (semi-quantitative reverse-transcription polymerase chain reactions (RT-PCR)). The ability of isoprenaline to increase in cAMP content in T-cells was also performed in the absence and presence of the non-selective PDE inhibitor, 3-isobutyl-1-methyl xanthine (IBMX), to assess the functional impact of PDE induction.

Pre-treatment of T-cells with 1 mM 8-Br-cAMP, 1 μM PGE_2 and 1 μM fenoterol for 24 h increased PDE activity 2.95-, 1.46- and 1.37-fold respectively (from 3.04 ± 0.14 to 5.57 ± 0.17, 8.96 ± 0.18 and 4.17 ± 0.22 pmol/min/10^6 cells respectively, n = 3). This effect was abolished by actinomycin D and cycloheximide suggesting that a PDE(s) was induced. Inhibition studies in cells exposed to 8-Br-cAMP for 24 h using rolipram (a PDE4 inhibitor) and Org 9935 (a PDE3 inhibitor) demonstrated that both PDE3 and PDE4 were induced. Semi-quantitative RT-PCR, detected mRNA for HSPDE4A5, 4D1, 4D2 and 4D3 in control and treated cells, but not HSPDE4B1 or HSPDE4C1. Kinetic analyses suggested that HSPDE4A5, 4D1 and/or 4D2 were the relevant isoforms as their transcripts were elevated (~ 1.7 to 3.5-fold) prior ($t_{1/2}$ ~ 80 min) to the increase in PDE4 activity ($t_{1/2}$ > 6 h). Western analysis, using an antibody (α-galK-hPDE1) that recognises all forms of PDE4, labelled two bands of immunoreactivity in vehicle-treated cells that, based on molecular weight, corresponded to HSPDE4A5 (125 kDa) and HSPDE4D3 (93 kDa). In contrast, while the intensity of the 93 and 125 kDa bands was unchanged in cells treated with 8-Br-cAMP, an additional 72 kDa band (HSPDE4D1) was labelled that was not detected after vehicle. To assess the functional impact of PDE induction, Jurkat T-cells were cultured for 24 h in medium supplemented with vehicle or a combination of rolipram (50 μM) and PGE_2 (1 μM). Each cell preparation was then exposed to isoprenaline (0.1 nM to 3 μM) in the absence and presence of IBMX (500 μM) and the cAMP accumulated after 60 sec was measured. In vehicle-treated cells, isoprenaline elicited a concentration-dependent increase in cAMP mass (from 0.54 to 2.77 pmol/10^6 cells at 3 μM; 5-fold elevation) with an EC_{50} of 17.1 nM. In contrast, the maximal increase in cAMP effected by isoprenaline in Jurkat T-cells in which PDE had been induced was very modest (from 0.39 to 0.48 pmol/10^6cells) and amounted to merely 2.9% of that elicited in vehicle-treated cells. In the presence of IBMX, isoprenaline-induced cAMP accumulation was increased 3.4- and 31.9-fold in control and 8-Br-cAMP-treated T-cells respectively; no change in EC_{50} was detected between the two cell preparations (15.6 and 29.9 nM respectively). Thus, the maximal increase in cAMP effected by isoprenaline in cells where PDE was induced was only 2.4-fold less than in vehicle-treated cells, compared to a 27.7-fold difference observed in the absence of IBMX. These data suggest that chronic treatment of Jurkat T-cells with cAMP-elevating agents compromises β_2-adrenoceptor-mediated cAMP accumulation in part by stimulating transcription of the HSPDE4D gene which is followed by an increase in protein for HSPDE4D1 and, possibly, HSPDE4D2. If similar effects occur *in vivo* then this could exacerbate airways inflammation by limiting the anti-inflammatory activity of endogenous prostanoids that act via Gs-coupled receptors.

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225P MODULATION OF BK_{Ca} CHANNELS BY THE DIPHENYLUREA, NS1608, IN RAT ACUTELY DISSOCIATED CORTICAL NEURONES

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Large conductance, calcium activated K^+ channels (BK_{Ca}) are found in most excitable cells throughout the mammalian peripheral and central nervous sytems (Latorre et al, 1989). Their large conductance and sensitivity to both calcium and voltage make them a potential pharmacological target for modulation of cell excitability. It has previously been reported that the substituted diphenylurea NS1608, can activate BK_{Ca} channels in various peripheral tissues including coronary arterial and smooth muscle cells (Hu et al, 1995, Hu and Kim, 1996). The purpose of the present study was to examine the effect of NS1608 on brain BK_{Ca} channel activity using whole-cell and inside-out patch clamp recordings from acutely dissociated rat cortical neurones.

The intracellular solutions comprised (mM): 140 KCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.2 with 2.7 $CaCl_2$ (100nM free intracellular Ca^{2+} $[Ca^{2+}]_i$ for whole cell recordings and either 4 or 4.9 $CaCl_2$ (1μM and 10μM $[Ca^{2+}]_i$ respectively) for inside-out patches. The extracellular solution contained (mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.2. for both configurations. All results are expressed as mean ± S.E.M.

In the whole-cell configuration, BK_{Ca} currents were generated by 10mV depolarising steps (200ms duration) from -60 to +100mV from a holding potential of 0mV. In 4 cells, application of 5μM NS1608 (3 minutes) increased the amplitude of steady state BK_{Ca} currents from 89 ± 50pA to 236 ± 69pA at +60mV ($p < 0.01$; n=4). This activation was partially reversible on washout (10 minutes).

Application of NS1608 (1-10μM) to the intracellular aspect of inside-out patches (3 minute exposure) increased BK_{Ca} channel activity (N_fP_o) with no change in maximal single channel conductance or voltage sensitivity (n = 15). 10μM NS1608 reversibly increased BK_{Ca} channel activity; N_fP_o increased from 0.33 ± 0.20 to 1.34 ± 0.53 at +40mV in 1μM $[Ca^{2+}]_i$ ($p < 0.05$, paired t-test; n = 5) and from 0.68 ± 0.26 to 1.66 ± 0.48 at -40mV in 10μM $[Ca^{2+}]_i$ ($p < 0.05$, paired t-test; n = 5). The degree of NS1608-induced activation was the same regardless of whether 1μM or 10μM $[Ca^{2+}]_i$ was present ($p>0.05$, unpaired t-test). The resulting channel activity underwent rapid transitions between maximal and subconductance states, thus appearing as a flickery activation. These data demonstrate that NS1608, at concentrations up to 10μM, is a potent activator of native rat cortical BK_{Ca} channels.

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226P β-AMYLOID TOXICITY ON CULTURED ASTROCYTES

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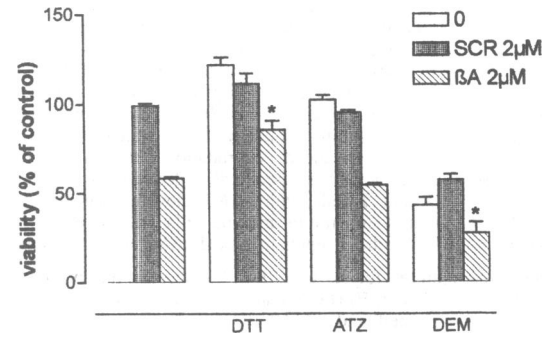
ßAmyloid (ßA) is a 39-42 amino acid peptide which abnormally aggregates forming senile plaques in Alzheimer's disease (AD) brains. ßA is cytotoxic to different clonal cell lines and primary neurons in culture by increasing hydrogen peroxide and altering calcium homeostasis. We have recently described that ßA 25-35 alters astrocyte morphology, increases GFAP staining and decreases viability (Salinero et al.,1997). In this work we have further characterized ßA toxicity on cultured astrocytes and the possible involvement of apoptosis in ßA induced cell death.

Primary astrocyte cultures were prepared from neonatal (P0) rat cortex according to McCarthy and de Vellis (1980). Mechanically dissociated cortices were seeded onto flasks and grown for 3 weeks in DMEM F12, containing 10% fetal calf serum (FCS) and 80 µg/ml gentamicine (37ºC; 5% CO_2/95% O_2). After overnight agitation the cultures were treated with trypsin-EDTA and cells were plated onto 96 well plates precoated with poly-L-Lysine, at a density of 10^4 cells per well. Cultures (DMEM 1% FCS) were treated with ßA 25-35, the smallest fragment with biological activity, or a peptide with a scrambled sequence (Neosystem, France).Viability was measured by the MTT assay and results are expressed as % of untreated cultures. DNA staining was performed with Hoechst 33258 and DNA breaks were detected by the TUNEL method after fixation (4% paraformaldehyde) of the cultures.

ßA decreased astrocyte viability dose-dependently in concentrations up to 8 µM. Higher concentrations did not result in a further effect. The scrambled (SCR) peptide showed no effect. In contrast to previous reports of ßA toxicity on neuronal cultures, Substance P (5 µM), catalase (400 µg/ml) or vitamin E (200 µM) did not alter ßA toxicity. However, the sulfhidryl reducing agent dithiothreitol (DTT, 2mM) significantly reduced ßA effect. Interestingly, cell viability in the presence of ßA was unchanged after blockade of catalase with aminotriazol (ATZ, 5 mM) and was further reduced after inhibition of glutathione peroxidase with diethyl maleate (DEM, 1mM). Cell death may be apoptotic, as judged by increased nuclear TUNEL staining (6h and 24h after addition of ßA 10 µM) and number of cells showing chromatin fragmentation (3 fold increase at 16h vs. untreated cultures).



*:significantly different vs. ßA alone (ANOVA, Student's t test)

In summary, reactive oxigen species also appear to be responsible of ßA-induced apoptotic-like cell death in astrocytes in culture. Impairment of astrocyte function may be detrimental in AD pathology.

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227P EFFECTS OF ET-1 ON INTRACELLULAR CYCLIC NUCLEOTIDE LEVELS IN RAT PULMONARY ARTERIES

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Endothelin-1 (ET-1) has been implicated in the control of the pulmonary arterial ciculation. Although release of ET-1 by pulmonary endothelial cells mediates vasoconstriction in the pulmonary resistance arteries, the second messenger system behind ET-1-mediated contraction is still unclear. The data presented here demonstrate the ability of endothelin receptors to interact with cyclic nucleotide second messenger systems in rat pulmonary arteries.
42 day old male Wistar rats (175g) were killed by an overdose of sodium pentobarbitone, the lungs removed and placed in ice-cold Krebs and the main extralobar and pulmonary resistance arteries were dissected. Whole pieces of tissue (typically between 3-6mg) were incubated at 37ºC for 15 minutes in Krebs containing the general phosphodiesterase inhibitor isobutylmethylxanthine (1mM) and ET-1 (0.1µM) where appropriate. The incubation was terminated upon addition of 4% (w/v) perchloric acid, the tissues homogenised and the samples left overnight at 4ºC. Next day the samples were sonicated for 15 minutes, centrifuged at 3000 rpm for 10 minutes and the supernatant neutralised with KOH/HEPES. Intracellular cyclic AMP was determined using the binding protein method described by Brown *et al.* (1972) whereas intracellular cyclic GMP levels were assessed by the radioimmunoassay method developed by Cailla *et al.* (1976).
The results are shown in Table 1. Stimulation of endothelin receptors by ET-1 in the main extralobar artery resulted in a consistant decrease in intracellular cyclic AMP of 44±15.1% (control = 100%, n=5) and a four fold increase in the levels of this cyclic nucleotide in the pulmonary resistance vessels (402.4±74.9%). In contrast, ET-1 caused a significant increase in intracellular cyclic GMP (190.1±25.3%) in the extralobar arteries but had no effect in the resistance vessels (120.6±14.1). This effect persisted in the absence of endothelium. These data confirm that there is ET-1 receptor heterogeneity present in the different arterial branches of the rat pulmonary system (MacLean and McCulloch, 1994) and that activation of these receptors results in differential modulation of cyclic nucleotides either by direct activation or indirectly via some still to be elucidated cross-talk mechanism. This work was funded by The Wellcome Trust
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Table 1. Effect of 0.1µM ET-1 on intracellular cyclic AMP and cyclic GMP levels in rat pulmonary artery.

Pulmonary artery		Intracellular cyclic nucleotide concentration (pmol/mg tissue) cyclic AMP	cyclic GMP
extralobar	control	4.69±1.76 (n=5)	82.2±21.7 (n=14)
	+ET-1	2.71±1.45 (n=5)	156.6±50* (n=14)
resistance	control	3.39±0.86 (n=7)	36.4±6.4 (n=11)
	+ET-1	11.5±2.46** (n=7)	43.9±9.1 (n=11)

n= number of animals. Results are expressed as mean±SEM. Statistical comparisons using paired Student's t test. *P< 0.05, **P< 0.01

228P ET_B RECEPTOR-MEDIATED VASOCONSTRICTION OF RABBIT PULMONARY RESISTANCE ARTERIES: EFFECT OF DEVELOPMENTAL AGE

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Endothelin (ET) has been implicated in many pathophysiologic conditions including persistant pulmonary hypertension of the newborn (PPHN). Elevated circulating ET-1 levels have been reported in infants with PPHN and a positive correlation with disease severity has been shown (Rosenberg *et al.*, 1993; Kumar *et al*, 1996). We have previously shown that ET-receptor mediated responses (Docherty & MacLean, 1995) and the role of ET_A receptors (Docherty & MacLean, 1996) in rabbit pulmonary resistance arteries (PRAs) alter with developmental age. In this study we intended to investigate the involvement of ET_B receptors in ET-1-evoked vasoconstrictions.

Fetal (2 day preterm), neonatal and adult NZW rabbits were killed with sodium pentobarbitone. Small PRAs were dissected out and mounted as ring preparations (2mm length) on a wire myograph (under ~125mg tension) in Krebs (at 37°C) bubbled with 3% O_2/6%CO_2 balance N_2 for the fetal vessels and 16% O_2 for all others. Cumulative concentration response curves (CCRCs) to ET-1 (1pM-0.3μM) were carried out in the absence or presence of 1μM BQ788 (selective ET_B receptor antagonist).

The results are shown in Table 1. Similar sensitivity to ET-1 was noted in PRAs from fetal, 0-24 hours and 4 day old rabbits, which in turn were significantly more sensitive than vessels from 7 day old and adult rabbits. ET-1 evoked maximum responses of a similar magnitude at all age points studied (~94%). The presence of BQ788 caused a marked rightward shift of ET-1-induced vasoconstrictions in PRAs from fetal, 0-24 hour and 4 day old rabbits however, failed to inhibit responses in vessels from 7 day old and adult rabbits. Furthermore, this antagonist caused a marked augmentation of the maximum ET-1 response, but only at the age points where an inhibition was noted.

The results indicate a hypersensitivity to ET receptor stimulation at a time when plasma ET-1 levels have been shown to be elevated (Levy et al, 1995). We have previously shown that ET_B vasoconstrictor receptors are predominant in rabbit PRAs. This was indicated further by the inhibitory effect of BQ788 in fetal and newborn responses. The failure of BQ788 to inhibit ET-1 evoked responses in the 7 day old and adult may indicate an alteration in ET_B receptor subtype with increasing age. If this hypersensitivity occurs in man, it could only contribute to the high pulmonary resistance prevalent in PPHN.

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Table 1 Effect of BQ788 on ET-1 response in fetal and neonatal rabbit pulmonary resistence arteries.

Developmental age	pEC50				Maximum response (% 50mM KCl)	
	ET-1	n/n	+ 1μM BQ788	n/n	ET-1	+ 1μM BQ788
Fetal	8.7±0.2+	6/6	7.6±0.1**	6/6	95.3±9	140.1±15.8*
0-24 hrs	8.8±0.2+	6/6	8.0±0.1**	6/6	84.0±14	169.6±17.7**
4 days	8.6±0.1+	7/6	8.2±0.0**	6/6	101.7±16.8	163.9±13.4*
7 days	8.0±0.2	6/6	8.3±0.2	7/7	92.7±11.1	115.4±19.9
Adult	7.9±0.3	8/6	7.8±0.1	6/5	93.8±9.6	112±4.7

Statistical comparisons were made by one way analysis of variance: control ET-1 compared to presence BQ788, *p<0.05; **p<0.01; control ET-1 pEC50 compared to 7 day and adult, p<0.05. Values are mean ± SEM. n/n = number of ring preparations/number of animals. ET-1, endothelin-1.

229P RAPID DEVELOPMENT OF PULMONARY HYPERTENSION AND RESPONSES TO ENDOTHELIN-1 IN INDUCIBLE NITRIC OXIDE SYNTHASE KNOCKOUT MICE

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It has been demonstrated that both inducible nitric oxide synthase (iNOS) and endothelial NOS are upregulated by chronic hypoxia in rat lung (LeCras et al., 1996). However, a role of iNOS in the development of pulmonary hypertension (PHT) has not been confirmed. Here we investigate the development of PHT in iNOS knockout mice (MFI -/-, male, 25-40g). These mice and their wild-type controls (MFI+/+) were exposed to hypoxic/hypobaric (10% oxygen) conditions for 10 days. After sacrifice, the heart and lungs were excised and pulmonary resistance arteries (PRAs, ~150μ i.d.) dissected from the lungs. The right ventricle of the heart was weighed as was the left ventricle plus septum. Lungs were sectioned (4μ sections) and stained with Elastin Van Giesen stain. Using light microscopy, we examined the pulmonary arterioles of ~60-80μi.d. and counted those exhibiting vascular remodelling with a double elastic lamina and thickened media. The PRAs were set up on a wire myograph under tension equivalent to 15 mmHg or 35 mmHg (PHT mice). They were bubbled with 16% O_2/6%CO_2 balance N_2. Cumulative concentration-response curves (CCRCs) were constructed for endothelin-1 (ET-1) to examine functional changes. Results are summarised in Table 1. In the wild-type mice exposed to hypoxia, whilst there was significant remodelling of vessels, PHT was not severe enough to induce right ventricular hypertrophy (which is an accurate index of PHT). There was an increase in the maximum response to ET-1 in the PRAs but no change in sensitivity. The iNOS knockout mice exhibited greater vascular remodelling with right ventricular hypertrophy and a marked increase in sensitivity to ET-1. This demonstrates a more rapid development of PHT in the iNOS knockout mice. Absence of iNOS had no effect on normal responses to ET-1 in control mice. This study suggests that iNOS may play a protective role in the early development of PHT, perhaps by exerting an antiproliferative influence. It suggests that iNOS does not normally influence ET-1-induced vasoconstriction in PRAs

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Table 1. Effect of 10 days hypoxia on right ventricular hypertrophy, small vessel remodelling and responses to ET-1 in wild-type and iNOS knock-out mice (n=5).

Group	*RV/TV ratio*	*%Remodelled Vessels (number counted)*	*ET-1 pEC50*	*ET-1 Emax*
Control (+/+)	0.174 ± 0.013	2.2 ± 0.88 (295)	9.90 ± 0.20	147 ± 33
Hypoxic (+/+)	0.210 ± 0.016	23 ± 3.5 (255)***	10.4 ± 0.4	325 ± 69*
Control (-/-)	0.169 ± 0.018	7.2 ± 2.5 (229)	9.21 ± 0.25	191 ± 24
Hypoxic (-/-)	0.235 ± 0.012*	61.8 ±11.3 (219)**†	10.83 ± 0.3***309 ± 106	

(-/-): iNOS knockout mice; (+/+): wild-type controls; RV/TV: right ventricular/total ventricular weight; Emax: maximum response expressed as % of response to 50mM KCl. Statistical comparisons using unpaired Student's t test: compared with appropriate control *P<0.05; **P<0.01; P<0.001; compared with hypoxic (+/+) †P<0.05.

230P DEPRESSOR RESPONSES TO ENDOTHELIN-1 IN THE SUPERIOR COLLICULUS INVOLVE ET_B AND ADRENERGIC RECEPTORS

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Endothelin-1 (ET-1) microinjected to the superficial layer of the brain superior colliculus (SC) induces decreases in blood pressure (D'Amico et al., 1996). However, no study has been done on which receptor mediate(s) the response to endothelin-1 into the SC. Thus, using anaesthetised rats we have examined the effects of the endothelin receptor antagonists, FR 139317 (ET_A receptor selective), SB 209670 (ET_A/ET_B receptor non-selective) and BQ-788 (ET_B receptor selective antagonist) on the responses following administration of ET-1 to the superficial layer of the SC of anaesthetised male Wistar rats (250-200 g).

The rats were anaesthetised with urethane (1.2 g/kg i.p.) and their femoral arteries cannulated. The spontaneously breathing animals were then placed in a stereotaxic head frame and the dorsal surface of the brain exposed by a craniotomy to permit intracerebral microinjections. The coordinates of the atlas of Paxinos and Watson (1986) (measured in mm from the bregma: posteriorly, -8.0; laterally, 0.5; vertically, 3.2) were used to position the microsyringe. Statistical analysis was performed by using ANOVA.

ET-1 (10 pmol) (100 nl volume) decreased (32 ± 3%) the basal mean blood pressure (MABP; 109 ± 6 mmHg, n=6) associated with systemic and regional haemodynamic changes as measured by administration of ^{57}Co-labelled microspheres into the left ventricle. For instance, ET-1 decreased the total peripheral resistance by 39 ± 2% and decreased the vascular resistance in the skin (53 ± 7%), the muscles (44 ± 3%), the spleen (42 ± 3%), the left and right kidney (38 ± 4% and 45 ± 5%, respectively), the mesentery (43 ± 4%), the large and small intestine (35 ± 2% and 28 ± 4%, respectively); n=5. ET-1 also increased the blood flows and the percentage of cardiac output distributed to these organs. ET-1 did not affect the heart rate (ET-1, 394 ± 6 beats min^{-1}, n=4; control, 401 ± 7 beats min^{-1}, n=4).

Decreases in blood pressure induced by ET-1 were greatly reduced by pre-administration to the SC of BQ-788 (5 nmol/rat) or SB209670 (3 nmol/rat) (94 ± 5 and 98 ± 6%, $p<0.01$, n=6, respectively), while not affected by FR139317 (5 nmol/rat) (6 ± 3%, $p>0.05$, n=6). Decreases in blood pressure induced by ET-1 were also significantly ($p<0.05$) reduced by pre-administration to the SC of the α_1-adrenoceptor agonist phenylephrine (1 nmol) (40 ± 5%, n=4), β_1/β_2-adrenoceptor antagonist propranolol (3.4 nmol) (48 ± 11%, n=5), or β_1-adrenoceptor antagonist acebutolol (5 nmol) (50 ± 6%, n=4). Microinjections to the SC of isoprenaline (1 nmol), β_1/β_2-adrenoceptor agonist; dobutamine (4 nmol), β_1-adrenoceptor agonist; prazosin (2.4 nmol), α_1-adrenoceptor antagonist, increased significantly ($p<0.05$) the hypotensive effect induced by ET-1 (47 ± 6%, n=4; 50 ± 9%, n=4; 47 ± 7%, n=5, respectively). In contrast, decreases in blood pressure induced by ET-1 were not affected by pre-administration to SC of clonidine, α_2-adrenoceptor agonist, yohimbine, α_2-adrenoceptor antagonist, or salbutamol, β_2-adrenoceptor agonist.

Finally, treatment of the SC with ET antagonists or selective/non-selective adrenoceptor agonists/antagonists alone or prior to ET-1 did not cause any change in basal heart rate, with exception of isoprenaline which, alone or prior ET-1, caused a slight increase in heart rate (e.g. control, 397 ± 13 beats min^{-1}, n=4; isoprenaline + ET-1, 427 ± 9 beats min^{-1}, $p<0.05$, n=4).

In conclusion, ET_B receptors are the predominant mediators of the actions of endothelin-1 in the superior colliculs of the rat. These latter effects are also exerted with selective involvement of alpha$_1$- and beta$_1$-adrenoceptors.

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Paxinos, G.; Watson, C. (1986). *The rat Brain in the Stereotaxic Coordinates, 2nd edn.* San Diego: Academic Press.

231P HAEMODYNAMIC RESPONSES TO CHRONIC LIPOPOLYSACCHARIDE (LPS) INFUSION IN CONSCIOUS, HYPERTENSIVE TRANSGENIC ((mRen-2)27) RATS: INVOLVEMENT OF ENDOTHELIN

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Mullins *et al* (1990) created hypertensive, transgenic ((mRen-2)27) rats by inserting the mouse Ren-2 renin gene into the rat genome. In these animals, the hypertension and regional vasoconstrictions are reduced by the non-selective endothelin (ET) antagonist, SB 209670 (Gardiner *et al* 1995c). Since, in Long Evans rats, we have evidence for a substantial involvement of ET in the haemodynamic effects of LPS (Gardiner *et al* 1995a), here we have compared responses to LPS in hypertensive, transgenic (TG) rats and normotensive, control (Hannover Sprague-Dawley (SD)) rats, and assessed the effects of SB 209670. Male, heterozygous TG rats (4-5 months old, 400-500g) and homozygous, age-matched SD rats were chronically instrumented with pulsed Doppler probes and intravascular catheters (all surgery was carried out under sodium methohexitone anaesthesia, 40-60 $mgkg^{-1}$ i.p., supplemented as required). LPS (*E coli* serotype 0127 B8, Sigma) was infused (150 $\mu gkg^{-1}h^{-1}$ i.v.) continuously for 24h. At 24h, a primed i.v. infusion (10 μgkg^{-1} bolus, 600 $\mu gkg^{-1}h^{-1}$) of SB 209670 was begun and continued with LPS for 7h. Before LPS, mean arterial blood pressure (MAP) and renal, mesenteric and hindquarters vascular conductances (RVC, MVC, HVC, respectively), but not heart rate (HR), differed between the SD and TG rats: MAP (mmHg) SD 108 ± 3, TG 171 ± 2; RVC, MVC, HVC ([kHz $mmHg^{-1}$]10^3): SD 71 ± 7, 79 ± 7, 59 ± 6, TG 39 ± 3, 36 ± 3, 28 ± 3, respectively, all $P<0.05$ (Mann-Whitney U test); HR (beats min^{-1}): SD 359 ± 8, TG 326 ± 8). Responses to LPS and SB 209670 are shown in Table 1.

One h after the onset of LPS infusion, TG rats showed a marked fall in MAP and a renal vasodilatation, in contrast to SD rats. However, by 24h neither group was hypotensive, although renal vasodilatation was apparent in SD, but not TG, rats at this stage (Table 1). Following 7h co-infusion of LPS and SB 209670, there was a substantial reduction in MAP in TG rats, which was 5-fold greater than that seen in SD rats. The marked hypotensive effect of SB 209670 in TG rats was accompanied by significant vasodilatation in all 3 regions monitored, but particularly in the renal and mesenteric vascular beds. These results are consistent with a substantial involvement of ET in the maintenance of cardiovascular status in endotoxaemic TG, compared to SD, rats. Moreover, considering our results (Gardiner *et al* 1995b) on the hypotensive effects of SB 209670 in TG rats under normal conditions (Δ MAP -31 ± 4 mmHg over 7h), it appears that LPS markedly enhances the contribution of ET to cardiovascular regulation in TG rats.

Table 1. Changes (mean ± s.e. mean) in SD (n = 7) and TG (n = 8) rats during LPS infusion. The change at 31h was after co-infusion of LPS and SB 209670 for 7h. *P<0.05 for change (Wilcoxon test).

		1h	24h	31h
MAP	SD	-11 ± 3*	4 ± 3	-12 ± 3*
(mmHg)	TG	-35 ± 3*	-2 ± 4	-62 ± 3*
RVC	SD	11 ± 6	29 ± 10*	19 ± 8*
([kHz $mmHg^{-1}$]10^3)	TG	40 ± 2*	2 ± 3	26 ± 4*
MVC	SD	-7 ± 6	0 ± 3	14 ± 8
([kHz $mmHg^{-1}$]10^3)	TG	11 ± 2*	-6 ± 4	26 ± 6*
HVC	SD	16 ± 3*	14 ± 7	25 ± 9*
([kHz $mmHg^{-1}$]10^3)	TG	12 ± 2*	-3 ± 3	11 ± 3*

Thanks to Dr John Mullins for the SD and TG rats, and to Dr Eliot Ohlstein (SKB) for SB 209670.

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Gardiner, S.M. *et al.* (1995b) Br.J.Pharmacol., 116, 2005-2016
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232P ENDOTHELIN-B RECEPTOR-MEDIATED CONSTRICTION CAN BE UNCOVERED IN CEREBRAL PIAL ARTERIOLES

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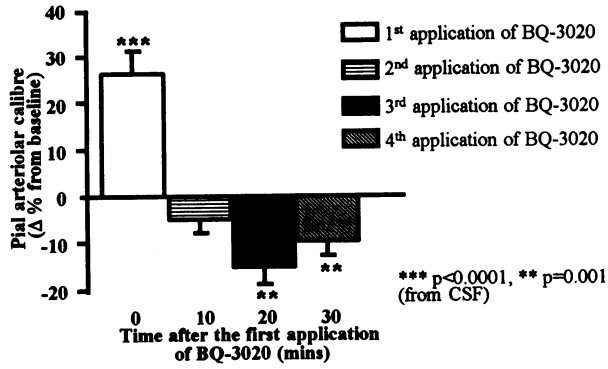
In the cerebrovascular circulation, endothelin (ET)-A receptor activation mediates marked prolonged vasoconstriction whereas ET-B receptor activation effects dilatation. Nevertheless, ET-B receptor induced constriction has been demonstrated in some vascular beds but not yet in the brain. Through the use of a selective ET-B receptor agonist (BQ-3020) and ET-B antagonist (BQ-788), these investigations aimed to investigate if ET-B receptor-mediated constriction could be uncovered in cerebral pial arterioles *in vivo*.

The experiments were carried out in six anaesthetized (α-chloralose) and ventilated (25% O_2 in air) cats. All key physiological parameters were monitored and maintained within physiological limits during the experiment. A left parietal craniotomy was performed to expose the pial arterioles overlying the parasagittal, suprasylvian and ectosylvian gyri. Artificial cerebrospinal fluid (CSF), BQ-3020 (ET-B agonist; 1 μM, N-acetyl-Leu Met Asp Lys Glu Ala Val Tyr Phe Ala His Leu Asp Ile Ile Trp), BQ-788 (ET-B antagonist; 0.001-1 μM, N-cis-2,6-dimethylpiperidinocarbonyl-L-gamma-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine), or potassium (10 mM) were applied into the perivascular space surrounding individual arterioles. The pial arteriolar calibre was measured using of a video image splitting device (Patel et al., 1996). The data are expressed as mean ± SEM. Statistical analysis was carried out using ANOVA followed by two-tailed Students's unpaired t-test with a Bonferroni correction factor. In each experiment, n=4-16 (n, the number of arterioles examined).
While the first application of the ET_B receptor agonist BQ-3020 (1 μM) elicited a dilatation of the arterioles, marked tachyphylaxis of vasodilatator responses was observed in response to a second application of BQ-3020. Furthermore, subsequent advential microapplications of the ET-B receptor agonist effected a significant constriction of cerebral arterioles (figure). In separate experiments, the arterioles did not display tachyphylaxis to repeated applications of potassium. The perivascular application of the ET-B receptor antagonist BQ-788 (0.001-1 μM) had no effect on arteriolar calibre per se but blocked both BQ-3020-induced dilatation (IC_{50}:5 nM) and vasoconstriction.



This investigation demonstrates, that repeated activation of ET-B receptors induces not only tachyphylaxis of the vasodilator response, but also demonstrates, for the first time, a constriction of cerebral resitance arterioles *in vivo*. The data suggest a potential role of ET-B receptors in pathophysiological situations where release of endogenous agonist is increased.

Patel et al., (1996) *Eur J Pharmacol* 307:41-48

233P VASCULAR ET-1 RECEPTORS IN A RAT MODEL OF CHRONIC HEART FAILURE

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Plasma endothelin-1(ET-1) levels are raised 2 to 3 fold in chronic heart failure (CHF), correlate with disease severity, and may contribute to exercise intolerance, outcome and increased peripheral vascular resistance in this condition. ET receptor antagonists therefore have a potential therapeutic role in the treatment of CHF.

This study was designed to investigate the receptors mediating the effects of ET-1 in small mesenteric arteries from rats with CHF induced by coronary artery ligation and sham-operated controls. The role of the ET_A receptor in ET-1 constriction was investigated using the selective ET_A receptor antagonist, BQ-123.

CHF was induced in male Wistar rats (200 - 250g) by ligation of the left anterior descending coronary artery (LAD) under pentobarbitone anaesthesia. In sham-operated rats, the ligature was placed under the LAD and pulled through. 5 weeks post-operation the rats were sacrificed after the left ventricular end diastolic pressure (LVEDP) had been measured to confirm the presence of CHF. The mesenteric bed was immediately excised and placed in Krebs-Henseleit solution. Third order mesenteric arteries (internal diameter 312±8.5μm) were dissected, mounted in a perfusion myograph and the intraluminal pressure raised to 60mmHg. The arteries were constantly superfused with oxygenated Krebs-Henseleit solution. After 1hr equilibration, vessels were challenged with 60mM KCl solution (x2) and the endothelium removed by the passage of an air bubble through the lumen. Denudation was confirmed by the loss of dilatation to acetylcholine (10^{-6}M) following pre-constriction by phenylephrine (10^{-5}M). A 30ml reperfusion circuit of Krebs-Henseleit solution was set up and vessels were superfused with Krebs-Henseleit solution control (n=5 per group) or BQ-123 (10^{-6}M, n=5) for 30 min. Cumulative concentration-response curves (CRCs) to ET-1 (10^{-13}- $3x10^{-8}$M) were constructed and internal lumen diameter measured continuously. The results are expressed as a % of maximum constriction to 60mM KCl.

All CHF rats had an LVEDP > 15mmHg. ET-1 produced concentration-dependent constrictions in vessels from both CHF and sham-operated rats. There was no difference in the sensitivity of the arteries in the two groups to ET-1 (CHF EC_{50} = 5.1±1.1 x 10^{-10}M vs sham-operated EC_{50} = 5.5±2.9 x 10^{-10}M; P=0.88 students t-test). BQ-123 inhibited responses to concentrations of ET-1 above 10^{-9}M, without affecting responses to lower concentrations, as previously described in vessels from normal Wistar rats (Mickley *et al.*, 1997). The EC_{50}s could not be calculated because maximum constriction was not reached in all the vessels studied.

Our results show that there is no alteration in the sensitivity to ET-1 in small mesenteric arteries from rats with CHF, 5 weeks post-infarct. Furthermore, at this time point, the relative involvement of ET_A and non-ET_A (ET_B) receptors in mediating ET-1 induced constriction is not altered relative to the control sham-operated group. The results suggest that, as in normal vessels (Mickley *et al.*, 1997), a combined ET_A/ET_B receptor antagonist would be most effective for inhibition of ET-1 induced constriction in CHF.

Mickley, E.J., Gray, G.A. & Webb, D.J. (1997). *Br. J. Pharmacol.*, **120**,1376-1382.

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234P EVIDENCE FOR AN ATYPICAL ENDOTHELIN RECEPTOR MEDIATING ENDOTHELIN-INDUCED MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT

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Endothelin (ET) is proposed to act via two receptor subtypes: an ET_A receptor at which endothelin-1 (ET-1) is more potent than endothelin-3 (ET-3) and an ET_B subtype which is isopeptide non-selective. ET-1 induces focal cerebral ischaemia when injected in close proximity to the rat middle cerebral artery (MCA) (Sharkey *et al*, 1993). In vitro studies suggest that the ET_A receptor subtype mediates the vasoconstrictor actions of ET within the cerebrovasculature (Feger *et al*, 1994). However, there is evidence for an atypical receptor on cerebral arteries (Salom *et al*, 1995). To further characterise the ET receptor mediating constriction of the MCA *in vivo*, we have examined the effects of microapplication of ET-3, ET-1 and of the selective ET_B receptor agonist IRL1620 (James *et al*, 1993) onto the rat MCA. We also evaluated the ability of the selective ET_A receptor antagonist, FR139317, to inhibit ET-1 and ET-3 mediated ischaemia.

Male Sprague-Dawley rats (280-320g; n = 91) were anaesthetised (halothane in N_2O:O_2) and placed in a stereotaxic frame, where normothermia was maintained by a heating blanket. Following craniectomy (Bregma: AP = 0.9mm, L = -5.2), a cannula was lowered 8.7mm below the skull, and ET-3, ET-1, IRL1620 (100pmols in 2µl) or vehicle injected. For co-injection studies, a dual cannula was used for injection of FR139317 (3nmols in 3µl; Sogabe *et al*, 1993) 10 minutes before ET-1 or ET-3 (100pmols in 2µl). Three days later, the rats were killed and the brain processed for quantitative histopathology (Sharkey *et al*, 1993). Laser Doppler flowmetry (LDF) was used to record cortical tissue perfusion velocity following ET-1 and ET-3-induced MCA occlusion. Rats were anaesthetised, and a catheter inserted into the right femoral artery for blood gas analysis. Rats were placed in a stereotaxic frame, and following a craniectomy, LDF recordings made from the parietal cortex for 15 min prior to injection of endothelin isopeptide (100pmols in 2µl), and for 3 hours afterwards. Physiological variables were kept within normal limits for LDF studies. Data are presented as mean ± s.e. mean and were analysed Students t-test and two-way ANOVA.

Perivascular injections of ET-3 and ET-1 onto the MCA maximally reduced cortical tissue perfusion by 68 and 78% respectively. Both ET-1 and ET-3 depressed blood flow by >50% throughout the 3 hour measurement period, and there were no statistically significant differences in maximal reduction or temporal profile of oligaemia produced by the two ET isopeptides. Histopathological analysis revealed that both ET-1 and ET-3 produced large ischaemic lesions within the vascular territory of the MCA. The volume of damage produced by ET-3 (128 ± 20mm^3) was not significantly different from that of ET-1. Similarly, the minimum effective dose required to produce ischaemia (33pmols) was the same. However, while pretreatment with FR139317 reduced ET-3 mediated ischaemic damage by > 90% it had no effect on ET-1 mediated lesions. Furthermore, tissue damage produced by the ET_B receptor agonist, IRL1620 (100pmols) was not significantly different from sham controls.

The equipotency of ET-1 and ET-3 in reducing cerebral blood flow and producing ischaemic brain damage is consistent with the presence of an ET_B mediated vasoconstriction of the MCA. However, the ability of FR139317 to block ET-3 but not ET-1 mediated ischaemia and the lack of efficacy of IRL1620, suggest the presence of an atypical endothelin receptor.

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Feger *et al* (1994) *J.Cereb.Blood Flow Metab.*, 14: 845-852.
Salom *et al* (1993) *Cerebrovasc.Brain Metab.Rev.*, 7: 131-152.
James *et al* (1993) *Cardiovasc.Drug Rev.*, 11: 253-270.

235P FURTHER DEVELOPMENT OF A NOVEL MODEL OF FOCAL CEREBRAL ISCHAEMIA TO INCLUDE CONTROLLED REPERFUSION

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Since vascular occlusion following stroke in humans is seldom permanent, animal models of stroke have been developed to incorporate controlled reperfusion (Overgaard, 1994). However, physical damage to the cerebral arteries incurred during some occlusion procedures, may confound interpretation of the pathophysiology (McAuley, 1995). Whilst the use of endothelin-1 (ET-1) to occlude the middle cerebral artery (MCA) of the rat circumvents mechanical damage to cerebral vessels, control over the duration of ischaemia is limited (Sharkey et al. 1993). Consequently we have modified an existing model of ET-1-induced MCA occlusion to permit controlled reperfusion.

Male Sprague-Dawley rats (280-320g; n = 33) were anaesthetised (halothane in N_2O : O_2), placed in a stereotaxic frame, and maintained normothermic by a heating blanket. Following craniectomy (Bregma: AP = 0.9mm, L = -5.2), a dual-cannula (24 gauge containing a 31 gauge needle) was lowered 8.7mm below skull and ET-3 (100 pmols in 2µl) was injected. Following 10 or 90 minutes, the specific ET_A receptor antagonist FR139317 (Sogabe *et al*, 1993) was injected (3nmols in 3µl), the cannula withdrawn, and the animal returned to a cage following recovery in an incubator. Three days later, rats were killed and the brain processed for quantitative histopathology (Sharkey *et al*, 1993). For laser Doppler flowmetry (LDF) studies, rats were anaesthetised as above, a catheter inserted into the right femoral artery for blood analysis, and placed in a stereotaxic frame. A craniectomy was then performed and LDF probes advanced to the dura over the parietal cortex for baseline recordings made. ET-3 (100 pmols in 2µl) was injected followed 10 minutes later by FR139317 (3nmols in 3µl), and recordings continued for a further 90 minutes. Data are presented as mean ± s.e. mean and analysed by Student's t-test and one-way analysis of variance.

ET-3-induced MCA occlusion resulted in a large reproducible infarct (132 ± 27mm^3; n = 10) restricted to the vascular territory of the MCA. Injection of FR139317, 10 minutes after the ET-3 resulted in a 91% reduction in ischaemic damage. By contrast, the volume of ischaemic damage following injection of FR139317 after a 90 minute delay, was not significantly different to the standard ET-3-induced MCA occlusion. Physiological parameters were maintained within normal limits for LDF studies. LDF determined that tissue perfusion velocity was reduced by > 60% 5 minutes following ET-3-induced MCA occlusion (n = 4). Injection of FR139317 after a 10 minute delay reversed this perfusion deficit to baseline levels within 5 minutes.

These data demonstrate that ET-induced MCA occlusion can be manipulated to produce a model of focal cerebral ischaemia with controlled reperfusion by the use of ET-3 instead of ET-1 and the injection of an ET_A receptor antagonist.

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McAuley, (1995). *Cerebrovasc. Brain Metab. Rev.* 7: 53-180
Sharkey *et al.* (1993). *J. Cereb. Blood Flow Metab.* 13: 865-871
Sogabe *et al.* (1993) *J. Pharmacol. Exp. Ther. 262*: 1040-1046

236P EFFECTS OF EXPOSURE TO REACTIVE OXYGEN SPECIES ON CONTRACTION AND RELAXATION RESPONSES IN THE RAT LEFT ANTERIOR DESCENDING CORONARY ARTERY

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Reactive oxygen species (ROS) have been suggested to be involved in the injury to blood vessels occurring after ischaemia and reperfusion, with ROS scavengers reducing damage (Seccombe & Schaff, 1995). This study examines the effects of exposure to two of these species on contraction and relaxation of the rat left anterior descending coronary artery (LAD).

Segments of LAD (2.0 mm long; normalised internal diameter 200-400 μm) from male Wistar rats (250-450 g) were mounted in a wire myograph (JP Trading, Aarhus, Denmark). The vessel was bathed with physiological solution (mM: NaCl 115.3, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5, glucose 11.1) equilibrated with 95% O_2/5% CO_2 at 37°C. Vessels were equilibrated for 60 min before normalisation (Mulvany & Halpern, 1977). After another 30 min, control responses were obtained before vessels were exposed either to the ROS generating system xanthine/xanthine oxidase (0.1 mM/100 mU ml^{-1}) or to hydrogen peroxide (H_2O_2; 1 mM) for up to 30 min. Vessels were washed at the end of the exposure and allowed a further 30 min to equilibrate before redetermining cumulative concentration-response curves. Statistical comparison was by analysis of variance.

Exposure to xanthine/xanthine oxidase for 30 min caused only a 10% decrease in the maximum contraction to 5-HT from 6.3 ± 0.2 mN (control) to 5.7 ± 0.1 mN ($P < 0.05$; n=5), and had no effect relaxation to carbachol (CCh; Table 2) or the adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) in vessels precontracted with a submaximal concentration (3 μM) of 5-HT.

Exposure to H_2O_2 (1 mM) for either 10 or 15 min did not alter contraction to 5-HT, whereas exposure for 20 min reduced the maximum response with only a small change in EC_{50} (Table 1). Exposure to 1 mM H_2O_2 for 30 min greatly reduced contraction to 5-HT. This effect was not limited to 5-HT; contraction to both endothelin-1 (ET-1) and KCl were also almost completely abolished following 30 min exposure.

Table 1 Contraction to 5-HT in rat LAD after H_2O_2 exposure

	Max response (mN)	EC_{50} (nM)	n
Control	2.5 ± 0.1	895 ± 111	8-12
10 min H_2O_2	2.8 ± 0.1	1417 ± 168	6-10
15 min H_2O_2	2.4 ± 0.1	1724 ± 43	7
20 min H_2O_2	1.4 ± 0.1 ***	6138 ± 1097 ***	7
30 min H_2O_2	0.2 ± 0.1 ***/†††	2806 ± 1724	4

***, †††: $P < 0.001$ relative to control and 20 min exposure, respectively.

Ten min exposure to H_2O_2 had no significant effect on relaxation to CCh but exposure for 15 min slightly potentiated the maximum relaxation (Table 2). Neither 10 or 15 min exposure to H_2O_2 had any significant effect on relaxation to NECA.

Table 2 Relaxation to carbachol in 5-HT-precontracted rat LAD

	Max relaxation (% of 5-HT induced tone)	EC_{50} (nM)	n
Control	61.8 ± 2.0	988 ± 475	10-14
10 min H_2O_2	65.1 ± 1.2	1587 ± 140	6-7
15 min H_2O_2	82.4 ± 1.0 ***	1307 ± 77	8

***: $P < 0.001$ relative to both control & 10 min exposure.

These results suggest that neither xanthine oxidase (which produces superoxide and H_2O_2) nor H_2O_2 are particularly injurious to the LAD, altering responses only at high concentrations and long exposures. This is in contrast to results following exposure to peroxynitrite, which caused damage after short exposures (Otley *et al.*, 1997).

CEO is a Medical Research Council Research Student.

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Seccombe, J.F. & Schaff, H.V. (1995). *Ann. Thorac. Surg.*, 60, 778-788.

237P THE ANGIOTENSIN II-INDUCED VASOCONSTRICTION DURING NO BLOCKADE IN THE RENAL VASCULATURE: DUAL AT_1/AT_2-SENSITIVITY AND COUPLING TO ENDOTHELIAL P450 SYSTEM

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Although the renovascular actions of angiotensin II (AII) are primarily mediated by the AT_1 receptor subtype, AT_2 binding sites have also been evidenced in the renal vasculature. In other respects, NO is known to buffer renal effects of vasoconstrictors (Navar *et al.* 1996). We queried whether the NO-masked, AII-induced vasoconstriction (AII-VC) occurs via multiple receptor subtypes.

Isolated perfused rat (200-240 g) kidneys, (IPK) were perfused at constant flow with Krebs-gelatine buffer, in the presence of 0.1 mM indomethacin. A common pressure baseline of 90 mm Hg was used. The IPK were perfused throughout with/without the NO-synthase inhibitors L-NAME (0.1 mM) or L-NOARG (1 mM), or soluble guanylyl cyclase inhibitor LY83583 (10 μM), alone or in combination with either AII receptor antagonists, L15809 (AT_1), PD123319 (AT_2) or CGP42112 (AT_2) or 10 μM α-naphtoflavone (NF), a P450 inhibitor. After 60 min equilibration, 0.1 nM AII was infused close to the renal artery over a fixed period of 6 min. Values are means ± sem.

Perfusing IPK with L-NAME, L-NOARG or LY83583, comparably unmasked ($p<0.05$) VC to 0.1nM AII: (in mmHg): control = 3±2 (n=8); L-NAME = 59±7 (n=5); L-NOARG = 49±1 (n=3); LY83583 = 59±2 (n=3). Though L158809 fully inhibited this AII-VC (IC_{50}~0.025nM),a substantial fraction (64%) of the AII-VC was also sensitive to nanomolar concentrations of PD123319 (IC_{50}~0.5nM) (Fig. 1). CGP42112 at 5 nM also decreased by 52% this AII-VC. We then damaged endothelium by pretreating IPK with either CHAPS (0.2%, 30 sec). This treatment has previously been shown to blunt acetylcholine-induced vasodilation in IPK, whithout altering noradrenaline-VC (Massfelder *et al.*, 1996). CHAPS treatment decreased the L-NAME-potentiated AII-VC by 60% and 31% ($p<0.05$), respectively, suggesting the involvement of endothelium-derived vasoconstrictor compounds in the NO-masked AII-VC. Perfusion of IPK in the presence of NF inhibited by 85% ($p<0.05$) the L-NAME-unmasked AII-VC.

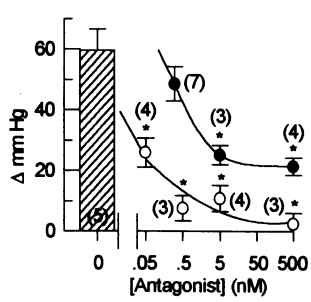

Fig.1. 0.1nM AII-VC in IPK during NO-blockade by 0.1 mM L-NAME, in absence (bar) or presence of increasing concentrations of L158-809 (○) or PD123319 (●). Values are means ± sem for the number of IPK indicated in parentheses. Asterisks indicate significant inhibitions ($P<0.05$ in ANOVA test) caused by AII receptor antagonists.

Taken together, the results suggest that NO-masked constrictions to subnanomolar concentrations of AII are mediated by classical AT_1 receptors and unidentified AT_1/AT_2-sensitive receptors. Moreover, the endothelial release of prostanoids derived from cytochrome P450 enzyme system mediates these NO-masked AII-VC, suggesting that tonic release of NO inhibits the activation of the endothelial PLA_2/P450 pathway in response to physiological concentrations of AII.

Navar, L.G., Inscho, E.W., Majid, D.S.A. *et al.* (1996) *Physiol. Rev.* 2, 425-536.
Massfelder, T., Stewart, A.F., Endlich, K. *et al.* (1996) *Kidney Int.* 50, 1591-1603.

238P EFFECT OF AGEING ON ENDOTHELIAL FUNCTION AND VASCULAR REACTIVITY IN RAT CONDUCTANCE AND RESISTANCE ARTERIES

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Endothelium-dependent vasorelaxation induced by acetylcholine (Ach) and the mechanisms of Ca^{2+} handling involved in contraction produced by noradrenaline (NA) were investigated in the aorta and in branch II or III of superior mesenteric arteries (SMA, internal diameter: 150-200 µm) from 14 (young adult) or 60-70 (old) week old male rats.

The vessels were mounted in organ chambers 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, $CaCl_2$ 1.25 (aorta) or 2.5 (SMA), glucose 5.5 continuously kept at 37°C and gassed with a mixture of 95% O_2, 5% CO_2 (pH 7.4). In nominally Ca^{2+}-free PSS, calcium was omitted and 0.5 mM EGTA was added. Contraction was recorded isometrically. For Ca^{2+} handling experiments, the endothelium was removed either by gently rubbing the intimal surface with curved forceps for the aorta or by intraluminal perfusion with 0.5% 3-[(3-Cholamidopropryl) dimethylammonio]-1-propanesulfonate for 30 s for the SMA. For relaxation experiments, arteries with functional endothelium were pre-contracted to 80% of maximal contraction produced by NA, the NA concentration being 0.3 µM for the aorta and 3 µM for the SMA. Results are expressed as a percentage of maximal contractile response to NA (contraction experiments) or percentage of the pre-contraction level (relaxation experiments). They are given as mean ± s.e.mean of at least 5 experiments. Analysis of variance (MANOVA) was used for statistical analysis.

In vessels with endothelium, the level of pre-contraction with NA was 2.4 ± 0.26 and 2.0 ± 0.26 g mg^{-1} dry tissue in the aorta and 3.2 ± 0.35 and 3.2 ± 0.32 mN mm^{-1} in the SMA from young adult and old rats (not significantly different with age). Acetylcholine (Ach, from 1 nM to 3 µM) produced relaxations in a concentration dependent manner in the aorta and the SMA with functional endothelium from young adult and old rats. In the former, the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (NOLA, 30 µM) but not the cyclo-oxygenase (COX) inhibitor, indomethacin (10 µM), significantly (P<0.001) attenuated the Ach-induced relaxation of both type of arteries, and the combination of NOLA with indomethacin did not produce further inhibition. The maximal relaxation to Ach was decreased from 91 ± 3% to 38 ± 9% (P<0.01) in the aorta and from 79 ± 6% and 28 ± 6.8% (P<0.01) in the SMA from young adult as compared to old rats. In the latter, NOLA alone abolished the Ach relaxation in the aorta but had no effect on the response to Ach in the SMA. Indomethacin significantly potentiated the response to Ach in both arteries (P<0.05). It prevented the inhibitory effect of NOLA on the relaxation elicited by Ach in the aorta from old rats (P<0.05). By contrast, indomethacin failed to potentiate the Ach relaxation in the SMA from old rats. The combination of both NOLA and indomethacin did not affect the Ach response in the SMA from old rats.

In the absence of functional endothelium, the maximal response to NA (1 and 10 µM, for aorta and SMA respectively) was significantly reduced in the aorta but not in the SMA of old rats. In young adult and old rats, the response to NA was 3.6 ± 0.4 and 2.1 ± 0.14 g mg^{-1} dry tissue (P<0.05) in the aorta and 3.9 ± 0.35 and 3.8 ± 0.32 mN mm^{-1} in the SMA.

In young adult rats, NA produced transient contraction in Ca^{2+} free medium in both type of arteries (21 ± 1.8% and 35 ± 4.5% respectively). In Ca^{2+}-free medium, ryanodine (RY), at a concentration (10 µM) blocking the Ca^{2+}-induced Ca^{2+} release, almost abolished the responses to NA in the aorta but did not affect the same response in the SMA. After depletion of intracellular Ca^{2+} stores with NA, addition of exogenous $CaCl_2$ (1 mM) produced a large contraction in the continuous presence of NA in both arteries being 84 ± 4.4% and 90 ± 5.5% in the aorta and the SMA respectively. Under the same conditions, the response to exogenous $CaCl_2$ was not significantly affected by RY in both arteries.

In old rats, in Ca^{2+}-free medium, NA produced a greater contraction in the aorta (40 ± 2.7%, P<0.01) but not in the SMA (35 ± 6%, N.S.) as compared to that obtained in vessels from young adult rats. In Ca^{2+} free medium, RY greatly reduced the response to NA in both arteries to 8 ± 2.1% (P<0.001) in the aorta and 11 ± 2.8 (P<0.01) in the SMA. The contraction produced by exogenous $CaCl_2$ (1 mM) was not significantly affected by RY in the aorta but it was reduced by 23% (P<0.05) in the SMA.

The above results suggest that the decrease in Ach-induced vasorelaxation with aging involves decreased NO-mediated vasodilatation and increased generation of vasoconstrictor product(s) from COX. COX activity appears to be required for NO-mediated vasorelaxation in the aorta (but not in the SMA) of old rats only. In old rats, the RY-sensitive component of contraction is enhanced, indicating an increased contribution of Ca^{2+}-induced Ca^{2+} release to the response to NA.

239P EFFECTS OF THE CALCIUM CHANNEL BLOCKER MIBEFRADIL ON SYSTEMIC HAEMODYNAMICS AND LEFT VENTRICULAR REMODELLING IN RATS WITH HEART FAILURE

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Left ventricular (LV) remodeling has been shown to play a key role in the progression of LV dysfunction into chronic heart failure (CHF). We have shown previously that the calcium antagonist mibefradil (Mib) increases survival in a rat model of CHF (Mulder *et al.*, 1997). The aim of the present study was to evaluate whether Mib affects systemic and cardiac hemodynamics, as well as LV remodeling in CHF.

CHF was induced by coronary artery ligation in rats. Seven days after ligation, rats were randomized into two groups (n=12 per group), treated with placebo or Mib. Twelve sham rats were used as controls. Mib (15 mg/kg/day for 60 days) was given as food admix. At the end of the study, systolic blood pressure (SBP, mm Hg) and heart rate (HR, beats/ min) were measured in conscious rats (plethysmography), In pentobarbital-anaesthetised rats, left ventricular (LV) end diastolic pressure (LVEDP, mm Hg) and LV dP/dt (10^3 mm Hg/sec) were measured using a Millar catheter.

Transthoracic Doppler echocardiographic studies were performed in methohexital-anaesthetised rats, using an echocardiographic system equipped with a 7 Mhz transducer (Acuson 128 XP/10C). A two-dimensional short axis view of the left ventricle was obtained at the level of the papillary muscle, in order to record M-mode tracings. End-diastolic (LVEDD, mm) and end-systolic (LVESD, mm) LV diameters were measured by the American Society of Echocardiology leading-edge method. LV outflow velocity was measured by pulsed-wave Doppler, in order to calculate cardiac output (CO, ml/min/m^2) and systolic ejection volume (SEV, 10^{-2} ml/min/m^2/beat). Values are expressed as mean ± sem.

Table 1. Haemodynamic data in anaesthetised rats.

	SBP	HR	LVEDP	dP/dt
Sham	122±1	392±12	3.3±0.3	9.3±0.4
CHF	110±3*	390±9	17.9±1.7*	7.1±0.4*
CHF+Mib	102±3*†	363±9*†	6.9±1.1*†	7.0±0.3*

(*: p<0.05 vs sham; †: p<0.05 vs CHF ; ANOVA).

Table 1 shows that Mib reduced systolic blood pressure, heart rate and LVEDP, without affecting LV dP/dt.

Table 2. Echocardiographic data in anaesthetised rats.

	LVEDD	LVESD	SEV	CO
Sham	6.5±0.3	3.4±0.3	2.0±0.1	6.9±0.2
CHF	10.2±0.2*	8.4±0.3*	1.6±0.1*	5.2±0.3*
CHF+Mib	9.4±0.2*†	7.4±0.4*†	1.8±0.1†	5.9±0.3*†

(*: p<0.05 vs sham; †: p<0.05 vs CHF ; repeated ANOVA).

Table 2 shows that, in a context of diminished cardiac preload, afterload and heart rate, Mib limits LV diastolic and systolic dilations and increases both cardiac output and systolic ejection volume.

This beneficial cardiac remodeling, together with the systemic and cardiac hemodynamic effects, probably contribute to the mibefradil-induced improvement in survival.

Mulder, P., et al. (1997) *J. Am. Coll. Card.* **29**: 416-421.

240P EFFECTS OF THE PAF ANTAGONIST NUPAFANT HCl (BB-2113) ON ARRHYTHMIAS AND REPOLARISATION DURING MYOCARDIAL ISCHAEMIA IN RAT HEARTS *IN VITRO*

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Platelet activating factor (PAF) exacerbates cardiac injury during ischaemia (Flores *et al.*, 1990). The PAF antagonist BN-50739 (BN) can reduce the incidence of ischaemia-induced ventricular fibrillation (VF) in isolated rat hearts (Koltai *et al.*, 1991). We have compared BN with the new PAF antagonist, nupafant HCl (BB-2113; (S)-N-(1-ethoxymethyl-3-methylbutyl)-N-methyl-α-(2-methyl-1H-imidazolo[4,5-c]pyridin-1-yl)-p-toluenesulphonamide hydrochloride) (BB). BB binds with high affinity to human platelet membrane PAF receptors; in this assay (Whittaker *et al.*, 1992) its affinity is 18 fold that of PAF (IC_{50} 0.06±0.003 nM versus 1.1±0.3 nM for PAF). BB reverses PAF-induced hypotension in the anaesthetised rat (Whittaker *et al.*, 1992), ED_{50} 0.8±0.02 μg/kg i.v..

Male Wistar rats (250-300g) were anaesthetised with 60mg/kg pentobarbitone (i.p.) and heparinised with 250 iu sodium heparin (i.p.). Hearts were excised and perfused in the Langendorff mode (n=12/group) with modified Krebs' (Rees *et al.*, 1993) or similar solution containing 10μM BN or 10μM BB. Treatment was randomised and analysis blinded. Drug administration began 5min before 30min left regional ischaemia. Heart rate (HR), QT_{90} (QT at 90% repolarisation), arrhythmias, coronary flow (CF) and other variables were measured by published methods (Rees *et al.*, 1993). Data were analysed by ANOVA and Dunnett's test or Mainland's contingency tables (Rees *et al.*, 1993); *P<0.05 versus control.

BN and BB reduced VF incidence during the entire 30min period of ischaemia, and BN reduced the incidence of VF during the first 15min of ischaemia (Table 1). Both BB and BN widened QT_{90} before ischaemia (Table 2). This effect persisted during ischaemia, and was significant at most time-points in both groups (Table 2). BN increased CF in the uninvolved zone (e.g., after 10 min of ischaemia, from 9.5±0.8 ml min^{-1} g^{-1} in controls to 13.8±1.1 ml min^{-1} g^{-1}; P<0.05) whereas BB had no effect on CF (data not shown). Neither drug affected HR (not shown).

Table 1. VF Incidence (% per group)

Period of Ischaemia:		*0-10min*	*11-15min*	*16-30min*	*total*
Group:	Control	0	42	67	75
	BN	0	0*	17*	17*
	Control	0	58	58	83
	BB	0	17	25	25*

Table 2. QT at 90% Repolarisation (msec)

Time:		*-1min*	*5min*	*10min*	*30min*
Group:	Control	53±3	75±5	75±3	69±2
	BN	73±3*	90±3*	94±4*	94±4*
	Control	53±1	72±2	68±3	65±2
	BB	61±2*	80±3	79±3*	74±3*

BN and BB were found to suppress ischaemia induced VF. This was accompanied by ECG changes reminiscent of K^+ channel blocking class III agents (Rees *et al.*, 1993). These changes may be sufficient to account for the effects on VF, although it is not clear whether they were a consequence of PAF antagonism or of direct effects on K^+ channels.

Flores, N.A., & Sheridan, D.J., (1990) *Br. J. Pharmacol.* **101**, 734-738.
Koltai, M. *et al.*, (1991) *Cardiovasc. Res.* **25**, 391-397.
Rees, S.A. & Curtis, M.J. (1993) *Circulation* **87**:1979-1989
Whittaker, M. *et al.*, (1992) *Pharmacol. Comm.* **1**, 251-257

241P SITE OF ANTIARRHYTHMIC ACTION OF NUPAFANT HCl (BB-2113), ASSESSED BY SELECTIVE REGIONAL MYOCARDIAL PERFUSION IN THE RAT HEART *IN VITRO*

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The aim of the present study was to examine the site of anti-arrhythmic action of nupafant HCl (BB), a new PAF antagonist that protects against ischaemia-induced ventricular fibrillation (VF) (Baker *et al.*, 1997).

Male Wistar rats (250-300g) were anaesthetised with pento-barbitone 60 mg kg^{-1} (i.p.). Hearts were excised and perfused with modified Krebs' (Rees *et al.*, 1995) or similar solution containing 0.1, 1 or 10μM BB in the dual coronary perfusion mode (Avkiran *et al.*, 1991), which allows solution to be delivered selectively under constant pressure (70mmHg) to the left and right ventricular coronary beds. Drug administration was begun 10min before the left bed was made ischaemic by unilateral cessation of perfusion for 30min. When drug was present in the solution meant for the right coronary bed, its delivery to the right bed was maintained throughout ischaemia. The antiarrhythmic PAF antagonist, BN-50739 (BN) (Koltai *et al.*, 1991), delivered at 10μM to both beds, was used as a positive control. The ECG was recorded from an electrode inserted into the left ventricle. Data were analysed by ANOVA, Dunnett's test and Mainland's contingency tables (Rees *et al.*, 1995); *P<0.05 versus control.

BB reduced VF incidence during the first 20min of ischaemia when administered to the left coronary bed, but had no effect when delivered to the right bed (table 1). It did not affect QT interval at 90% repolarisation (QT_{90}) when delivered to one bed only (e.g., at 10 min of ischaemia; Table 1). When delivered to both coronary beds, BB reduced VF incidence during the entire 30min period and widened QT_{90} interval. BN, delivered to both beds, had a similar effect on VF and QT_{90} (Table 1).

Table 1. VF Incidence (% per group)

Period of Ischaemia:		*0-5 min*	*6-20 min*	*21-30 min*	*0-30 min*	QT_{90} +10min
Drug to:	Control	0	100	100	100	67±4
left	0.1μM BB	0	92	91	92	68±4
bed	1μM BB	0	92	82	92	68±3
n=12	10μM BB	0	58*	75	75	65±1
right	Control	0	100	100	100	77±5
bed	1μM BB	0	67	100	100	67±2
n=6	10μM BB	0	83	83	83	64±7
both	Control	0	100	91	100	65±2
beds	10μM BB	0	50*	42*	50*	95±3*
n=12	10μM BN	0	42*	42*	58*	79±3*

Maintained drug delivery to the involved (left) region via collateral vessels may explain why BB-2113 has a more sustained effect on VF when delivered to the entire heart. A similar observation has been made with other drugs that widen QT interval when examined in the same model (Rees *et. al.*, 1995). The present data indicate that the drug's protection is afforded largely by an action occurring at a site in the involved region. This may reflect antagonism of endogenous PAF, which has been proposed to be a mediator of ischaemia-induced VF (Flores & Sheridan, 1990).

Avkiran, M., Curtis, M.J. (1991) *Am. J. Physiol.* **261**, H2082-H2090.
Baker, K.E. *et al.*, (1997) *Br. J. Pharmacol.* (this issue)
Flores, N.A., & Sheridan, D.J., (1990) *Br. J. Pharmacol.* **101**, 734-738.
Koltai, M. *et al.*, (1991) *Cardiovasc. Res.* **25**, 391-397.
Rees, S.A., Curtis, M.J. (1995) *J. Mol. Cell. Cardiol.* **27**, 2595-2605.

242P CONTRIBUTION OF HUMORAL AND SYMPATHETIC SYSTEMS TO THE LOW-FREQUENCY FLUCTUATIONS OF BLOOD PRESSURE AFTER SEVERE HAEMORRHAGE

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The aim of this study was to generate hemorrhage-triggered fluctuations in blood pressure (BP) at low-frequency (LF, <0.2 Hz) in conscious rats (n=10 in each group) and investigate with spectral analysis the relative roles of hemorrhage-activated catecholamines, the renin-angiotensin system (RAS) and arginine-vasopressin (AVP) on the generation of these fluctuations. The individual contribution of these factors was assessed using a combination of the selective antagonists, prazosin (1 mg/kg i.v.), losartan (10 mg/kg i.v.) and Manning compound (AVPX, 10 µg/kg i.v.). Comparisons during each protocol were assessed by a two-way analysis of variance with repeated measures. Experiments on living animals were performed according to Executive Order N°89-02683. At rest, systolic BP (SBP) LF fluctuations were increased by losartan (92%, P<0.05). The mid-frequency (0.2-0.6 Hz, MF) oscillations of SBP and diastolic BP (DBP) were decreased by prazosin (50%, P<0.05 and 67%, P<0.01). The high-frequency (respiratory, HF) oscillations of SBP were increased by prazosin (227%, P<0.05). After severe hemorrhage (20 ml/kg), the spontaneous BP recovery was characterised by the occurrence of slow fluctuations of SBP and DBP centered ~0.065 Hz and by increases of MF (89 %, P<0.01) oscillations of SBP. The stimulating effect of a blood loss on the HF component of SBP was already documented by Baujard *et al.* (1996). The occurrence of the SBP LF fluctuations was prevented when α_1-adrenergic activity was blocked by prazosin. These oscillations were always present in spite of inhibition of angiotensin II and were increased after inhibition of the AVP activity. Pretreatment with the specific inhibitors used in these studies favoured the amplifying effect of hemorrhage on HF fluctuations while they prevented the postbleeding increase in MF oscillations.

In conclusion, the present results show an association between the dependence of the postbleeding BP level upon catecholamines and the occurrence of slow fluctuations of BP. The buffering role of AVP suggest the establishment of a hierarchy between humoral systems in the genesis of the LF oscillations of BP, with the slow oscillations being generated by the main pressor system and being dampened by the other systems. The postbleeding rise in the MF component of SBP variability could be considered as reflecting the activations of both the sympathetic vasomotor drive and the RAS. The postbleeding increase in the HF component of BP variability was dampened by the activation of the humoral systems. These effects may reflect the low preload state due to hypovolemia.

Baujard C., Ponchon P. & Elghozi J.L. *Fundam. Clin. Pharmacol.* 10, 511-517, 1996.

243P SYSTEMIC AND REGIONAL HAEMODYNAMICS ASSESSMENT IN RATS WITH FLUORESCENT MICROSPHERES: A VALIDATION STUDY

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The radioactive microsphere technique is currently regarded as the reference method for blood flows measurements in experimental cardiovascular research. Since the use of radioactivity is becoming increasingly problematical, our goal was to develop and validate a reliable alternative to radioactive microspheres (RM) by using commercially available fluorescent microspheres (FM) for the determination of cardiac output and regional blood flows in rats.

Validation of the FM method was performed in three steps: (a) comparison of cardiac index (CI) and regional blood flow (RBF) values obtained by the FM and RM methods used simultaneously in the anaesthetized rat, (b) determination of the repeatability of systemic and regional haemodynamic measurements after two successive injections of FM in the conscious rat, and (c) evaluation of the ability of the FM method to detect and quantify systemic and regional vasodilating effects (using dipyridamole) in the conscious rat.

A mixture of RM (^{103}Ru, 15±5 µm, NEN) and FM (blue or yellow-green fluorescent label, 15±5 µm, Bioseb) was injected into the left ventricle of anaesthetized (sodium pentobarbitone, 50 mg/kg, i.p) male Wistar rats (300-360 g). To allow calculation of absolute CI and RBF values, the total injected radioactivity or fluorescence was determined and a reference blood sample was withdrawn from the femoral artery. Tissues (heart, kidney, brain and sample from skeletal muscle) and reference blood samples were weighed and counted for radioactivity in a gamma counter. Afterwards, they were digested in 16N (blood) or 4N (tissues) potassium hydroxide at room temperature for 24 hours. FM were physically separated by negative pressure filtration and the fluorescent dye was extracted with 2-ethoxyethyl acetate. Finally, fluorescence was quantified by spectrofluorimetry at the optimal excitation and emission wave lengths of each dye. Linearity of fluorescence intensity as a function of FM concentrations was verified. Recovery of FM was assessed by adding an internal standard (blue-green) to blood or tissue samples containing no FM, and its average value was 90±3% (s.d.).Excitation or emission spectra obtained for the different dyes after processing blood or tissue samples were compared to those determined directly in 2-ethoxyethyl acetate, and no spectral alteration was found.

CI values (range: 242-513 ml/min.kg) obtained with FM correlated with those obtained with RM : r = 0.819, p<0.001, slope = 1.04±0.17, intercept = -52.5±61.2 (n=20), and agreement was found between FM and RM (error 95% confidence interval for one pair = ±125.4). RBF values (range: 0.1-8 ml/min.g) obtained by FM and RM were also correlated: r = 0.991, p<0.001, slope = 0.83±0.01, intercept = 0.1±0.1 (n=109). Thus, as FM underestimated RBF as compared to RM, agreement assessment was performed after conversion of FM into estimated RM values. Agreement was dependent upon RBF values: error 95% confidence intervals for one pair were 0.07-0.14 and 3.53-7.09 for 0.1 and 5 ml/min.g, respectively. Two injections of FM at a 10 min interval in conscious animals (n=9) did not significantly alter CI (363.5±75.3 vs 346.4±127.2 ml/min.kg, NS), renal (5.5±1.0 vs 4.9±0.9 ml/min.g, NS) or left ventricular (5.6±1.8 vs 6.0±1.3 ml/min.g, NS) blood flow values. Corresponding error 95% confidence intervals were ±267.4, ±2.2 and ±3.1, respectively. Finally, dipyridamole infusion (2 and 4mg/kg.min for 10 min, n=9 per group) induced dose-dependent increases in CI (+19% and +34%) and in right (+169% and +311%) and left ventricular (+137% and +225%) blood flows whereas renal blood flow was not significantly altered (-4% and +1%).

These data indicate that the FM technique (a) is reliable and in agreement with the radioactive reference method, (b) provides repeatable measurements of systemic and regional haemodynamics and (c) allows detection, regional selectivity assessment and quantification of vasodilating effects in rats.

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Sumatriptan (SUM), a 5-HT$_{1B/D}$ receptor agonist, when administered systemically induces vasoconstriction and increases in mean arterial pressure (MAP) in several species, whereas it elicits hypotension in the rat. This study was designed to investigate the receptors involved in the hemodynamic effects of 5-HT$_{1B/D}$ receptor agonists in pentobarbitone anaesthetized rats (n=6-17 *per* group). Cumulative intravenous infusions of rizatriptan (RIZ) and SUM (from 0.63 to 2500μg/kg; each dose over 5min) induced dose-dependent and marked hypotension [-42±6 and -34±4mmHg respectively; both P<0.05 by one way ANOVA followed by Dunnett's test *vs.* vehicle (VEH): +5±3mmHg] and bradycardia (-85±16 and -44±12beats/min respectively; both P<0.05 *vs.* VEH: +16±6beats/min). Zolmitriptan evoked only moderate hypotension at the highest dose (-19±9mmHg; P<0.05 *vs.* VEH). Pretreatment with a high dose of the 5-HT$_{1B/D}$ receptor antagonist, GR 127935 (0.63mg/kg i.v.), failed to antagonize the hypotension and bradycardia evoked by SUM (-35±6mmHg and -52±19beats/min respectively; both P=NS *vs.* SUM in untreated rats), but moderately antagonized the hypotension and bradycardia evoked by RIZ (-20±5mmHg and -30±17beats/min respectively; both P< 0.05 *vs.* VEH and *vs.* RIZ in untreated rats). Furthermore, pretreatment with the selective 5-HT$_{1A}$ receptor antagonist, WAY 100635 (0.16 and 0.63mg/kg i.v.), dose-dependently antagonized the hemodynamic responses to RIZ which were almost abolished by the higher dose of WAY 100635 (-4±3mmHg and-15±8beats/min; both P=NS *vs* VEH and P<0.05 *vs.* RIZ in untreated rats). A slight but statistically significant reduction in MAP subsisted at the highest dose of SUM (-13±4mmHg following pretreatment with the higher dose of WAY 100635; P<0.05 *vs.* VEH). We next determined whether the hypotension and bradycardia evoked by RIZ and SUM were mediated by activation of receptors located within the central nervous system. In pithed rats with angiotensin II-normalized MAP, RIZ failed to evoke hypotension or bradycardia (+5±4mmHg and -6±16beats/min respectively; both P=NS *vs.*VEH and P<0.05 *vs.* RIZ in untreated rats). Similarly, SUM failed to induce bradycardia in pithed rats (+5±6beats/min; P=NS *vs.*VEH and P<0.05 *vs.* SUM in untreated rats) whereas a slight but statistically significant reduction in MAP, compared to controls, subsisted at the highest dose (-9±9mmHg; P<0.05 *vs.* both VEH and SUM in untreated rats). Furthermore, in bilaterally vagotomized and atropinized (1mg/kg i.v.) rats, the reduction in MAP and heart rate evoked by RIZ (-31±4mmHg and -64±9beats/min respectively; both P<0.05 *vs.*VEH and P=NS *vs.* RIZ in untreated rats) and SUM (-47±8mmHg and -56±10beats/min respectively; both P<0.05 *vs.*VEH and P=NS *vs.* SUM in untreated rats) were not statistically significantly different from those observed in untreated anaesthetized rats. In conclusion, the 5-HT$_{1B/D}$ receptor agonists, RIZ and SUM, elicit hypotension and bradycardia in the rat predominantly via activation of central 5-HT$_{1A}$ receptors which in turn reduce sympathetic nervous system activity.

245P PARATHYROID HORMONE-RELATED PROTEIN (PTHrP)-INDUCED VASODILATION IN ISOLATED PERFUSED KIDNEY OF SPONTANOUSLY HYPERTENSIVE RATS

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PTHrP is expressed throughout the renovascular system, vasodilates the renal vasculature, increases glomerular filtration rate and stimulates renin release from juxtaglomerular cells (Philbrick *et al.*, 1996). Mechanical stretch and experimental hypertension have been shown to stimulate expression of PTHrP in smooth muscles (Noda *et al.*, 1994; Takahashi *et al.*, 1995), suggesting a negative feedback control of vascular tone by PTHrP in hypertension. We compared the vasodilatory properties of PTHrP in isolated kidneys from spontaneously hypertensive (SHR) and Wistar-Kyoto rats (WKR).

Isolated rat (200-240 g) kidneys (IK) were perfused at constant flow, the flow being adjusted to reach perfusion pressure of 80 mmHg. After 1 hr equilibration, noradrenaline (NA) was injected for 15 sec every 2 min in order to induce repetitive and transient vasocontrictions of about 40 mmHg. The vasodilatory action of PTHrP was assessed by the abilty of continuously infused PTHrP(1-36) (1 to 30 nM) to reduce NA constriction.

In IK of WKR and SHR, 7.9±1.3 and 3.5± 0.5 µM (p<0.05, n=9 and 7) NE were required to induce 37.2±1.5 and 36.9±1.4 mmHg vasoconstriction, respectively (Fig. 1). PTHrP, decreased NA-induced constriction with similar EC_{50} values but lower E_{max} in SHR. Thus, 30 nM PTHrP(1-36) decreased by 70% the NA-induced-constriction in WKR and only by 30% in SHR (p<0.05). Endogenous vasoactive PTHrP is believed to be overproduced in SHR and could therefore be responsible for the lower response to exogenous PTHrP in SHR. We measured vascular resistances (RVR) in IK perfused for 1 hr without or with PTHrP(7-34) (40nM), a PTH/PTHrP receptor antagonist which has been shown to inhibit PTHrP-induced vasodilation in IK of Wistar rats (Musso et al., 1989). PTHrP antagonist neither altered RVR (in mmHg.ml^{-1}.min.g^{-1}) in WKR (6.9±0.5, n=9 *vs.* 7.2±0.6, n=5) nor in SHR (9.6±0.9, n=7 *vs.* 9.3±0.7, n=4).

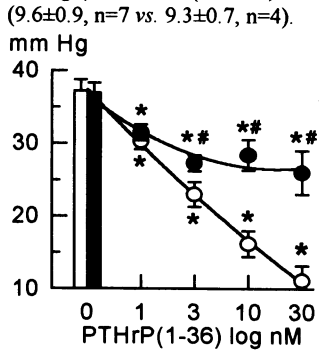

Fig. 1 NA-induced vaso-constrictions in absence (bars) and presence of 1 to 30 nM PTHrP(1-36) (curves) in IPK of SHR (filled) and WKR (open). Values are means ± SEM. *Significant effect of PTHrP (*t* test p<0.05). #p<0.05 between SHR and WKR (ANOVA p<0.05).

In conclusion, these results strongly suggest that PTHrP does not act as an endogenous renal vasodilator in both WKR and SHR. However, the vasodilatory action of PTHrP is blunted in the kidney of genetically hypertensive rats, possibly due to downregulation or desensitization of PTH/PTHrP receptors. Therefore, a negative feedback control of renovascular tone by PTHrP in hypertension appears to be unlikely.

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246P P2X RECEPTOR ACTIVATION EVOKES A BEZOLD-JARISCH-LIKE REFLEX IN ANAESTHETISED RATS

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During studies on rat articular nociceptors (Dowd et al, 1997) we observed that the P2X purinoceptor agonist αβ–methylene-ATP (αβMe) caused bradycardia, hypotension and apnoea, which are characteristics of a Bezold-Jarisch-like (BJ) vagal reflex (see Krayer, 1961). It has been shown that activation of P2X receptors depolarises rat isolated vagal afferents and nodose neurones (see Khakh et al, 1995). We tested the hypothesis that a B-J reflex can be evoked in anaesthetised rats by activation of P2X receptors associated with vagal afferents.

Male Wistar rats (376±19g, n=18) were anaesthetised with pentobarbitone (60 $mg.kg^{-1}$ i.p., supplemented hourly i.v.), and the trachea, femoral arteries and right jugular vein cannulated. Ventilation was monitored using an electrospirometer, arterial blood pressure via a pressure transducer, and the output signals recorded on a MacLab-8; bradycardia (Δ $beats.min^{-1}$), apnoea (duration, s), and hyperventilation (Δ $ml.min^{-1}$) were measured. Action potentials were recorded from sensory nerves using bipolar wire electrodes. Drugs were dissolved in 0.9% w/v NaCl and injected i.v. as a bolus over 2s at intervals of 5-10 min.

ATP (0.2-5 μmol i.v.) or αβMe (0.6-594 nmol i.v.) evoked short-lasting (1-10s) dose-related bradycardia with associated hypotension, and apnoea; high doses caused desensitisation. Transient hyperventilation (1-5s) preceded the apnoea. Bilateral mid-cervical vagotomy significantly reduced the bradycardia and apnoea; cutting the carotid sinus nerves (CSN) abolished the hyperventilation (Table 1). The P2 purinoceptor antagonist PPADS antagonised the reflex apnoea and bradycardia, but not the hyperventilation. Neural recordings showed that αβMe excited vagal inflation (e.g. 40 nmol: basal 11.8 increased to 25.6 $impulses.s^{-1}$, 5s duration) and arterial chemoreceptor (40 nmol: basal 3.4 increased to 14.2 $impulses.s^{-1}$, 2.4s duration) afferents.

Table 1. Apparent ED_{50} values for αβMe (moles) before and after denervation or PPADS (10 $mg.kg^{-1}$). Mean ± s.e.mean *P<0.05, Mann-Whitney test versus control in *n* experiments.

	Bradycardia		Apnoea		Hyperventilation	
Control	$1.5±0.4x10^{-8}$	*12*	$4.9±0.9x10^{-8}$	*13*	$2.3±0.6x10^{-8}$	*15*
Vagi cut	$>1x10^{-6}$ *	*5*	$3.4±1.4x10^{-7}$ *	*5*	$3.5±1.6x10^{-9}$ *	*5*
CSN cut	$4.4±2.8x10^{-8}$	*3*	$4.2±1.0x10^{-8}$	*4*	$>1x10^{-6}$ *	*4*
PPADS	$4.4±2.8x10^{-7}$ *	*3*	$>1x10^{-6}$ *	*3*	$2.4±1.6x10^{-9}$ *	*3*

We conclude that activation of P2X purinoceptors on vagal afferents elicits a BJ reflex in rats; activation of P2X receptors in the carotid bodies causes hyperventilation. PPADS antagonises vagal but not chemoreceptor responses to αβMe. The BJ reflex could be useful for the functional study and characterisation of drugs acting at P2X receptors, and may be of patho-physiological importance if evoked by endogenous ATP.

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247P STAPHYLOKINASE ACTIVATES PLATELETS THROUGH CLEAVAGE OF THE THROMBIN PROTEASE-ACTIVATED RECEPTOR 1

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The treatment of acute myocardial infarction with the plasminogen (Plg) activating drug streptokinase (Sk) results in platelet activation *in vivo* (Fitzgerald *et al.*, 1988). This may contribute to the failed reperfusion and acute reocclusion which limits the effectiveness of Sk. However the mechanism of this platelet activation is unknown. Staphylokinase (Sta) is a bacterial Plg activator undergoing evaluation for use in man (Collen & Lijnen, 1994). Similarly to Sk, Sta is not intrinsically active but must form a complex with Plg in the blood. We recently reported a novel potential mechanism for Sk-induced platelet activation, dependent on both cleavage of the platelet receptor for thrombin (Protease-Activated Receptor 1 (PAR1)) by Sk-Plg complex, and on anti-Sk-Fc receptor interactions (McRedmond *et al.*, 1996). We investigated whether Sta could also result in platelet activation by a similar mechanism to Sk.

Platelet aggregation was measured by light transmittance in human platelet-rich plasma, and concomitant thromboxane (TX) A_2 production assayed using a commercial enzyme immunoassay. In the presence of a subthreshold concentration of the platelet agonist ADP, Sta (30 nM) caused an irreversible platelet aggregation in 5 out of 7 subjects tested, which was accompanied by increased TXA_2 production (Table 1). This aggregation and TXA_2 production was inhibited by pretreatment with the monoclonal antibody 2/389, which binds to PAR1 and prevents receptor cleavage and activation by thrombin. Direct thrombin inhibition with the highly specific agent hirudin had no effect, suggesting that an agent other than thrombin caused platelet activation by cleaving PAR1. Incubation of Sta-Plg complex with a synthetic peptide corresponding to the PAR1 cleavage site resulted in cleavage at the authentic site required for receptor activation and generation of the expected peptide fragments, as judged by HPLC analysis. This suggests that Sta-Plg may activate platelets through a novel cleavage of PAR1.

Table 1. Platelet aggregation and Thromboxane A_2 production in human platelet-rich plasma.

	% Aggregation			TXA_2 (ng/ml)		
Treatment	Mean	s.e. mean	P *vs.* No Sta	Mean	s.e. mean	P *vs.* No Sta
No Sta	9.3	3.0		13.32	3.33	
Sta	43.0	10.9	.097	55.06	13.98	.0023
IV3 + Sta	57.7	3.3	.0012	68.43	12.18	.0023

Data were analysed by Kruskal-Wallis ANOVA, followed by Mann-Whitney test.

Streptococcal and Staphylococcal infections result in the presence in the general population of low levels of antibodies binding to Sk and Sta, respectively. We investigated the dependence of Sta-induced platelet activation on anti-Sta immune complexes using the monoclonal antibody IV3, which prevents interactions of IgG with the Fc receptor on platelets. Paradoxically, pretreatment with IV3 enhanced aggregation and increased TXA_2 production in all individuals tested, including those who showed little response to Sta alone (Table 1). The mechanism of Sta-induced platelet activation is therefore distinct from that previously reported for Sk-induced activation.

Recombinant Staphylokinase was a kind gift of Prof. D. Collen, University of Leuven, Belgium. Monoclonal antibody 2/389 was a gift of Dr. S.R. Stone, University of Cambridge.
Supported by a grant from the Irish Heart Foundation.

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248P ANGIOTENSIN-INDUCED CALCIUM RELEASE IN SMOOTH MUSCLE CELLS FROM HYPERTENSIVE RATS IS CONTROLLED BY ACTIN AND MICROTUBULE NETWORK

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The effect of angiotensin II (AII) in vascular smooth muscle cells (SMC) is known to be mediated by G proteins' signaling pathway (Rosendorff C., 1996). On the other hand, cytoskeletal elements have been suggested to participate in the signaling events associated with G protein activation.

The aim of this study is to assess the role of the cytoskeleton on the mobilisation of intracellular Ca^{++} (Ca_i^{++}) induced by AII in SMC from rat aorta (male, mean weight: 150g). As G proteins are implicated in cardiovascular dysfunction observed in hypertension, we have assessed this role in spontaneous hypertensive rats (SHR) as compared to normotensive Wistar Kyoto (WKY).

Cytochalasine D (2 µM) and nocodazole (5 µM) were used to study the implication of the cytoskeleton in mediating AII (10^{-6} M) release of Ca_i^{++} in cultured SMC. Ca_i^{++} was assessed in Fura-2 loaded cells (Molecular Probe, USA), using fluorescent imaging microscopy (Newcastle Photometric System, UK). Experiments were performed in presence (Ca^{++}: 1mM) and in absence (EGTA: 0.3mM) of external Ca^{++}. Results are expressed as % of control values. Comparisons were performed with ANOVA followed by Student's *t*-test for unpaired data. Statistical significance corresponded to $p<0.05$.

AII induces a transient release in Ca_i^{++} that reflects: a) release of Ca^{++} from internal stores; b) Ca^{++} influx from external media; c) Ca^{++} recapture in internal stores; d) Ca^{++} buffering capacity. In the hypertensive strain (SHR), cytochalasine D decreases by 52% both the amplitude and the amount of Ca^{++} released by A2 ($p<0.05$) (Fig. 1-A). This decrease is consequent to a significant alteration of Ca^{++} release from internal stores (assessed in the absence of external Ca^{++}). In contrast, in the normotensive rat (WKY), cytochalasine D has no effect on the A2-induced release of Ca_i^{++} (Fig. 1-A).

On the other hand, nocodazole induces a significant decrease in the Ca_i^{++} released by AII both in the SHR (-53%, $p\leq 0.05$) and WKY (-44%, $p<0.05$) (Fig. 1-B), consequent to a significant inhibition of Ca^{++} release from internal stores, (assessed in the absence of external Ca^{++}).

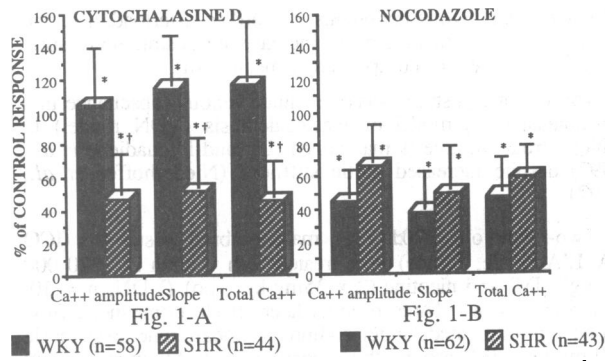

Fig. 1. The effect of cytochalasine (1-A) and nocodazole (1-B) is assessed on the AII-induced transient Ca_i^{++} release. Ca_i^{++} peak, slope of Ca_i^{++} increase and area under the curve are represented as amplitude, slope and total Ca^{++} respectively. * $p<0.05$ vs Control response; † $p<0.05$ SHR vs WKY

In conclusion, these results suggest that in the SHR, the actin and microtubular networks are implicated in the A2-induced signalling cascade, leading to Ca^{++} release from internal stores. In contrast, in the WKY, only microtubules network is related to the effect of AII.These novel findings could be relevent to the reported differences in mechanical properties of large vessels observed in hypertension as compared to control.

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249P CANNABINOID CB1 RECEPTOR AND ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION IN GUINEA-PIG CAROTID, RAT MESENTERIC AND PORCINE CORONARY ARTERIES

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The purpose of these experiments was to determine wether or not the endothelium-dependent hyperpolarizations of the vascular smooth muscle cells (observed in the presence of inhibitors of nitric oxide synthase and cyclooxygenase) can be attributed to the production of an endogeneous cannabinoid. Membrane potential was recorded in the isolated guinea-pig carotid, rat mesenteric and porcine coronary arteries using the intracellular microelectrode technique.

In the guinea-pig carotid artery, acetylcholine (1 µM) evoked an endothelium-dependent hyperpolarization of 18.8 ± 0.7 mV (n = 15) under control conditions. The cannabinoid receptor antagonist, SR 141716 (10 nM to 10 µM), produced a concentration-dependent direct hyperpolarizing effect (up to 10 mV at 10 µM) and a significant inhibition of the acetylcholine-induced hyperpolarization. Anandamide (0.1 to 3 µM) did not influence the membrane potential. At the concentration of 30 µM the cannabinoid agonist induced a non-reproducible weak hyperpolarization with a slow time course (5.6 ± 1.3 mV, n = 10). SR 141716 (1 µM) did not affect the hyperpolarization induced by 30 µM anandamide (5.3 ± 1.5 mV, n = 3).

In the rat mesenteric arteries, SR 141716 (1 µM) did not modify either the resting membrane potential of smooth muscle cells or the endothelium-dependent hyperpolarization induced by acetylcholine (1 µM) (17.3 ± 1.8 mV, n = 4 and 17.8 ± 2.6 mV, n = 4, in control and presence of SR 141716, respectively). Anandamide (30 µM) induced a weak hyperpolarization of the smooth muscle cells (9.9 ± 1.8mV, n = 7) which was not reproducible whereas acetylcholine was still able to hyperpolarize the tissue. The hyperpolarization induced by anandamide was significantly potentiated by a treatment with SR 141716 (1 µM; 17.3 ± 2.3 mV, n = 4). HU-210 (30 µM), a synthetic CB_1 receptor agonist, and palmitoylethanolamide (30 µM), a CB_2 receptor agonist did not influence the membrane potential of the rat mesenteric artery smooth muscle cells.

In the rat mesenteric arteries, the endothelium-dependent hyperpolarization induced by acetylcholine (1 µM) (19.0 ± 1.7 mV, n = 6) was not altered by glibenclamide (1 µM; 16.5 ± 3.5 mV, n = 2). However, the combination of charybdotoxin (0.1 µM) plus apamin (0.5 µM) abolished the acetylcholine-induced hyperpolarization and in these conditions, acetylcholine evoked a depolarization (9.0 ± 4.0 mV, n = 2). Conversely, the hyperpolarization induced by anandamide (30 µM) (9.9 ± 1.8 mV, n = 7) was inhibited by glibenclamide (4.0 ± 0.6 mV, n = 3) whereas it was significantly potentiated by the combination of charybdotoxin plus apamin (17.3 ± 3.2 mV, n = 3).

In the porcine coronary artery, anandamide up to 30 µM did not hyperpolarize the smooth muscle cells. The endothelium-dependent hyperpolarization and relaxation induced by bradykinin were not influenced by SR 141716 (1µM).

These results indicate that, the endothelium-dependent hyperpolarizations observed in the guinea-pig carotid, rat mesenteric and porcine coronary arteries, are not related to CB_1 cannabinoid receptor activation.

250P VENOUS CAPACITANCE IS NOT MODIFIED IN A RAT MODEL OF AORTIC STIFFNESS

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In essential hypertensive patients with aortic stiffness, stroke volume may be maintained - despite the increase in cardiac afterload - by a compensatory decrease in venous capacitance leading to an increase in venous return and cardiac filling pressure (Safar and London, 1987). However, it is not clear which of the two components of cardiac afterload, *i.e.* the increase in aortic stiffness or the increase in transmural pressure, stimulates the venous compensatory mechanism.

The aim of this study was to evaluate venous capacitance in a normotensive rat model of elastocalcinosis (VDN model) in which stroke volume is maintained (Lartaud-Idjouadiene *et al.*, 1997) despite increased aortic stiffness (Niederhoffer *et al.*, 1997).

Two-month old (220±12 g), male, outbred Wistar rats (ICO SA, L'Arbresle, France) were treated with vitamin D_3 (270 000 $IU.kg^{-1}$, IM) and nicotine (2 x 25$mg.kg^{-1}$, po) (VDN, n = 10; Controls, n = 8). Three months later an electromagnetic flow probe was implanted, under sodium pentobarbitone anaesthesia (60 $mg.kg^{-1}$, IP), around the ascending aorta for measurement of stroke volume (SV, µl). Five days later thoracic and abdominal aorta were chronically cannulated under halothane (1%)-oxygen anaesthesia with polyethylene cannula (0.96/0.58 mm) for measurement of mean aortic blood pressure (MAP, mmHg) and aortic pulse wave velocity (PWV, $cm.s^{-1}$). The thoracic and abdominal vena cava were also cannulated with silastic tubes (0.64/1.19 mm) for measurement of central venous pressure (CVP, mmHg) and venous infusion. One day later awake rats were connected to a sine-wave electromagnetic flowmeter and to low-volume pressure transducers.

Haemodynamic parameters were measured under baseline conditions. Total venous capacitance (VC, $ml.kg^{-1}.mmHg^{-1}$) was calculated as the reciprocal of the slope relating CVP to change in blood volume (London et al., 1978) following acute volume overload (40 $ml.kg^{-1}$ of PBS in 1 min). Results are expressed as means ± s.e.m. (Table 1) and compared with Student's t-test.

Table 1 : Haemodynamic parameters measured in control and VDN rats (* : $P < 0.05$ VDN *vs* Controls).

	Controls	VDN
MAP (mmHg)	115±3	119±4
PWV ($cm.s^{-1}$)	517±39	807±61*
SV (µl)	223±18	211±13
baseline CVP (mmHg)	1.9±0.9	1.6±0.8
VC ($ml.kg^{-1}.mmHg^{-1}$)	3.1±0.3	2.9±0.2

Venous capacitance was not modified in VDN rats despite aortic stiffness yet SV was maintained. Our results suggest that SV may be maintained by cardiovascular mechanisms other than a compensatory decrease in VC in this normotensive rat model of aortic stiffness.

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251P THE ROLE OF TRYPTOPHAN RESIDUES IN THE 5-HT_3 RECEPTOR BINDING SITE

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The 5-HT_3 receptor is a member of the super-family of ligand-gated ion channels which includes the nicotinic acetylcholine (nACh), glycine and $GABA_A$ receptors. These proteins contain an N-terminal ligand binding site which has been observed in a cleft ~30A above the membrane for the nAChR (Unwin 1995). The residues lining the cleft have not been identified but are likely to contain residues identified by photoaffinity labelling and mutagenesis to be important in ligand binding. It is the aim of the present study to identify amino acids involved in 5-HT_3 receptor ligand binding using mutagenic analysis.

Tryptophan has been implicated in 5-HT_3 receptor ligand binding by chemical modification (Miquel *et al.*, 1991). To identify the critical tryptophan (W) residues in the ligand binding site of the 5-HT_3 receptor, six of these residues in the N-terminal extracellular domain were mutated to tyrosine and/or serine.

Mutant constructs were generated using the Kunkel method (Kunkel, 1985) with 5-HT_3 receptor cDNA in the vector pRC/CMV. The altered cDNA was transiently or stably expressed in HEK293 cells via the calcium phosphate precipitation method. Radioligand binding, whole-cell patch clamp and immunofluorescent localisation of the resulting mutants were used to characterise the mutant receptors.

Substitution of W95, 102, 121 & 214 with tyrosine (Y) and/or serine (S) all resulted in receptors which lacked [^{3}H]granisetron (antagonist) and [^{3}H]meta-chlorophenylbiguanide (mCPBG, agonist) binding sites. These mutants also lacked functional responses to 5-HT and mCPBG. However, immuno-fluorescence studies revealed that the receptors were expressed and had a similar pattern of expression to the wild type receptor. Mutations of amino acids in adjacent positions and at W60 produced wild type-like receptors.

The W90Y mutation resulted in a functional receptor with altered radioligand binding properties ($p<0.05$): K_ds for [^{3}H]granisetron binding were 0.28 ± 0.01nM for W90Y and 0.051 ± 0.003nM for WT, and K_ds for [^{3}H]mCPBG binding were 8.2 ± 0.7nM and 1.48 ± 0.08nM respectively (mean ± SEM, n>5). Electrophysiological properties were also altered ($p<0.05$): EC_{50}s for 5-HT were 8.0 ± 0.3µM for W90Y and 2.1 ± 0.4µM for WT, and EC_{50}s for mCPBG were 2.0 ± 0.1µM and 0.81 ± 0.09µM respectively (mean ± SEM, n>5). Hill coefficients for either agonist with W90Y and WT were not significantly different from 2. The mutation W90S resulted in loss of function.

In conclusion, these data support the functional involvement of W90 in the binding of 5-HT_3 receptor ligands. The specific disruption of ligand binding caused by mutations to the more C-terminal tryptophan residues may indicate that these residues line a cleft homologous to that observed in the nAChR and are involved in the accessibility of the binding site to ligands.

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252P SUBSITUTION OF A CHARGED RESIDUE, LYSINE 282, IN THE M2 DOMAIN OF THE 5-HT$_3$ RECEPTOR AFFECTS THE RATE OF DESENSITISATION

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The 5-HT$_3$ receptor is a ligand-gated ion channel (LGIC) which, together with the acetylcholine, glycine and GABA$_A$ receptors, form an ion channel superfamily. The receptor proteins are all pentameric assemblies of subunits arranged around an axial channel. Each subunit consists of four transmembrane domains (M1 to M4) with M2 lining the wall of the channel. The defined transmembrane domains of this LGIC superfamily cloned to date consist entirely of hydrophobic and polar amino acids. The only exception is the 5-HT$_3$ receptor: a lysine residue (K282) is located towards the cytoplasmic side of M2. To investigate the role of this amino acid in normal receptor function we generated four substitutions: arginine (R), glutamine (Q), serine (S) and glycine (G) which form a series where charge and side chain length decrease.

The coding sequence of the 5-HT$_3$ receptor from N1E 115 neuroblastoma cells was subcloned into the expression vector pRcCMV. Mutants were generated by site directed mutagenesis, stably expressed in HEK293 cells and studied using whole cell patch-clamp. Patch electrodes were filled with the following solution (in mM) $CsCl_2$ 140, $MgCl_2$ 1, EGTA 10, HEPES 10, pH 7.2 with CsOH. Cells were perfused with an extracellular solution: NaCl 130, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 2, HEPES 10, Glucose 30, pH 7.2 with NaOH, except when studying receptor desensitisation when a low (10nM) calcium solution was used to avoid any secondary effects of the mutants upon calcium permeability.

All of the mutants were functional. 5-HT (30μM)-evoked currents in transfected HEK293 cells were completely and reversibly blocked by the 5-HT$_3$-selective antagonist granisetron (10nM, n=3). Dose-response curves revealed a small but significant increase in affinity for 5-HT which was independent of the substitution made at this position (Table 1); reversal potentials for the mutants did not differ significantly from WT (Table 1). There was, however, a large change in the rate of agonist-induced desensitisation, that was dependent on the amino acid at position 282 (R>G>S>Q;Table 1).

In conclusion the data cannot be explained by the decrease in charge or side chain length, suggesting a specific role for K and indicating that K282 is at a location in the channel which is intimately involved in the mechanism of 5-HT$_3$ receptor desensitisation.

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Table 1. Electrophysiological parameters of WT and mutant 5-HT$_3$ receptors.

Receptor	EC_{50}, μM	nH	Erev, mV	$T_{1/2}$, second
WT	2.34 ± 0.06(4)	2.87 ± 0.35(4)	-5.3 ± 2.0(4)	5.0 ± 0.5 (12)
K282R	1.50 ± 0.08(4)*	2.97 ± 0.18(4)	-2.6 ± 0.4(4)	26.6 ± 2.8 (11)*
K282Q	1.47 ± 0.06(4)*	2.77 ± 0.47(4)	-6.4 ± 0.9(4)	8.2 ± 0.8 (11)*
K282S	1.47 ± 0.04(4)*	2.39 ± 0.34(4)	-4.4 ± 2.3(4)	11.2 ± 1.2 (10)*
K282G	1.39 ± 0.10(4)*	2.73 ± 0.27(4)	-4.0 ± 1.9(4)	20.3 ± 2.0 (11)*

Results are mean ± s.e.m., (n) * indicates results which differ significantly from WT (t test, $p< 0.05$)

253P RECOMBINANT H5-HT$_{1B}$ AND H5-HT$_{1D}$ RECEPTORS STABLY EXPRESSED IN C6 GLIOMA CELLS ARE COUPLED TO CALCIUM-DEPENDENT POTASSIUM CHANNELS

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Reduced neuronal firing is currently considered to contribute to the therapeutic migraine abortive action of 5-HT$_{1B/D}$ receptor agonists (Hoskin *et al*, 1996). Both 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors are negatively coupled to adenylate cyclase via an inhibitory guanine nucleotide-binding protein, but the cellular mechanisms which modulate neuronal excitability following receptor activation are unknown. We sought to determine the link between receptor activation and excitability in C6 glioma cells stably expressing cloned h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors by means of the patch-clamp method.

Outward membrane currents were examined in non-transfected and in transfected C6 glioma cells using the whole-cell configuration. The internal pipette solution contained (in mM): 115 K-aspartate, 10 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 2 KH_2PO_4, 5 Tris-GTP, 4 Mg-ATP, 10 HEPES, 10 D(+)-glucose, pH 7.4 (KOH). The external solution contained (in mM): 135 choline chloride, 1.1 $MgCl_2$, 0.8 $CaCl_2$, 0.1 atropine sulphate, 10 HEPES, 10 D(+)-glucose, pH 7.4 (KOH).

Activation of h5-HT$_{1B}$ or h5-HT$_{1D}$ receptors stably expressed in voltage-clamped C6 glioma cells produced an outward current whose reversal potential (-80 mV) coincided with the Nernst potential for potassium ions. Tetraethylammonium chloride (10 mM) significantly reduced I_K by 28.4±2.4% ($p<0.05$; n=5). I_K was also markedly reduced by dialysing cells with EGTA (5 mM) in the internal medium indicating calcium-dependency. External application of isoprenaline (1 μM) or dibutyryl-cAMP (10 μM), respectively reduced $I_{K/Ca}$ amplitude by 23.7 ± 9.6% ($p<0.05$; n=5) and 23.9 ± 6.4% ($p<0.05$; n=6) suggesting that $I_{K/Ca}$ in C6 glioma cells is negatively regulated by cyclic AMP. In C6 glioma cells expressing either cloned h5-HT$_{1B}$ or h5-HT$_{1D}$ receptors, activation of either receptor with sumatriptan increased $I_{K/Ca}$ (max. increase: 19.4±7.2%, n=6, $p<0.05$ and 25.1±3.9%, n=6, $p<0.001$, respectively), with half maximal increases (EC_{50}) in $I_{K/Ca}$ of 56.3 and 68.7 nM, respectively. The absence of sumatriptan-induced increases in $I_{K/Ca}$ in non-transfected C6 cells confirmed a specific involvement of the respective membrane h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors in transfected C6 cells. In the presence of GR 127935 (0.1 μM), a 5-HT$_{1B/D}$ receptor antagonist (Clitherow *et al*, 1994), sumatriptan (1 μM) failed to significantly increase $I_{K/Ca}$ in transfected C6 cells, thus providing further evidence that sumatriptan-evoked responses were indeed mediated by h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors, respectively. In the presence of dibutyryl cAMP (10 μM), EGTA (5 mM) or a nominally Ca^{2+}-free medium in the patch pipette, $I_{K/Ca}$ was no longer enhanced by sumatriptan (1 μM; -15.2±6.7%, NS; -1.2±0.9%, NS; -5.3±3.7%, NS, respectively). Collectively these results indicate that both h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors are positively coupled to Ca^{2+}-dependent K^+ channels, which in turn are negatively regulated by cAMP.

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254P MODIFICATION OF APOMORPHINE-INDUCED CHANGES IN LOCAL CEREBRAL GLUCOSE UTILISATION BY THE NK_1 RECEPTOR ANTAGONIST GR205171

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The tachykinin NK_1 receptor antagonist GR205171 has previously been reported to possess broad spectrum anti-emetic activity (Gardner *et al.*, 1996). However, the neural circuits recruited in the actions of this NK_1 anatgonist are unknown. The aim of the present study was to identify neuranatomical sites important in the abilty of GR205171 to modify apomorphine-induced changes in local cerebral glucose utilisation (LCGU) using quantitative 2-deoxyglucose autoradiography.

Experiments were carried out on male Lister Hooded rats (300 - 500g; n = 6/group) which were briefly anaesthetised to allow surgical insertion of polythene cannulae into the femoral artery and vein. Rats were randomly assigned to 1 of 6 treatment groups and were either pre-treated with vehicle or GR205171 (0.3 mg kg^{-1} s.c.) 30 minutes prior to treatment with apomorphine (0.25 mg kg^{-1} or 0.5 mg kg^{-1} s.c.) or vehicle which was administered 15 minutes before an i.v. bolus injection of [^{14}C]-2-deoxyglucose (125μCi kg^{-1}). The dose of GR205171 chosen reflects that which blocked apomorphine (0.25 mg kg^{-1} s.c.) -induced conditioned taste aversions in rats (McAllister and Pratt, 1996). LCGU was measured using the fully quantitative [^{14}C]-2-deoxyglucose autoradiographic technique in accordance with the original methodology as first described by Sokoloff *et al.*, (1977). For each experimental animal LCGU values for each of the brain structures measured were calculated as the mean ± S.E.M.. Statisitical significance was assessed using one-way ANOVA, followed by the Newman-Keuls test where appropriate. GR205171 (0.3 mg kg^{-1}) was found to modify apomorphine (0.5 mg kg^{-1}) -induced changes in LCGU in 17 out of 35 brain sites examined. These areas were mainly within the basal ganglia, limbic system and brain stem. For example apomorphine (0.5 mg kg^{-1}) significantly increased glucose use in the NTS (control = 64 ± 2; apomorphine = 95 ± 2μmol/100g/min; $p<0.05$) and the caudate nucleus (control = 89 ± 1; apomorphine = 122 ± 2 μmol/100g/min; $p<0.05$). Pre-treatment with GR205171 significantly modified these values to 106 ± 4 and 78 ± 3 μmol/100g/min respectively ($p<0.05$). However GR205171 (0.3 mg kg^{-1}) alone caused an increase in a number of brain areas including the nucleus accumbens where glucose use was increased by 27% indicating an endogenous role of substance P. The sites funtionally affected within the basal ganglia may be important in the ability of GR205171 to depress stereotypy induced by apomorphine in rats, whereas the effect on LCGU in the NTS may reflect an important site of action regarding the aversive effects of NK_1 antagonists.

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255P ALTERATIONS IN $GABA_B$ RECEPTOR mRNA EXPRESSION IN HIPPOCAMPAL SCLEROSIS ASSOCIATED WITH HUMAN TEMPORAL LOBE EPILEPSY

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We have previously reported an overall loss of $GABA_B$ receptors from all regions of hippocampi taken from patients undergoing temporal lobe resection for intractable temporal lobe epilepsy (TLE) (Billinton *et al.*, 1997). Further to this, we have examined for changes in $GABA_B$ receptor mRNA expression, and compared this with neuronal density in samples of resected hippocampi from patients (n=13, mean age 35 years) with intractable TLE and associated hippocampal sclerosis (HS), and in control tissue from neurologically normal post-mortem (PM) control brain (n=9, mean age 74 years) using *in situ* hybridization.

Samples of hippocampi were frozen in embedding matrix on dry ice within 20 min of surgery and stored at -80°C. Control hippocampi were frozen 5.5-28 hours post mortem. Control tissue pH was obtained. Cryostat sections (10 μm) were prepared at -15°C to -20°C and mounted onto charged microscope slides. Sections were fixed with 4% paraformaldehyde in ice cold phosphate buffered saline (PBS) pH 7.2 for 5 min, washed in fresh PBS, dehydrated using increasing concentrations of ethanol and stored in 95% ethanol until assay. Oligonucleotides were labelled with ^{35}S and diluted to a concentration of 6.7 x 10^6 dpm/ml in hybridization buffer. Sections were removed from alcohol, allowed to dry and hybridized overnight at 42°C. Sections were then washed in standard saline citrate (SSC) for 2 x 30 min at 55°C, rinsed in 1 x SSC and 0.1 x SSC (1 min at room temp), dehydrated using increasing concentrations of ethanol, and air dried before being apposed to [^{3}H]-sensitive film for 13 days. Resulting images were analysed on an MCID M4, and optical density converted to attmol/mg tissue using ^{35}S brain paste standards. Six hippocampal subregions were identified with cresyl violet stain in adjacent sections. Non-specific hybridization was demonstrated in the presence of 100-fold excess unlabelled oligonucleotide. Neuronal density values for five hippocampal subregions were obtained from paraffin embedded samples using a 3D cell counting method (Williams & Rakic, 1988; HS n=10; PM n=5).

The level of expression of $GABA_B$ receptor mRNA was significantly increased in the subiculum (180 ± 9%; Student's *t*-test, $p<0.001$) However, overall expression was not significantly different in any other region. A significant decrease in neuronal density was observed in all regions (reduced to 11 ±3% - 77 ±10% of control). The subiculum was not assessed for neuronal density. When corrected for neuronal loss, a significant increase in mRNA level was seen in both CA1 (1569 ± 274%, $p<0.05$) and dentate gyrus (693 ± 202%, $p<0.05$).

These data suggest an upregulation of $GABA_B$ receptor mRNA expression in subregions CA1 and dentate gyrus in TLE, but an alternative explanation is that neurons expressing $GABA_B$ mRNA are selectively preserved in HS. However the difference in age between PM control and HS groups, which may affect the levels of mRNA being detected, cannot be ignored.

Post-mortem brain tissue was provided by Dept. Pathology, University of Birmingham, and the Parkinson's Disease Society Brain Bank, Institute of Neurology, London,

AB is an MRC/CASE student.

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256P REGIONALLY SELECTIVE CHANGES IN THE DISPLACEMENT OF α-DENDROTOXIN BINDING BY CHARYBDOTOXIN AND TOXIN K IN THE RAT SEPTOHIPPOCAMPAL PATHWAY DURING AGEING

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The existence of α-dendrotoxin (α-DTX) binding complex subtypes was demonstrated by Awan & Dolly, 1991. It has been postulated that these subtypes exist due to the formation of homo- and heteromultimeric voltage-dependent K^+ channels. The aim of this study was to determine if α-DTX binding complexes exist within structures of the septohippocampal pathway and to investigate whether any changes occur in ageing.

The method was modified from Awan & Dolly, 1991. 15μm brain sections were cut at -22°C from young (3mths, 250-300g) and aged (22mths, 450-550g) male hooded Long Evans rats. Binding was determined by incubating sections with either 1nM ^{125}I-α-DTX alone (total) or 1nM ^{125}I-α-DTX in the presence of 1μM DTX (non-specific), 1μM charybdotoxin (CTX) or 1μM toxin K (to reveal subtypes) in Krebs phosphate buffer containing 1mg/ml BSA at room temperature for 45 minutes. Sections were then washed in three changes of Krebs at 4°C and dried prior to exposure to X-ray film with ^{125}I brain paste standards. The resultant autoradiograms were quantified using computer based densitometry. Results were analysed using two-way ANOVA with repeated measures.

Table 1 shows that toxin K produced less displacement of α-DTX binding than CTX in the medial septal nucleus (MSN), the dentate gyrus granule cell layer (DG gcl) and the molecular layers of the CA1, CA2 & CA3 regions of the hippocampus (HCA1, HCA2 & HCA3 mol). This suggests the presence of binding complex subtypes. In the majority of structures, there was no effect of age on α-DTX binding. However, the ability of toxin K to displace α-DTX was significantly less in aged tissue compared to young tissue in the HCA1 and HCA2 mol. These data suggest that there may be selective changes in K^+ channel formation with age, particularly within the hippocampus, which alters toxin binding affinity. One possible explanation for this is that during ageing there is a change in the formation of homo-and heteromultimeric channels.

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TABLE 1: Specific binding of ^{125}-I-α-DTX in the absence and presence of competing toxins. All data are expressed as mean±sem (n=6). *p<0.05 young vs aged; ‡p<0.05 α-DTX + Toxin K vs α-DTX + CTX (young); † p<0.05 α-DTX + Toxin K vs α-DTX + CTX (aged).

	α-DTX (fmol/mg tissue)		α-DTX + CTX(fmol/mg tissue)		α-DTX + Toxin K(fmol/mg tissue)	
Brain Region	young	aged	young	aged	young	aged
MSN	13.51±0.36	9.75±0.24*	1.76±0.07	1.80±0.10	5.77±0.20‡	5.62±0.54†
DG gcl	5.25±0.29	4.91±0.24	0.74±0.05	0.73±0.14	1.70±0.27‡	2.19±0.10†
HCA1 mol	7.58±0.57	6.90±0.49	1.16±0.05	1.31±0.13	3.17±0.18‡	4.18±0.18†*
HCA2 mol	6.91±0.43	6.16±0.34	1.46±0.09	1.22±0.09	3.09±0.13‡	3.78±0.13†*
HCA3 mol	7.29±0.44	6.52±0.41	1.28±0.12	1.84±0.05	2.83±0.31‡	3.53±0.29†

257P LEUKOTRIENE B_4 ACTIVATES THE NADPH OXIDASE VIA A *LYN* TYROSINE KINASE SIGNALLING PATHWAY INDEPENDENT OF ERK1/2, JNK46/54 AND p38 MAP KINASES

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Eosinophils have the potential to destroy normal healthy tissue due to their ability to undergo a massive increase in O_2 consumption in response to a diverse array of stimuli culminating in the generation of highly reactive, O_2-derived free radicals. This process is catalysed by a multi-component enzyme complex known as the NADPH oxidase (E.C. 1.23.45.3). At present, little is known of the intracellular mechanisms underlying the activation of the eosinophil NADPH oxidase complex. We have previously excluded a major role for intracellular Ca^{2+}, protein kinase C (PKC), phospholipase D (PLD) and phosphatidylinositol 3-kinase (PI 3-kinase) during LTB_4-induced NADPH oxidase activation (Perkins *et al.*, 1995). In this study, we have extending these investigations by examining the possible roles of the MAP kinases, extracellular regulated kinases 1/2 (ERK1/2), c-jun N terminal kinases 46/54 (JNK46/54) and the p38 MAP kinase and/or the *src* family of tyrosine kinases.

Guinea-pig peritoneal eosinophils (>95% purity) were separated from contaminating macrophage/monocytes using a discontinuous Percoll density gradients. Following LTB_4 stimulation, H_2O_2 generation by the NADPH oxidase was quantified using the horse radish peroxidase-catalysed oxidation of scopoletin. Activation of ERK1/2 and JNK46/54 was monitored using an 'in gel' renaturation assay that employed myelin basic protein and recombinant GST-c-jun (1-135) as the substrates, respectively. p38 MAP kinase stimulation was determined by Western blot with antibodies to the phosphorylated enzymes. Activation of the *src*-kinases was assessed by determining tyrosine phosphorylation. This involved immunoprecipitation of total cellular phosphotyrosine proteins using an agarose conjugated anti-phosphotyrosine monoclonal antibody, 4G10 and subsequent detection of the respective *src*-family tyrosine kinases by Western blot.

LTB_4 evoked a rapid and transient generation of H_2O_2 that was concentration-dependent (EC_{50} = 89 ± 11 nM (n=4)) and antagonised by the LTB_4-receptor blocking drug, U-75,302 (IC_{50} = 386 ± 69 nM (n=4)). Exposure of eosinophils to a maximally effective concentration of LTB_4 (1 μM) resulted in a rapid activation of extracellular regulated kinase-1/2 (ERK-1/2) and p38 MAP kinase; c-jun N-terminal kinases 46 and 54 (JNK-46/54) were not activated under identical experimental conditions. Kinetic studies revealed that the time-course of H_2O_2 production ($t_{½}$ = 16 ± 4 sec (n=4)) preceded activation of ERK-1/2 ($t_{½}$ = 26 ± 4 sec (n=3)) and p38 MAP kinase ($t_{½}$ = 34 ± 5 sec (n=3)). Furthermore, neither the ERK-1/2 inhibitor, PD098059 (10 μM) nor the p38 inhibitor, SB203580 (20 μM) attenuated H_2O_2 production suggesting that these MAP kinases were not central to NADPH oxidase activation. Western immunoblot analyses of eosinophil lysates reproducibly identified the *src*-like tyrosine kinases *lyn*, *hck*, *fgr* and *lck* whereas *src*, *yes*, *blk* and *fyn* were apparently absent. LTB_4 (1 μM) promoted a rapid and transient tyrosine phosphorylation of *lyn*, but not *hck*, *fgr* or *lck*, with a time-course that preceded the generation of H_2O_2. The tyrosine kinase inhibitor, lavendustin A (30 μM), attenuated *lyn* tyrosine phosphorylation and H_2O_2 production but had no significant affect upon LTB_4-induced activation of either ERK-1/2 or p38 MAP kinase. These data suggest that NADPH oxidase activation is mediated via a *lyn* tyrosine kinase-dependent mechanism that is divorced from the stimulation of ERK-1/2, JNK-46/54 and p38 MAP kinases. These studies demonstrate also that LTB_4 activates ERK-1/2 and p38 MAP kinase by a mechanism that does not involve *lyn* tyrosine kinase.

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258P ACTIVATION OF EXTRACELLULAR SIGNAL-REGULATED KINASES 1 AND 2 IN HUMAN EOSINOPHILS: DISSOCIATION FROM SPONTANEOUS APOPTOSIS AND THE ENHANCED SURVIVAL OF CELLS AFFORDED BY IL-5

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Compelling evidence is available that eosinophils play a central role in the induction and perpetuation of allergic inflammatory responses which are characteristic features of asthma and atopic dermatitis. The ability of eosinophils to survive in the lung is believed to be due to the release of pro-inflammatory cytokines such as interleukin-5 (IL-5) which prevent the natural process of apoptosis (programmed cell death). Despite the knowledge that IL-5 enhances eosinophil survival by preventing apoptosis, the cellular signalling pathways responsible for producing this effect are currently undefined. One possibility is that mitogen-activated protein (MAP) kinases play an important role. Indeed, inhibition of extracellular signal-regulated kinases (ERK) have been implicated in the survival-promoting actions of nerve growth factor and fibroblast growth factor-2 in PC-12 pheochromocytoma and L929 fibrosarcoma cell lines, respectively (Xia *et al.*, 1995; Gardner & Johnson, 1996). Moreover, IL-5 has been shown to activate ERK2 in human eosinophils (Pazdrak *et al.*, 1995; Bates *et al.*, 1996). Thus, the purpose of this study was to evaluate the role of p42 and p44 MAP kinases (ERK2 and ERK1 respectively) in spontaneous apoptosis of human eosinophils and in the ability of IL-5 to prevent this response.

Eosinophils were isolated from venous blood by Ficoll-Paque centrifugation followed by negative selection with anti-CD16 mAb microbeads. The resulting cell population contained >98% eosinophils. Apoptosis was determined by flow cytometry of eosinophils stained with propidium iodide, and confirmed morphologically. Cytosolic proteins were extracted and used for western blot and "in-gel" phosphorylation assays. For western blotting, proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with specific antibodies that recognise the unphosphorylated and phosphorylated forms of ERK1 and ERK2, which were detected by ECL. For the "in-gel" phosphorylation assays, proteins were size fractionated by SDS-PAGE in gels containing myelin basic protein ($MBP)_2$. Following renaturation of the proteins, the gels were incubated with [γ^{-}]ATP and phosphorylation of MBP allowed to proceed for 1 h. Non-specifically-bound radioactivity was washed away and the remaining activity detected by exposure to film.

Using a combination of flow cytometry and morphological analyses, IL-5 (100 fM - 1 nM) increased the survival of human eosinophils. This effect was concentration-dependent and was the result of inhibiting those processes which govern apoptosis. For example, after culture of eosinophils for 48 h in the absence IL-5, 29.8 ± 8.3% (n = 9) of the cells were apoptotic whereas in the presence of IL-5 (10 pM) only 8.7 ± 1.1% (n = 9) showed apoptotic characteristics. Consistent with data reported by Bates et al. (1996), a high concentration of IL-5 (1 nM) (approximately 100-times greater than that required to enhance maximally eosinophil survival), activated ERK1 and ERK2 (assessed by western blot analyses and "in gel" renaturation assays) in a time-dependent manner with a maximal effect seen at 30 min. However, neither ERK1 nor ERK2 were activated at a lower concentration of IL-5 (10 pM) where survival was still markedly enhanced. A selective inhibitor of ERK1 and ERK2, PD 098059 (0.1-30 μM), did not affect spontaneous eosinophil apoptosis or the enhanced survival of cells effected by IL-5 (100 fM - 1 nM) when measurements were made at 48 h, but inhibited by 59.7 ± 7.4% (n = 3) the activation of ERK1/ERK2. Brief exposure (3 to 180 min) of eosinophils to IL-5 (10 pM), before culture for 48 h, inhibited apoptosis maximally by only 20.1 ± 4.4% (n = 3), whereas in the continuous presence of IL-5 (10 pM) 86.6 ± 0.6% (n = 3) of the cells remained viable. Thus, transient exposure of eosinophils to IL-5 is insufficient to maintain eosinophils viable. Taken together, these data demonstrate that although IL-5 can activate ERK1 and ERK2 in human eosinophils, they are not involved in the processes that govern spontaneous apoptosis or the enhanced cell survival afforded by IL-5.

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259P [D-Arg^6,D-Trp^{7,9},N^{me}Phe^8]-SUBSTANCE P (ANTAGONIST G) INDUCES APOPTOSIS IN SMALL CELL LUNG CANCER CELLS VIA A NOVEL MECHANISM INVOLVING ACTIVATION OF jun KINASE

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Antagonist G ([D-Arg^6, D-Trp^{7,9}, N^{me}Phe^8]-substance P (6-11)) is a hexapeptide which inhibits small cell lung cancer (SCLC) cell growth *in vitro* and *in vivo* and is currently under Phase I clinical investigation for the treatment of small cell lung cancer (Sethi, *et al.*, 1992; Langdon *et al.*, 1996). Its proposed mechanism of action is due to inhibition of cellular signalling via antagonism of a broad range of neuropeptide receptors (Sethi *et al.*, 1992), however there is some evidence that this class of compound interacts directly with G-proteins (Mukai *et al.*, 1992). The present study aims to establish whether antagonist G can induce apoptosis in SCLC cell lines *in vitro* and examines the effect of antagonist G on c-jun N terminal kinase (JNK) an enzyme known to be activated by a number of other apoptotic inducing stimuli in other cell types. In addition the effect of antagonist G on G-protein activity will be assessed by [^{35}S]-GTPγS binding to SCLC membranes.

The SCLC cell line NCI-H69 was used in these studies. Cells were cultured in RPMI-1640 medium with 10% foetal calf serum and quiesced for 24 h in serum free RPMI with 0.25% bovine serum albumin prior to use. In apoptosis studies quiesced cells (1x10^6) were incubated in serum free RPMI with antagonist G at 37°C for 24 h. Apoptosis was assessed morphologically as described (Tallet *et al.*, 1996). For JNK activity quiesced cells (1x10^7) were incubated with antagonist G for 60 min at 37°C. Cell lysates were prepared and JNK measured by immunocomplex assay using 3 μg anti-JNK full length polyclonal antibody and 1 μg GST-c-jun(1-79) (Santa Cruz; Coso *et al.*, 1995).

[^{35}S]-GTPγS binding was measured as described (Wieland & Jakobs, 1994).

In the present study we show that antagonist G (25 μM) stimulates the basal rate of apoptosis in SCLC cells from 10.5 ± 4.2% to 37.1 ± 5.4% (n=4) with an EC$_{50}$ of 3.0 ± 0.08 μM, (n=4). In addition we show that antagonist G (25 μM) caused a 2.9 fold increase in basal JNK activity (EC$_{50}$ = 3.2 ± 0.09 μM, n=4). We also show that antagonist G inhibits basal [^{35}S]-GTPγS binding to washed SCLC cell membranes (IC$_{50}$ 0.8 ± 0.02 μM, n=4) in the absence of neuropeptide.

We therefore suggest that the ability of antagonist G to induce apoptosis and stimulate JNK occurs via a neuropeptide-independent mechanism and not by neuropeptide receptor antagonism. The effect of antagonist G on [^{35}S]-GTPγS binding suggest that antagonist G has potent neuropeptide-independent effects in SCLC cells mediated at the G-protein level. Whether this effect mediates its effects on JNK and apoptosis requires further study.

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260P LIPOPOLYSACCHARIDE INDUCES DISSEMINATED ENDOTHELIAL APOPTOSIS REQUIRING CERAMIDE GENERATION

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The endotoxic shock syndrome is characterized by systemic inflammation, multiple organ damage, circulatory collapse and death. Excessive systemic release of tumor necrosis factor (TNF)α and other cytokines purportedly mediates this process. However, the primary tissue target remains unidentified. The present studies provide evidence that endotoxic shock results from disseminated endothelial apoptosis. Injection of lipopolysaccharide (LPS), and its putative effector TNFα, into C57BL/6 mice induced apoptosis in endothelium of intestine, lung, fat and thymus after 6 hours, preceding non-endothelial tissue damage. LPS or TNFα injection was followed within one hour by tissue generation of the pro-apoptotic lipid ceramide. TNF-binding protein, which protects against LPS-induced death, blocked LPS-induced ceramide generation and endothelial apoptosis, suggesting systemic TNF is required for both responses. Acid sphingomyelinase knockout mice displayed defects in LPS-induced endothelial apoptosis and animal death, defining a role for ceramide in mediating the endotoxic response. Further, intravenous injection of basic fibroblast growth factor, which acts as an intravascular survival factor for endothelial cells, protected mice against LPS-induced endothelial apoptosis and animal death. These investigations demonstrate that LPS induces a disseminated form of endothelial apoptosis, mediated sequentially by TNF and ceramide generation, and suggest that this cascade is mandatory for evolution of the endotoxic syndrome.

261P SIGNAL TRANSDUCTION PATHWAY INVOLVED IN PACLITAXEL-INDUCED CELL CYCLE ARREST AND APOPTOSIS

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Introduction: Taxoids are effective drugs in the treatment of several tumor type particularly ovarian and breast cancers (1). The cytotoxic effect of paclitaxel is related to its ability to stabilize microtubules and to enhance microtubule assembly (2). These pacitaxel effects on microtubule dynamics could account for a cell cycle block in mitosis (3). Paclitaxel can also induce apoptosis (4). It has been shown that paclitaxel activates raf-kinase and increases expression of an inhibitor of cyclin-dependent kinase, p21 (5). The aim of the present work was to study the relationships between signal transduction pathway induced by paclitaxel and cellular responses to this compound : cell cycle arrest and apoptosis.

Methods Paclitaxel cytotoxic effect was measured by clonogenic assay. Dual analysis of DNA content (propidium iodide staining) and p21 content (immunofluorescence staining) were made by flow cytometry. Apoptosis was assessed by TUNEL technique. $p34^{cdc2}$ immunoprecipitates were assayed for presence of p21 protein by western blotting or for histone kinase activity.

Results : MCF-7 breast adenocarcinoma cells and NIH-OVCAR-3 ovarian adenocarcinoma cells are highly sensitive to paclitaxel cytotoxic effect (respective IC_{50} 0.9+/-0.1 nM and 0.7+/-0.2 nM). In both cell lines, paclitaxel treatment results in a time and dose-dependent accumulation of cells with a G2/M DNA content, whereas apoptosis is only observed in the NIH-OVCAR-3 cell line. In this cell line, overexpression of the antiapoptotic gene bcl-2 inhibits paclitaxel-induced DNA fragmentation without affecting the ability of this compound to block the cell cycle in mitosis, suggesting that, mitotic arrest is not sufficient for paclitaxel-induced apoptosis. It has been previously found that paclitaxel treatment results in an elevation of p21 protein level and that this paclitaxel effect requires raf-kinase activation (5). An activation of raf-kinase and Map-kinase, which occurs 12h after paclitaxel addition, is detected in MCF-7 and NIH-OVCAR-3 cells, whereas, a concomittant increase in p21 protein level is only found in MCF-7 cells. Kinetic studies indicate that p21 protein elevation is subsequent to the mitotic arrest and occurs in cells arrested with a G2/M DNA content. In MCF-7 cells, the increase in p21 is correlated with the exit from abnormal mitosis which results in formation of cells with micronuclei. Indeed in this cell line, a correlation exists between elevation of p21 expression and inhibition of $p34^{cdc2}$ activity. In addition, high amount of p21 protein is associated with inactive $p34^{cdc2}$/cyclin B protein complex following paclitaxel treatment. In addition, p21 antisense oligonucleotides inhibit paclitaxel induction of p21 and significantly decreases MCF-7 cell survival after paclitaxel treatment. In NIH-OVCAR-3 cells, defective in basal and paclitaxel-induced p21 expression, treatment with paclitaxel results in a prolonged activation of $p34^{cdc2}$ and a delayed mitotic exit associated with apoptotic cell death.

Conclusion : These data suggest that p21 is not required for paclitaxel-induced mitotic arrest but argue in favor of a role of p21 in facilitating the progression through mitosis and enhancing cell survival after spindle damage induced by paclitaxel treatment. A role of p21 as a repressor of paclitaxel-induced apoptosis is now under investigation.

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262P POLYAMINE REGULATION OF ETOPOSIDE-INDUCED APOPTOSIS IN HL-60 CELLS

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Polyamines are required for optimal rates of cell growth, for stabilisation of cellular macromolecules and for protein synthesis (Marton & Pegg, 1995). They also have a role in cell death with high concentrations being cytotoxic and lower concentrations preventing glucocorticoid induced DNA fragmentation. The aim of this study was to determine the effects of polyamine depletion or addition on etoposide induced apoptosis in human leukaemic HL-60 cells.

HL-60 cells were grown ± spermine 25μM (+1mM aminoguanidine) or 1mM α-difluormethylornithine (DFMO), an inhibitor of polyamine biosynthesis, for 48 h. Apoptosis was measured by DNA fragmentation (ELISA), gel electrophoresis, flow cytometry and polyamines by HPLC.

Exogenous polyamine addition at the same time as etoposide decreased DNA fragmentation in HL-60 cells to varying extents with spermine being the most effective. A 48 h exposure to polyamines increased intracellular polyamine concentrations by 60% and decreased DNA fragmentation compared to control cells (Table 1). This was paralled by a decrease in the percentage of apoptotic cells measured by flow cytometry. Polyamine depletion by DFMO also decreased DNA fragmentation (Table 1) and the percentage of apoptotic cells. In both cases although DNA fragmentation was decreased, apoptosis was not prevented as a characteristic DNA ladder was detected upon gel electrophoresis. The reduction in the percentage of apoptotic cells and DNA fragmentation suggests that modulation of polyamine content delays the time course of apoptosis in HL-60 cells treated with etoposide. One explanation of these conflicting results is that the effects of both polyamine addition and depletion are mediated through ornithine decarboxylase (ODC). ODC has been shown to transform NIH 3T3 cells (Auvinen *et al.*, 1992) and also mediate apoptosis in IL-3 dependent cells (Packham & Cleveland 1994). Since DFMO inhibits ODC and high levels of polyamines induce antizyme which degrades ODC it may be that it is ODC which is important for apoptosis and that inhibition of ODC by what ever mechanism will delay DNA fragmentation.

Table 1: Effect of 1mM DFMO or 25μM spermine (+ 1mM aminoguanidine) on DNA fragmentation in HL-60 cells

Time (h)	etop	etop + DFMO (% of DMSO 0.2% control)	etop	etop + spm
12	550	280	321	0
24	273	131	741	210

Values are calculated from means of 2 experiments with 3 replicates

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263P DEVELOPMENT OF A HIGH-THROUGHPUT IMMUNOBLOTTING SYSTEM SUITABLE FOR THE DETECTION AND QUANTIFICATION OF PROTEINS IN HUMAN TISSUE

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Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970) and western blotting techniques (Towbin *et al.*, 1979) with immunodetection are a useful way of detecting the presence of specific proteins in tissue extracts. These methods tend to be labour intensive and can lack sensitivity when studying proteins that are N-terminal glycosylated, such as G-protein-coupled receptors (GPCRs). In the present study we describe an alternative method, which has been developed to provide a rapid measure of protein expression in a large range of protein extracts.

Protein extracts were prepared by homogenisation of tissue in a buffer containing 50mM HEPES (pH 7.4); 1mM EDTA; 1mM EGTA; 250mM sucrose; 0.2mM PMSF, using an UltraTurrax homogeniser. Protein in the tissue extracts was solubilised by adding 1% SDS and incubating for 10 minutes at room temperature. Cell debris was removed by high speed centrifugation (40,000x*g* for 15 minutes at room temperature). Protein concentration was determined using a Bicinchoninic acid protein assay with BSA as a standard. Proteins were blotted onto a 0.2μm nitrocellulose membrane using a 96-well filtration manifold system. Protein solutions were allowed to filter through the membrane under gravity and any residual volume drawn through after 1 hour under low vacuum. Endogenous alkaline phosphatase activity was inhibited on the blots through treatment with trichloroacetic acid and sulfosalicylic acid. Acid treatment did not affect the subsequent immunodetection of proteins. The blot was incubated with PBS, 1% low fat dried milk (Marvel) to block non-specific sites, and then probed with a protein specific antibody. Detection of bound antibody was achieved using enhanced chemifluorescence and visualisation with a fluorimager (Molecular Dynamics). The resultant image was quantified using ImageQuant software (Molecular Dynamics) which provides accurate densitometry over 5 log units. Control blots were treated with a non-immune, isotype-matched immunoglobulin. The results from the control blots were used to normalise the results for specific antibodies to account for autofluoresence and residual alkaline phosphatase activity in some protein extracts.

Validation of this technique was achieved through study of the expression of paxillin, a cytoskeletal protein. It was studied in human ileum and duodenum using a monoclonal anti-paxillin antibody which is a mouse IgG1 isotype (Transduction Laboratories). Quantification of paxillin was possible over a range of protein loadings in both tissues (0.0625-8μg; Figure 1). The results clearly demonstrate that the expression of paxillin is substantially higher in the ileum than in the duodenum. They also illustrate that quantification of a specific protein in an extract of human tissue is possible using this method.

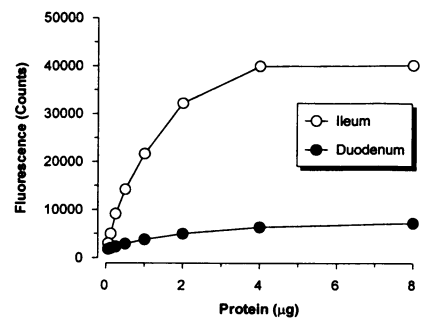

Figure 1. Quantification of paxillin in human ileum and duodenum protein extracts.

Further studies will assess the general applicability of the 96-well blotting methodology against low abundance, more therapeutically relevant human proteins such as GPCRs.

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264P ALLERGIC INFLAMMATION IN THE RAT URINARY BLADDER: INVOLVEMENT OF MAST CELLS AND C-FIBRES

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The aetiological basis for interstitial cystitis (IC), a chronic inflammatory disease, is unclear. It has been proposed that IC may develop in response to an immune reaction, and supporting this immunoglobulin and complement deposits have been identified in affected bladders. Furthermore there have been several reports associating systemic allergic disease, such as asthma, with IC (Hellstrom *et al.* 1979). Thus models which mimic such immunologically-derived responses would be of potential value in further elucidation of the mechanisms involved in the processes of IC development as well as for investigation of therapeutic interventions. In light of this we have developed a model of bladder allergy in the rat.

Sensitisation of female wistar rats (150-175g) was achieved by i.p. injection of a mixture of 1mg ovalbumin (OA) and 100mg $Al(OH)_3$ suspended in 1 ml of saline. Fourteen days later rats were either killed by dislocation of the neck, bladders removed and mounted in an organ bath for challenge with OA *in vitro* or anaesthetised with pentobarbital (45 $mgkg^{-1}$) and challenged with OA *in vivo* (intravesical administration of 10mg in 0.3 ml saline) or saline control. At various times after *in vivo* challenge the extent of plasma protein extravasation (PPE) was determined by the estimation of intravenously administered Evans Blue dye leakage into the bladder (oedema expressed as $ngmg^{-1}$ tissue). The effect of various selective antagonists or enzyme inhibitors on the OA responses were investigated.

OA caused a time-dependent PPE (n=11-18) in the urinary bladder which was biphasic with peak responses at 2-4h (125.6±29.9) and 24h (154.4±57.3). The 2 h PPE response was abolished by systemic capsaicin pretreatment (50 mg kg^{-1} s.c. 4 days prior to use) (n=16, $p<0.05$) whilst the response at 24h was unaffected (n=14). Prior degranulation of mast cells (compound 48/80 5 mg kg^{-1}, s.c. for 3 consecutive days) significantly inhibited (29±5 $ngmg^{-1}$ tissue n=8, $p<0.05$) the PPE response at the 2h timepoint compared to control (89±30 $ngmg^{-1}$ tissue n=8). The tachykinin NK-1 receptor antagonist, SR140333 (0.1 µmol kg^{-1}i.v., 1h pretreatment) inhibited the 2h PPE response (n=10, $p<0.05$) whilst the tachykinin NK-2 receptor antagonist MEN 11420 (0.1 µmol kg^{-1}) had no significant inhibitory effect (n=8). The bradykinin B_2 receptor antagonist, HOE 140 (0.1 µmol kg^{-1}) similarly to SR 140333 blocked the 2h PPE response to OA (n=10, $p<0.05$) whereas the selective B_1 receptor antagonist B9858 (0.1 µmol kg^{-1}) had no significant effect (n=8). Inhibition of cyclooxygenase (COX) achieved by pretreatment with the COX inihibitor dexketoprofen (2 $mgkg^{-1}$) also blocked the PPE response.

In the isolated urinary bladder OA (1 $mgml^{-1}$) produced a contractile response which was modified by certain drug treatments as shown in table 1 below.

Table 1 Modulation of OA-induced contraction in vitro

Pretreatment	Contraction to OA (% of NKA)	significance andn=animals
Control	37.4 ± 3.1	n=7
Capsaicin (10µM, 20 min)	32.1 ± 4.2	NS, n=7
Compound 48/80 (in vivo as above)	22.0 ± 4.8	p<0.05, n=7
Capsaicin + compound 48/80	12.7 ± 3.2	p<0.01, n=7
ONO1078 (1µM, 15 min)	33.5 ± 4.9	NS, n=8
HOE 140 (1 µM, 15 min)	37.0 ± 5.4	NS, n=5

These results show that both the early inflammatory response and alterations in smooth muscle reactivity to OA challenge in actively sensitised animals is dependent on mast cell degranulation and the activation of sensory C-fibres. Furthermore this model of allergic cystitis may be a convenient model to investigate both the processes involved and potential novel therapies in the treatment of interstitial cystitis.

AA is a BHF intermediate Fellow

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265P INHIBITION OF NEUTROPHIL:ENDOTHELIUM INTERACTION IN MURINE POST-CAPILLARY VENULES BY LIPOCORTIN 1

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Neutrophil (PMN)-derived lipocortin 1 (LC1) inhibits PMN emigration in *in vitro* assays and exerts anti-migratory effects in several murine models of PMN extravasation (Perretti *et al.*, 1993, 1996); its action is mimicked by a peptide spanning its N-terminus, i.e. peptide Ac2-26. We have tested here peptide Ac2-26 and LC1 actions on PMN extravasation across inflamed murine mesenteric post-capillary venules.

Male Swiss Albino mice (12-15 g) were fasted overnight and injected i.p. with either 0.5 ml saline or with 1 mg zymosan. Animals received 200 µg s.c. of peptide Ac2-26 or 200 µl phosphate-buffered saline (PBS) 30 min prior to the challenge. The mesenteric microvascular bed was mounted onto a microscope (Zeiss Axioskop "FS") equipped with a x40 water immersion objective lens. Images were projected onto a Sony Triniton colour video monitor by a video camera and recorded for subsequent off-line analysis. At 4 h post-zymosan injection, the number of adherent cells was determined in 1-3 randomly selected vessels of at least 100 µm in length and the number of emigrated leucocytes were monitored within 50 µm of the vessel wall. In some cases, mesenteries were exposed 2 h after zymosan and vessels with 5-6 adherent cells selected: PBS (100 µl), peptide Ac2-26 (200 µg) or human recombinant LC1 (25 µg) were then administered i.v. and the fate of these adherent leucocytes monitored up to 10 min. In other cases, peritoneal cavities were washed 2 or 4 h after zymosan (200 µg peptide Ac2-26 or 200 µl PBS being given i.v. at 2 h) and the number of accumulated PMN quantified following specific staining. Statistical differences between experimental groups were determined by ANOVA followed by the Bonferroni test.

Zymosan challenge produced an increase at 4 h in adherent and emigrated leucocytes: 6.2 ± 0.7 vs. 2.0 ± 0.3 adherent cells, and 9.8 ± 1.7 vs. 2.1 ± 0.2 extravasated cells in zymosan- and saline-treated mice, respectively (n=6, $P<0.05$). Treatment of mice with peptide Ac2-26 reduced the extent of cell adhesion and emigration by 43% and 55%, respectively (n=7; $P<0.05$) Using the protocol to monitor the fate of adherent leucocytes, injection of sterile PBS 2 h post-zymosan produced modest effects, such that 80% of adherent leucocytes emigrated through the vessel wall and minimal cellular detachment was observed (4.6 ± 0.8 vs. 6.2 ± 0.5 adherent cells at 6 min post treatment vs. time 0; not significant). In contrast, peptide Ac2-26 caused an extensive detachment of leucocytes (80% over the 10 min of obvservation) with fast kinetics: 1.4 ± 0.4 vs. 5.6 ± 0.5 adherent cells at 6 min post-treatment vs. time 0; n=5, $P<0.05$). A similar effect was seen with LC1, and only 0.5 ± 0.3 leucocytes were still adherent at 6 min post-treatment (vs. 6.0 ± 0.4 adherent cells at the starting time; n=4, $P<0.05$). The 'therapeutic' administration of peptide Ac2-26 was also tested in the peritonitis model: peptide Ac2-26 or PBS were given i.v. at 2 h post-zymosan (a time-point where 2.2 ± 0.1 $x10^6$ PMN could be recovered from the peritoneal cavity, n=12), and cell influx measured at 4 h with 13 ± 0.9 $x10^6$ vs. 7.3 ± 0.7 $x10^6$ PMN in the PBS and peptide Ac2-2-6 group, respectively (53% of inhibition on net values, n=12, $P< 0.05$).

In conclusion, acute administration of peptide Ac2-26 produces a rapid alteration of leucocyte post-adhesion events, in mesenteric post-capillary venules, leading to a reduced cell influx in acute inflammation.

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266P EVIDENCE THAT CANNABINOID-INDUCED INHIBITION OF EVOKED CONTRACTIONS OF THE GUINEA-PIG MYENTERIC PLEXUS PREPARATION CAN BE MODULATED BY Ca^{2+} AND cAMP

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Cannabinoid receptor agonists inhibit electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine (MPLM), most probably by acting through prejunctional cannabinoid CB_1 receptors to reduce acetylcholine release (Coutts & Pertwee, 1997). Several effector systems have been proposed for CB_1 receptors. Among these are N-type Ca^{2+} channels and adenylate cyclase, to both of which CB_1 receptors are thought to be negatively coupled through $G_{i/o}$ proteins. The present experiments were directed at further validating an involvement of CB_1 receptors in cannabinoid-induced inhibition of evoked contractions of MPLM. This was achieved by establishing whether the inhibitory effect on MPLM of the CB_1 receptor agonist, (+)-WIN55212, could be appropriately modulated by altering Ca^{2+} concentration in the surrounding medium or by adding forskolin, 8-bromo-cAMP or 3-isobutyl-1-methylxanthine (IBMX). Some experiments were performed with normorphine instead of (+)-WIN55212.

Strips of MPLM were dissected from the small intestines of male albino Dunkin-Hartley guinea-pigs (280-800g) and suspended in 4 ml organ baths containing modified Krebs solution kept at 37° C and bubbled with 95% O_2 and 5% CO_2 (Coutts & Pertwee, 1997). Tissues were stimulated continuously with 0.5 ms pulses and supramaximal voltage at 0.1Hz. Once isometric contractions were constant, preparations were pretreated with with forskolin, 8-bromo cAMP or IBMX, 30 min before the construction of cumulative dose-response curves to (+)WIN 55212. These were compared with control dose-response curves of this cannabinoid. In addition, (+)-WIN 55212 and normorphine dose-response curves were constructed in Krebs solution containing 0.64 mM or 5.08 mM Ca^{2+} instead of the usual 2.54 mM. Values have been expressed as means and limits of error as s.e.mean. Data were subject to analysis of variance (ANOVA) followed by Dunnett's two-tailed test. A P value of < 0.05 was considered to be significant.

Maximum inhibition of contractile responses by (+)-WIN55212 was 70.79±3.45% (n=8). This was significantly reduced to 35.8±6.6% by forskolin (100 nM; n=6), to 36.55±3.9% by 8-bromo-cAMP (100 nM; n=6) and to 42.4±3.4% by IBMX (100 nM; n=7) ($P< 0.01$). At this concentration, none of these pretreatment drugs had any significant effect on the control twitch tension or on the contractions due to exogenous acetylcholine (n=3-5). Decreasing the Ca^{2+} concentration to 0.64 mM, produced a significant increase in the maximum inhibitory effect of (+)-WIN55212 from 55.9±4.9% to 76.44±5.07% ($P<0.01$; n=8) and a slight leftward shift of the log concentration-response curve of the agonist. Increasing the Ca^{2+} concentration to 5.08 mM, resulted in a significant reduction the maximum inhibitory effect of (+)-WIN55212 to 40.7±3.0% (n=10; $P<0.05$). Similarly, the maximum inhibitory effect of normorphine, which was 59.4±5.3% in the presence of 2.54 mM Ca^{2+}, rose significantly in the presence of 0.64 mM Ca^{2+} (to 77.9±4.43%; n=10, $P< 0.01$) and fell significantly in the presence of 5.08 mM (to 41.95±4.99%; n=10, $P< 0.01$). These results lend further support to the hypothesis that cannabinoid-induced inhibition of electrically-evoked contractions of MPLM is mediated by cannabinoid CB_1 receptors.

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267P EFFECTS OF TACHYKININS AND CAPSAICIN ON THE MECHANICAL AND ELECTRICAL ACTIVITY OF THE GUINEA-PIG ISOLATED TRACHEA

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The effects of tachykinins and capsaicin were studied in the isolated trachea of the guinea-pig by measuring intracellular membrane potential and isometric tension. Experiments were carried out in the presence of phosphoramidon (10 µM) and captopril (1 µM). These inhibitors did not alter the resting membrane potential of the tracheal smooth muscle cells which averaged -51 mV. Most preparations presented spontaneous slow wave activity. Tetraethylammonium (TEA), a potassium channel blocker (8 mM), depolarized the membrane potential to -44 mV and induced a rhythmic activity.
In control solution, substance P [Nle^{10}]-neurokinin A(4-10) (10 nM -1 µM), and capsaicin (0.1 and 1 µM) induced concentration-dependent depolarizations which were statistically significant at the highest dose tested (depolarization by 1 µM: 8, 11 and 16 mV respectively). In the presence of TEA, the three substances induced depolarizations which were statistically significant at the highest dose tested for substance P (1 µM) and at 0.1 and 1 µM for both [Nle^{10}]-neurokinin A(4-10) and capsaicin (depolarization by 1 µ M: 11, 17 and 10 mV for substance P, [Nle^{10}]neurokinin A(4-10) and capsaicin, respectively). In the presence or absence of TEA, [$MePhe^7$]-neurokinin B (10 nM - 1 µM) did not induce any significant changes in membrane potential. The depolarizing effects of substance P and [Nle^{10}]-neurokinin A(4-10) (1 µM) were blocked specifically by the specific antagonist of NK_1 and NK_2 receptors: SR 140333 (0.1 µM) and SR 48968 (0.1 µM), respectively. The effects of capsaicin (1 µM) were partially inhibited by each antagonist and fully blocked by their combination.

Substance P (1 nM - 100 µM), [Nle^{10}]-neurokinin A(4-10) (0.1 nM - 10 µM), [$MePhe^7$]-neurokinin B and capsaicin (0.1 µM - 10 µM) evoked concentration-dependent contractions. The contractions to substance P were significantly inhibited by SR 140333 (apparent pA_2 value 7.5 ± 0.2; n = 6, slope of the Schild plot constrained to unity) but unaffected by SR 48968 (10 nM - 1 µM). On the other hand, the response to [Nle^{10}]-neurokinin A(4-10) was significantly inhibited by SR 48968 (apparent pA_2 value 8.5 ± 0.2; n = 6, slope of the Schild plot constrained to unity) and unaffected by SR 140333 at the same concentrations. Although SR 48968 (0.1 µM) alone did not influence the effects of substance P, it potentiated the inhibitory effect of SR 140333 (0.1 µM) (pK_B of SR 140333 individually: 7.6 ± 0.1, pK_B of the combination of SR 140333 + SR 48968: 8.9 ± 0.2; n = 6, $p < 0.05$) A similar synergistic effect of these two compounds was observed in the inhibition of the contractile response to [Nle^{10}]-neurokinin A(4-10) (pK_B of SR 48968, 0.1 µM, individually: 8.5 ± 0.2, pK_B of the combination of SR 48968 + SR 140333, 0.1 µM: 9.2 ± 0.2; n = 6, $p < 0.05$. Neither SR 140333 nor SR 48968 (0.1 µM) individually influenced the contractions to [$MePhe^7$]-neurokinin B and capsaicin. However, the combination of the two antagonists abolished the contractions to either peptide.

These results demonstrate that the stimulation of both NK_1 and NK_2 tachykinin-receptors induced contractions and depolarizations of the guinea-pig tracheal smooth muscle and that both receptors are stimulated during the endogenous release of tachykinins by capsaicin. There is no evidence for a major role of NK_3 receptors in the contraction of the isolated guinea-pig trachea.

268P DIFFERENCES IN THE PHARMACOLOGICAL PROFILES OF HUMAN LUNG MEMBRANES FROM SMOKERS AND NON-SMOKERS

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Cigarette smoking reduces lung function in man and may be associated with chronic obstructive lung diseases such as bronchitis and emphysema. However, there is evidence that exposure to cigarette smoke is also associated with an increased incidence of asthma (Weitzman *et al.*, 1990), and that it exacerbates the symptoms of the disease (Menon *et al.*, 1991). As the mechanism of the 'pro-asthmatic' effect of smoking is unclear, the present study was designed to investigate whether smokers' lungs show any impairment in their responsiveness to agents which elevate cAMP.

Human lung samples were obtained *post mortem* (PM). Tissue from three non-smokers (male, aged 17 yr, PM delay 9.45 h; male, aged 29 yr, PM delay 8 h; female, aged 39 yr, PM delay 12 h) and three smokers (male, aged 40 yr, PM delay 7.05 h; female, aged 53 yr, PM delay 7.5 h; male, aged 54 yr, PM delay 12.45 h) was used. Membranes were prepared as described by Merritt *et al.*, 1991. Briefly, lung tissue was homogenized in a Waring blender in 0.29M sucrose/25mM Tris-HCl buffer (pH 7.4, 4°C) and centrifuged for 10 min at 1000x*g*. The resultant supernatant was used directly in the study or stored at -80°C. The study design was essentially as described by Keen & Nahorski, 1988. Membranes (300 - 400μg) were incubated in V-bottomed 96-well plates for 15 min at 37°C in buffer containing 25mM HEPES, 64mM Tris maleate (pH 7.4), 0.5mM EGTA, 100mM NaCl, 1mM IBMX, 0.1mM GTP, 1mM ATP and test agonists. The reaction was terminated by microwave for 15s, after which the samples were centrifuged for 15 min at 2000x*g*. The amount of cAMP in the sample was determined using a cAMP binding protein assay. Data are expressed as mean values ± s. e. mean (n ≥ 8 from three donors).

Concentration-effect curves for the prostanoid IP-receptor agonists, iloprost and cicaprost (0.1nM - 10μM), the β-adrenoceptor agonist, isoprenaline (0.1nM - 100μM), and the direct activator of adenylate cyclase, forskolin (0.1nM - 100μM), were constructed. Basal levels of cAMP in lung membranes from smokers (13.15 pmol ± 0.34) were 2 to 3-fold lower than those seen for non-smokers (35.45 pmol ± 0.85).

In lung membranes from non-smokers, all four compounds caused concentration-related increases in cAMP production (pEC_{50} values; iloprost 6.86 ± 0.08; cicaprost 6.97 ± 0.04; forskolin 4.92 ± 0.18; isoprenaline 5.84 ± 0.19; maximum cAMP levels; iloprost 93.49 pmol ± 4.32; cicaprost 71.52 pmol ± 2.43; forskolin 106.64 pmol ± 7.01; isoprenaline 60.66 ± 2.76). In lung membranes from smokers, over the same concentration range, iloprost, cicaprost and forskolin again caused concentration-related increases in cAMP (pEC_{50} values; iloprost 6.96 ± 0.06; cicaprost 7.09 ± 0.12; forskolin 5.41 ± 0.16), however, maximum increases in cAMP were lower than those obtained in lungs from non-smokers (maximum cAMP levels; iloprost 27.01 pmol ± 1.28; cicaprost 36.44 pmol ± 1.35; forskolin 23.86 pmol ± 0.71). Interestingly, in lung membranes from smokers, isoprenaline was without effect on cAMP levels, even at the highest concentration tested (100μM).

These results suggest that smoking reduces both basal and agonist-induced elevation of cAMP in lung membranes. The inhibition of agonist-induced activity was most marked with isoprenaline. It is possible that smoking causes a chronic elevation of endogenous mediators which result in increases in cAMP, leading to a down-regulation of receptors and/or cAMP signal transduction. In this regard, smoking has been shown to elevate circulating catecholamine levels (Grassi *et al.*, 1994). However, an alternative explanation is that a contaminant is present in smokers' lungs which interferes with cAMP detection.

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269P TUMOUR NECROSIS FACTOR-α PRIMES EOSINOPHIL ADHESION TO HUMAN BRONCHIAL EPITHELIAL CELLS

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Increased concentrations of tumour necrosis factor-α (TNF-α) have been detected in the bronchiolar lavage fluid of symptomatic asthmatics (Barnes, 1994) and may, in part, mediate eosinophil/airway epithelial cell adhesion seen in asthma. Cytokines such as interleukin (IL)-5 and IL-3 have been shown to prime eosinophil function (Koenderman *et al.*, 1996) in that they have little effect alone but increase the response of a second stimulus. We hypothesised that TNF-α may also prime eosinophil function and in the present study we investigated the effects of TNF-α priming on C5a-stimulated eosinophil adhesion to normal human bronchial epithelial cells (NHBEC).

NHBEC (Clonetics, San Diego, USA) were maintained in basal epithelial growth medium supplemented with antibiotics and monolayers of NHBEC were grown to confluence on 96-well plates. Human eosinophils isolated from peripheral blood of mildly atopic adult donors and labelled with a fluorescent dye (Calcein-AM, 10μM; Burke-Gaffney and Hellewell, 1996), were incubated for 30 min with NHBEC in the presence of TNF (10ng ml^{-1}) or Krebs-Ringer-Phosphate-Dextrose buffer (KRPD). C5a (10^{-7}M) or KRPD were added to NHBEC/ eosinophil co-culture for a further 30 min and adhesion measured at 60 min. Results were expressed as mean ± s.e mean of percent adherent cells over total cells (1×10^5) added per well, determined by fluorescence.

Basal adhesion of unstimulated eosinophils to NHBEC (16.3±1.9%,n=6) was not significantly increased following exposure of eosinophils during the adhesion assay to TNF-α (15.1±2.3%,n=4). Incubation of eosinophils with NHBEC and KRPD followed by C5a significantly ($P<0.05$) increased eosinophil adhesion to NHBEC (31.1±2.8%, n=6); this was further increased when eosinophils were primed with TNF-α (46.9±8.5%, n=4; $P<0.05$). Adhesion was also increased when eosinophils were pre-incubated with NHBEC (15 min), then exposed to TNF-α (15 min) and finally to C5a (30 min; 71.1±6.1%, n=3). When measured at 30 min, C5a significantly ($P<0.01$) increased eosinophil adhesion from 9.2±1.3% to 14.7±0.7% (n=4) but pre-exposure of eosinophils to TNF-α in an eppendorf tube (30 min) did not further increase this adhesion (15.0±2.2%). Monoclonal antibodies against CD18 (6.5E, 20μg ml^{-1}) or ICAM-1 (11C8-I, 10μg ml^{-1}), but not VLA-4 (2B4, 20μg ml^{-1}), abolished the increased adhesion due to TNF-α priming.

These results show that TNF-α primes C5a-stimulated eosinophil adhesion to NHBEC. The primed adhesion was CD18/ICAM-1-dependent and contact with the epithelial monolayer facilitated priming. We speculate that TNF-α priming of eosinophil adhesion may play a role in facilitating eosinophil-epithelial interactions in airway inflammatory diseases such as asthma.

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270P [Arg^6, D-Trp^{7,9}, N-MePhe^8]-SUBSTANCE P (ANTAGONIST G) POTENTIATES OEDEMA IN RABBIT SKIN

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Antagonist G is a substance P analogue which is an effective inhibitor of small cell carcinoma of the lung (Jones *et al.*, 1996). However, its therapeutic potential is limited by its toxicity. The aim of this project was to determine whether the toxic effects of antagonist G were related to oedema formation, using the rabbit skin model (Williams, 1979).

New Zealand white rabbits (males, 2-3 kg) were anaesthetised with Sagatal (30 mg/kg), the back shaved and marked out with 4 latin squares of 15 injection sites. ^{125}I-Albumin (5 μCi) made up in Evans blue dye solution (2.5%) was injected 5 min before intradermal injections of inflammatory mediators. Antagonist G (50 nmol/site) was either co-injected or given 5 min before bradykinin (BK) or n-formyl-methionyl-leucyl-phenylalanine (fMLP) for 30 min. Injection sites (16 mm) were counted in a gamma counter and results converted to μl plasma. Table 1 shows that antagonist G (50 nmol/site) induced oedema compared with the vehicle control (27 ± 1 and 17 ± 1 μl plasma respectively, n=8, $p<0.001$) and significantly (Anova, $p<0.001$) potentiated oedema formation by BK and fMLP. Antagonist G was not significantly potentiated by PGE_2 (1 μg), giving exudation of 26 ± 1 and 27 ± 2 μl plasma respectively. The inflammatory effect of antagonist G was not observed with either of its two major metabolites (Jones *et al.*, 1996).

These data suggest that the toxic effects of antagonist G may be related to oedema formation. This is unlikely to involve substance P since the latter does not induce oedema in rabbit skin (Brain & Williams, 1985).

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Table 1. Oedema formation in the rabbit skin (mean ± sem μl plasma n=16 sites): effect of antagonist G

	saline	BK (0.1 nmol)	BK (0.3 nmol)	BK (1 nmol)
control response	11 ± 2	30 ± 7	37 ± 9	49 ± 8
+ antagonist G (pre-) *	19 ± 2	51 ± 6	54 ± 8	68 ± 9
control response	17 ± 1	52 ± 7	69 ± 9	82 ± 9
+ antagonist G (co-) *	27 ± 1	73 ± 5	93 ± 6	93 ±10
	saline	fMLP (20 ng)	fMLP (100 ng)	
control response	17 ± 1	21 ± 2	25 ± 1	(* P< 0.001 Anova)
+ antagonist G (co-) *	26 ± 1	39 ± 3	37 ± 1	

271P LPS-INDUCED PLASMA LEAKAGE IN RAT SKIN IS MODULATED BY INDUCIBLE NITRIC OXIDE SYNTHASE THROUGH THE ACTIVATION OF TRANSCRIPTION FACTOR NF-κB

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Rats challenged with lipopolysaccharide (LPS) produce large amounts of nitric oxide (NO) following the induction of the inducible NO-synthase (iNOS) in several tissue and organs (Làszlò et al., 1995). Recent studies have shown that iNOS expression is regulated at transcriptional level by the transcription nuclear factor-κB (NF-κB) (Schreck et al.,1992).

In this study we investigated the role of NO in a model of LPS-induced plasma leakage in rat skin using the non-selective NOS inhibitor N^G-nitro-L-arginine methylester (L-NAME) and the more selective iNOS inhibitor S-methyl isothiourea (SMT). Moreover, we studied the involvement of NF-κB in this model using pyrrolydinedithiocarbamate (PDTC), an inhibitor of NF-κB activation. Plasma leakage in the skin was measured in male Wistar rats (250-300g) anaesthesized with Nembutal (60 mg/kg i.p.). The back of rats was shaved and ^{125}I-labelled human serum albumine (HSA) (2 μCi/kg of body weight in 1% Evans blue solution) was injected i.v. LPS, L-NAME, SMT or saline (100 μl) were injected intradermally in duplicate immediately before ^{125}I-HSA administration. PDTC was injected locally 30 min before stimulus. After 30 min to 2 h terminal blood samples were taken by intracardiac puncture. The injection sites were punched out, samples were counted in a gamma-counter and the results were converted to l μl of plasma. In some experiments, the skin samples from rats were cut and nuclear mini-extracts were prepared according to the procedure described by Schreiber et al. (1989). NF-κB activation was evaluated by a gel shift assay. In the same experiments cytosolic proteins from skin homogenates were electrophoresed, transferred onto nitrocellulose membrane and incubated with an anti-iNOS antibody. The immune complexes were visualised by the ECL chemiluminescence method. All the results are expressed as mean± S.E.M. (n=6-12 rats). Statistical comparisons were made by one-way ANOVA followed by Bonferroni's test *P<0.05. LPS (1, 10, 100 μg/site) produced a dose-related increase in plasma leakage (18.2±3.2; 27.2±2.9*; 40.4±9.6* μl/site) as compared to saline control (11.4±2.2 μl/site). This increase was maximal at 2h; therefore this time point and the dose of 10 μg/site LPS was used in all other experiments. L-NAME and SMT (0.01, 0.1, 1.0 μmol/site) reduced plasma leakage in a dose-related manner (L-NAME: 26.0±5.5, 20.2±1.6, 18.0±2.0* μl/site; SMT: 19.5±1.5*, 17.0±1.6*, 15.0±2.3* μl/site) compared to LPS alone. At the lowest concentration used (0.01 μmol/site), SMT significantly reduced plasma leakage by 30.0±1.2%, while L-NAME was not effective. PDTC (0.01, 0.1, 1.0 μmol/site) inhibited plasma leakage by 9.0±0.6, 33.0±4.0*, 51±2%* respectively and suppressed LPS induced NF-κB activation. Western blot analysis showed significant levels of iNOS protein in the skin samples of LPS-treated rats, as compared to basal levels present in saline-injected rats. PDTC (0.1-1.0 μmol/site) dose-dependently decreased iNOS protein expression induced by LPS. Our results indicate that LPS-induced plasma leakage in rat skin is modulated by NO mainly produced by the iNOS isoform. Furthermore, the suppression of oedema formation by PDTC, an inhibitor of NF-κB activation, is correlated to the inhibition of iNOS protein expression.

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272P HETEROGENEOUS LEVAMISOLE RECEPTORS IN *OESOPHAGOSTOMUM DENTATUM* AND ANTHELMINTIC RESISTANCE

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Resistance to anthelmintics drugs has increased as a problem as a result of continued treatment of nematode parasites of man and animals and requires further investigation. Levamisole is a selective nicotinic agonist producing spastic paralysis of nematodes but now resistance has become a serious issue. Properties of nicotinic acetylcholine receptors (nAChRs) activated by the anthelmintic levamisole were investigated using cell-attached and isolated inside-out muscle patch recordings from levamisole-sensitive and -resistant isolates of the nodular worm of the pig, *Oesophagostomum dentatum* using the methods of Robertson and Martin (1993). Levamisole-resistant isolates were produced by first infecting pigs with levamisole-sensitive isolates and the passaging 10 generations of *O. dentatum* through pigs, and on each passage treating with an increased dose of levamisole and culturing eggs and larvae from the surviving adults (Varady *et al.*, 1997). In levamisole-sensitive isolates, the conductances of the different receptors activated by 10μM levamisole varied but were in the range 28.5-45.6 pS. The mean open-times at -50 to -75mV also varied and were in the range 1.69-4.43 ms. The distributions of conductances and open-times of the channels showed that the receptors could not be described as a single homogeneous population. In a population of 13 channels, there were at least two channel subtypes: one type, *G35*, had a mean conductance of 35 pS and open-time of 1.6 ms; another subtype, *G45*, had a mean conductance of 44.6 pS and open-time of 2.7 ms. The distribution of the properties of 8 levamisole receptors recorded from resistant nematodes and activated by 10 μM levamisole also suggested heterogeneity (conductance range, 24.6-46.7 pS; mean open-times 0.73-2.89 ms at -50 to -75mV). On average the channels from the resistant-isolates carried less current than the channels from the sensitive-isolates. Molecular and genetic experiments on the free-living nematode, *C. elegans*, have identified some of the genes encoding the levamisole receptor in nematodes. The genes include: *unc-38* encoding an α-subunit of the nAChR and *unc-29 & lev-1* encoding β-subunits of the nAChR. Although the pharmacological profile of vertebrate neuronal nAChRs may vary with the molecular structure of the α-subunit, they are also influenced by the β-subunit structure because the agonist binding sites are thought to be formed at specific subunit interfaces with the α-subunit and adjacent β-subunit. Different combinations of the receptor subunits may account for the heterogeneity of the nAChRs seen in *O. dentatum* and a modification of the combinations could relate to the development of levamisole resistance in parasitic nematodes.

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273P MODULATION OF $GABA_A$ RECEPTORS AND INHIBITORY SYNAPTIC CURRENTS IN CULTURED RAT CORTICAL NEURONES BY THE ENDOGENOUS BRAIN LIPID *CIS*-9,10-OCTADECENOAMIDE (COA)

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Introduction *Cis*-9,10 octadecenoamide (cOA) was first isolated from the cerebrospinal fluid of sleep deprived cats and induced physiological sleep when injected, i.p., into laboratory rats (Cravatt *et al*, 1995). The mechanism of action of cOA is unknown although it has been shown to modulate recombinant $5HT_2$ receptors expressed in Xenopus oocytes (Huidobro-Toro & Harris, 1996). Here we examine its potential to modulate the $GABA_A$ receptor which is an acknowleged target for many depressant/hypnotic drugs.

Methods Oleic acid was treated with oxalyl chloride in CH_2Cl_2. The amide was formed by treatment of the acid chloride with concentrated NH_4OH and purified as a single band by column chromatography (M.P.76°C). Whole-cell recordings were obtained from pyramidal cells in cultured cortical neurones: exogenous GABA was applied via a Y-tube as 0.1-2s pulses, using established techniques and saline solutions (Lees &Leach, 1993). cOA was dissolved initially in dimethylsulphoxide (DMSO) or ethanol. All extracellular solutions contained 0.1% DMSO or ethanol, 0.033-0.1% bovine serum albumin (to facilitate dissolution of the lipid) with, or without, cOA. Curves were fit by non-linear regression to a 3-term logistic equation (Graph Pad software). Data are cited as mean ± s.e.mean and were analysed statistically as indicated below.

Results All cells were clamped at -30 or -45mV. Peak currents evoked by brief, 10μM GABA pulses were enhanced by 20μM cOA (the modulatory effect reached equilibrium within 10-12 min) in all cells examined (1.47 ± 0.07 fold, repeated measures ANOVA with Dunnett's test, $p<0.05$, n=5): the responses were fully reversible. Qualitatively similar results were obtained using either ethanol or DMSO as solvent. At the same agonist concentration (10μM is < GABA EC_{50}), in a further 35 cells this modulatory action was shown to be dose-dependent: the saturable sigmoid curve indicating an apparent threshold of 3.2μM for cOA and an EC_{50} of 15.3 ± 1.6μM. Almost all cells in cortical cultures exhibited spontaneous synaptic activity in physiological saline (see Lees and Leach, 1993) at 22-24°C. 50nM tetrodotoxin blocked the majority of the large (0.1-1nA) excitatory and inhibitory currents (n=3), indicating that they were evoked/multiquantal events. At 32-64μM cOA, both the frequency and the mean amplitude of spontaneous synaptic currents were markedly and reversibly depressed (n=8). 20μM cOA reversibly enhanced the duration of spontaneous outward currents in the cultured networks (n=3). Preliminary analysis indicates that averaged outward currents (50-100 events aligned at the mid point of their rising phase, using WCP software from J.Dempster, Univ. of Strathclyde) were best fit by two exponentials and that cOA selectively enhanced the slow component (by more than 2 fold).

Conclusions These data suggest that cOA may represent an endogenous (humoral or membrane associated) modulator of the $GABA_A$ receptor and inhibitory synaptic currents in the mammalian CNS.

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274P $GABA_B$ RECEPTOR-MEDIATED MODULATION OF MUSCARINIC ACETYLCHOLINE RECEPTOR-MEDIATED SYNAPTIC RESPONSES IN THE RAT HIPPOCAMPUS

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The hippocampus receives a dense cholinergic input from the medial septal nucleus, which is believed to have an important role in learning and memory processes *in vivo*. Stimulation of this pathway *in vitro* has been shown to evoke muscarinic acetylcholine receptor (mAChR)-mediated responses in hippocampal pyramidal neurones. Recently we have shown that adenosine A_1 receptors, which couple to similar effector systems to γ-aminobutyric acid $(GABA)_B$ receptors, modulate mAChR-mediated responses (Morton & Davies, 1997). Here we report the effects of $GABA_B$ receptor activation on mAChR-mediated synaptic responses in the CA1 region of the rat hippocampus.

Transverse hippocampal slices from 2- to 4-week-old female Cob-Wistar rats were prepared using standard techniques and maintained at 32 ± 2 °C in an interface recording chamber continuously perfused with artificial cerebrospinal fluid gassed with 95 % O_2/5 % CO_2. Intracellular current clamp recordings were made from CA1 pyramidal neurones using electrodes (70-110 MΩ) filled with potassium methylsulphate (2 M). In all experiments 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX; 2-4 μM), D-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116; 50 μM) and picrotoxin (50 μM) were included in the perfusing medium to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and $GABA_A$ receptors respectively. Under these conditions, reproducible $GABA_B$ receptor-mediated IPSPs ($IPSP_Bs$) followed by slow mAChR-mediated EPSPs ($EPSP_Ms$) could be evoked using a single stimulus delivered in the *stratum oriens*.

The selective $GABA_B$ receptor agonist baclofen (5 μM) occluded $IPSP_Bs$ and inhibited $EPSP_Ms$ by 71 ± 15 % (n = 4, mean ± s.e.mean). The selective $GABA_B$ receptor antagonist [1-(*S*)-3,4-(dichlorophenyl)ethyl] amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP 55845A; 1 μM), when applied alone, inhibited $IPSP_Bs$ and increased the amplitude of $EPSP_Ms$ to 253 ± 74 % of control responses (n = 3). In contrast, the GABA uptake inhibitor diphenylmethanone, 0-[2-(3-carboxy-1,2,5,6-tetrahydro-1-pyridinyl)ethyl]oxime, HCl (NNC 05-0711, 10 μM) increased the amplitude of $IPSP_Bs$ to 241 ± 38 % of control amplitudes and depressed $EPSP_Ms$ by 58 ± 10 % (n = 4). CGP 55845A (1 μM) reversed this inhibition of $EPSP_Ms$ and abolished the $IPSP_Bs$ (n = 2).

These data suggest that there is a $GABA_B$ receptor-mediated inhibition of cholinergic synaptic transmission which can be activated by endogenously released GABA.

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275P GABA UPTAKE IS REDUCED IN THE THALAMUS, BUT NOT THE CORTEX, OF AN ANIMAL MODEL OF ABSENCE EPILEPSY

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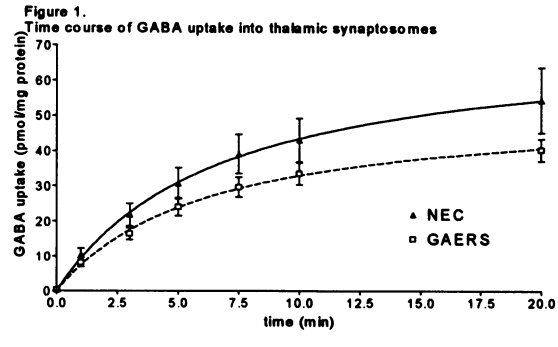
Genetic Absence Epilepsy Rats from Strasbourg (GAERS) are an animal model of absence epilepsy (Vergnes *et al*, 1982). During absence seizures, the EEG displays characteristic spike-and-wave discharges. These originate in the thalamus, and are thought to result from overactivity of the GABA system (Lui *et al*, 1991). Increased levels of extracellular GABA have been found in the thalamus of these animals (Richards *et al*, 1995). We decided to examine GABA uptake in GAERS, and in the non-epileptic control strain (NEC), to establish whether a reduction in uptake was responsible for the raised levels of extracellular GABA.

Uptake of [^{3}H]GABA into crude synaptosomal preparations was assayed by a filtration method. Female GAERS and NEC (13 weeks old) were killed by decapitation. Thalamic and cortical tissues were dissected over ice and homogenized in ice-cold sucrose (0.32M). The homogenates were centrifuged (1600g, 10min, 4°C), and the supernatant centrifuged (48000g, 10min, 4°C). The pellet was resuspended in ice-cold artificial cerebrospinal fluid (aCSF) (gassed with 95% O_2/5% CO_2; pH 7.2-7.4). 1ml samples of crude synaptosomes, containing 0.05-0.1mg protein, were incubated at 37°C in the presence of GABA (25nM, containing 2.5nM [^{3}H]GABA (specific activity 98.9Ci/mmol)), for 10min (thalamus) or 5min (cortex) (except time courses). Synaptosomes were recovered by rapid filtration with 10ml ice-cold aCSF. Filter radioactivity was assessed by scintillation counting. Non-carrier-mediated uptake was determined in the presence of 1mM nipecotic acid. Inhibitors were preincubated with synaptosomes for 15min. Protein content was determined by the method of Bradford (1976). Data are given as mean±s.e.mean. Statistical tests were unpaired, two-tailed Student's t tests, unless stated otherwise.

Time course studies revealed a significant reduction in thalamic GABA uptake in GAERS, compared to NEC (two-factor ANOVA on whole time course, $P<0.01$, n=5) (see Figure 1). Cortical uptake did not differ between the two strains (two-factor ANOVA on whole time course, n=5) (data not shown). In the presence of the GABA-transaminase (GABA-T) inhibitor, aminooxyacetic acid (AOAA) (100μM), GABA uptake was significantly higher in both cortical and thalamic preparations ($P<0.05$, n=4). The increase was similar for thalamic uptake in both strains (134±5% (NEC) and 139±5% (GAERS); % control rate; n=4). In contrast, AOAA caused a larger increase in cortical uptake in GAERS, compared to NEC (134±2% vs 123±4% respectively; n=4; $P<0.05$). Saturation analysis of GABA uptake (0.01-30μM, 2.5nM [^{3}H]GABA) revealed no differences in the kinetics of cortical uptake in both strains, (V_{max} = 2342±143 (NEC) and 1988±160 (GAERS) pmol $min^{-1}mg^{-1}$protein; K_d = 4.9±0.7μM (NEC) and 3.6±0.2μM (GAERS); n=5), nor did thalamic V_{max} values differ (504±57 (NEC) and 573±51 (GAERS) pmol $min^{-1}mg^{-1}$ protein; n=5). However, the thalamic K_d was significantly higher in GAERS compared to NEC (3.9±0.1 vs 2.8±0.5μM respectively; n=5; $P<0.05$).

Figure 1.
Time course of GABA uptake into thalamic synaptosomes



In conclusion, GABA uptake was reduced in thalamic synaptosomes from GAERS. This is probably a result of the reduced affinity of the transporter protein for GABA. It does not appear to be due to increased metabolism of GABA, as AOAA affects GAERS and NEC similarly. Cortical uptake did not differ between the two strains, although GABA-T activity may be higher in GAERS. The reduction in thalamic uptake may contribute to the higher extracellular GABA levels observed in GAERS, and to seizure generation.

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276P MODULATION OF RECOMBINANT $GABA_A$ RECEPTORS BY *CIS*-9,10-OCTADECENOAMIDE (COA): SUBUNIT DEPENDENCE AND FLUMAZENIL SENSITIVITY

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Introduction cOA was first isolated from the cerebrospinal fluid of sleep deprived cats and induced physiological sleep when injected, i.p., into laboratory rats (Cravatt *et al*, 1995). A significant depressant effect of cOA on human $GABA_A$ $\alpha_1\beta_1$ receptors has been reported: a marked potentiation of recombinant $5HT_2$ receptors was observed at nM concentrations (Huidoboro-Toro & Harris, 1996). Our pilots indicate that cOA enhances current through $GABA_A$ receptors in cultured cortical cells. Here we examine whether the response is subunit-dependent or sensitive to the benzodiazepine antagonist flumazenil.

Methods Recombinant human receptors were expressed in stage V-VI *Xenopus* oocytes by blind injection of cDNA's (provided by Dr P Whiting, MSD, UK) into the nucleus of de-folliculated cells. Two-electrode voltage clamp, 1-5 days later, was used to measure responses to superfused GABA and the effects of modulatory compounds at equilibrium (at room temperature). Flumazenil (Roche, Basel) and cOA were dissolved in dimethylsulphoxide (DMSO). Extracellular solutions contained constant levels of ($\leq$0.1%) DMSO, 0.033-0.1% bovine serum albumin (to facilitate dissolution of the lipid) with, or without, cOA. Data, presented as mean $\pm$ s.e.mean, were analysed by paired, two-tailed Students t-test using Prism software (Graph Pad, USA).

Results Human $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2L}$ subunit combinations were expressed, resulting in large currents (in the μA range) within 1-2 days. Maximal current response in any given cell was determined (3mM GABA) and modulatory effects were characterised for responses to a fixed low concentration (within the range EC_{10-35}) of agonist. Changes in base-line conductance, in response to a -20mV jump from -40mV, for 200ms at 2.5Hz, were monitored as a stable indicator of receptor activation. 333nM diazepam significantly enhanced GABA evoked conductance in the $\alpha_1\beta_2\gamma_{2L}$ receptors (n=5, $p<0.001$, mean enhancement 2.8 $\pm$ 0.3 fold) but did not significantly alter responses in $\alpha_1\beta_2$ (n=5, $p>0.05$). Flumazenil dose-dependently reduced the modulatory response to diazepam in the $\alpha_1\beta_2\gamma_{2L}$ receptors (apparent IC_{50}, 112nM, n=5). 10μM cOA reduced GABA-evoked conductance (0.74 $\pm$ 0.04 fold) in $\alpha_1\beta_2$ receptors ($p<0.01$, n=9) but consistently enhanced (1.3 $\pm$ 0.04 fold) the response in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ ($p<0.001$, n=12). Neither response was significantly affected by 1μM flumazenil ($p>0.05$).

Conclusions The modulatory effects of cOA and diazepam were subunit-dependent (both required γ-subunit expression for enhancement of inhibitory conductance) but the endogenous sleep modulator was not antagonised by flumazenil. cOA may represent an endogenous modulator of $GABA_A$ receptor isoforms in the mammalian brain.
Acknowledgement: Funded by The Wellcome Trust.

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277P STREPTOZOTOCIN-INDUCED MECHANICAL HYPERALGESIA: *REDUCED SENSITIVITY TO BACLOFEN* ANTINOCICEPTION

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Systemic administration of streptozotocin (STZ) to rats induces classical symptoms of human diabetes (polyuria, polydipsia, weight loss, hyperglycemia) associated with chronic mechanical hyperalgesia. This hyperalgesia shows reduced sensitivity to morphine but is reversed by antidepressant drugs (Courteix et al.,1994). The GABAergic system has been implicated in other chronic hyperalgesic states, whereby changes in GABA content in spinal cord interneurones are concomitant with changes in the antinociceptive efficacy of the $GABA_B$ agonist, baclofen (Malcangio and Bowery,1996). Thus, the aim of this study was to investigate whether the hyperalgesia associated with STZ-induced neuropathy showed altered sensitivity to systemic administration of (±)baclofen compared to controls.

Single intraperitoneal injections of STZ (50 mg/kg) or saline to rats were given after an overnight fast. Five days later blood glucose was checked and only rats with concentrations above 15 mM were considered diabetic. Rat threshold to mechanical and thermal stimulation was monitored throughout the study by using the Randall-Selitto and Plantar tests, respectively (Ugo Basile, Italy). The former consisted of applying an increasing force (expressed in grams) to the hind paw of slightly restrained rats until struggling or paw withdrawal was recorded. The plantar test consisted of applying a thermal stimulus (infrared beam) to the hind paw of unrestrained rats and recording the paw withdrawal latency (expressed in seconds). Data were analyzed by ANOVA.

STZ induced significant ($p<0.01$) reduction (30-50%) in mechanical nociceptive threshold from 2 up to 8 weeks after injection. Thermal sensitivity between saline- and STZ-treated rats was not different up to 12 weeks after injection when a small, even though significant ($p<0.05$), reduction in threshold was recorded. Single administration of (±)baclofen (4 mg kg^{-1}, n=6; SIGMA) induced a significant increase in mechanical threshold in control rats (pre-injection value: 267.5±9.9 g; 30 min after injection value: 395.0±24.9 g; mean ± s.e.mean, $p<0.01$). This is the highest antinociceptive dose of (±)baclofen which is devoided of muscle-relaxant effects (Malcangio *et al.*,1991).
The same dose of (±) baclofen (4 mg kg^{-1}) injected to 6 week-diabetic rats (n=8) did not modify the reduced mechanical threshold (pre-injection value: 181.9±12.5 g; 30 min after injection value: 141.9 ±21.7 g). It was necessary to administer 16 mg kg^{-1} (±)baclofen to induce a significant ($p<0.01$) increase in STZ-treated rat threshold (322.5 ±48 g, n=6).

This study showed that (±)baclofen effectiveness was reduced in a rat model of chemically-induced neuropathy. We suggest that changes within the endogenous inhibitory systems of the spinal cord are likely to occur to counteract the increased excitability of nociceptors responsible for the ongoing hyperalgesic state.

Technical assistance from R. Fluck is acknowledged.

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278P EFFECTS OF CORTICOSTERONE ON THE ACTIONS OF EXCITATORY AMINO ACIDS IN DOPAMINE-SENSITIVE NEURONES IN THE VENTRAL TEGMENTAL AREA

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Glucocorticoid hormones have effects on CNS neurones, and recent studies have suggested these are of importance in drug dependence. However, there has been little investigation of the effects of these hormones on neuronal function related to dependence. We investigate the effects of corticosterone (the major glucocorticoid hormone in rats) on neurones in the ventral tegmental area (VTA). This area is thought to be involved in reward mechanisms and motivation, and has been implicated in dependence on alcohol, psychostimulants and other drugs.

Male hooded Lister rats (200-225g) were used throughout. Midbrain slices containing the VTA were prepared, two slices from each rat, after cervical dislocation. Recordings were made, after an settling period of 60 min, from slices perfused with aCSF at 2.3 ml/min, using the interface method of slice perfusion (one slice per chamber). Single unit recordings were made from dopamine sensitive-cells with firing frequencies less than 5 Hz and action potential duration over 2 ms. Studies on AMPA and kainic acid were carried out on separate slices, from different rats. Either AMPA 10 μM or 50 μM, or kainic acid 10 μM or 30 μM were added to the perfusion medium for 5 min, at 15 min intervals; recordings were obtained during the last 1 min of excitatory amino acid application. After baseline measurements, corticosterone was added at increasing concentrations, from 100 nM to 2 μM to one slice of each pair; 15 min contact with corticosterone was allowed before addition of AMPA or kainic acid. N values were 5 per treatment group for the AMPA study and 6 for kainic acid (one pair of slices per rat, one neurone per slice). Student's t-test compared the firing rates in the slices to which corticosterone was added with the rates in parallel recordings in the absence of corticosterone.

Addition of corticosterone to the bathing medium at 500 nM or 1 μM significantly increased the mean firing frequency when either AMPA or kainic acid was added, compared with recordings in the absence of corticosterone (Table 1). In the presence of 2 μM concentration of corticosterone, however, the firing rate in response to kainic acid was significantly reduced but the rate after addition of AMPA was significantly increased. Corticosterone did not alter the basal firing frequency of the cells. No evidence of changes in action potential duration was seen after any of the corticosterone concentrations.

The results indicate that selective alterations in firing rate in response to AMPA and to kainic acid occur in dopamine-responsive cells in the VTA at concentrations of corticosterone similar to those circulating in the body during times of stress. These may be related to the psychological effects of glucocorticoid hormones.

Table 1. Firing rates (Hz), mean ± s.e.m., cort = corticosterone; EAA = excitatory amino acid; kain. = kainic acid.
*P<0.05; **P<0.01 compared with controls

Basal, (no EAA) control slices	Basal, before addition cort		Controls	Cort 500 nM	Controls	Cort 1 μM	Controls	Cort 2 μM
2.1 ± 0.1	3.5 ± 0.3	AMPA 10 μM	3.2 ± 0.3	4.9 ± 0.3**	3.9 ± 0.5	7.6 ± 0.6**	3.6 ± 0.4	8.7 ± 1.8*
	5.1 ± 0.5	AMPA 50 μM	4.4 ±0.3	7.0 ± 0.3**	5.0 ± 0.2	10.5 ± 0.6**	4.5 ± 0.5	12.4 ± 1.9*
1.8 ± 0.3	3.2 ± 0.5	kain.10 μM	3.2 ± 0.5	4.7 ± 0.6	3.1 ± 0.4	6.8 ± 0.7**	2.9 ± 0.3	3.0 ±1.4
	5.0 ± 0.5	kain. 30 μM	5.3 ± 0.6	7.5 ± 0.5*	5.0 ± 0.5	11.0 ± 1.0**	5.1 ±0.4	0.8 ± 0.4**

279P INDUCTION OF C-FOS EXPRESSION IN CULTURED RAT STRIATAL NEURONES BY ACTIVATION OF DOPAMINE D_3 RECEPTORS

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Dopamine receptors exert a powerful long-term influence on neurones of the basal ganglia, exemplified by their modulation of the expression of immediate - early genes (IEGs) such as c-fos. Investigation of the physiological function of D3 dopamine receptors *in vivo* has been hampered by a lack of selective ligands. We examine here the effect of endogenous D3 dopamine receptor stimulation on IEG expression in a primary neuronal culture, where drugs can be applied in defined concentrations.

Cultures were prepared from rat E17 embryonic striatal tissue as described (Simpson and Morris, 1995), and maintained in B27-supplemented neurobasal medium (Gibco, Paisley, UK). On day 12, the cells were fixed in 4% paraformaldehyde 45 minutes after addition of drugs, and hybridised as described with a ^{35}S-labelled oligonucleotide specific for c-fos mRNA (Morris,1995; Simpson and Morris, 1995). The autoradiographic signal in drug-treated culture wells was expressed as a percentage of the signal in vehicle-treated wells. Each treatment was repeated on a number of different cultures. Affinities at cloned rat D3 and D2 receptors transfected into CHO cells were determined as described (Millan et al.,1995), employing ^{125}I-iodosulpiride (1nM) as a radioligand. The results are presented as means ± sem, and statistical analysis was performed using the Wilcoxon or Mann-Whitney U-tests.

The preferential D3 agonists 4αR10βR-(+)-trans-3,4,4α,10β-tetrahydro-4-n-propyl-2H5H[4,3-β]-1,4-oxazin-9-ol (PD128907, 30nM) (Pugsley *et al.*, 1995) and (+)-7- hydroxy-2-(N,N-di-n-propylamino)tetralin ((+)7-OHDPAT, 5nM), (Millan *et al.*, 1995) both increased c-fos expression, by 26% ± 7.6%* (n=5) and 37.8% ± 19.6%* (n=3) respectively (*p<0.05 versus vehicle treatment). (-) 7OHDPAT had no significant affect on c-fos mRNA levels at concentrations up to 100nM.(+)-[7-(N, N-dipropylamino)-5,6,7,8-tetrahydronaph-tho(2,3β)dihydro,2,3-furane] (S14297) (Millan et al., 1995) showed higher affinity at D3 (K_i = 7.2 ± 1.7 nM, n = 3) than D2 (K_i = 223 ± 57nM, n = 3) receptors. Its stereoisomer S17777 showed lower affinity at D3 (K_i = 407 ± 113 nM, n = 3) and D2 (K_i = >10,000 nM, n = 3) receptors. The increase in c-fos mRNA induced by 10nM PD128907 was reduced by 91.8% ± 5.3% (n = 6,*p<0.05 versus 10nM PD128907 alone) by 30nM S14297, but was only reduced by 24.5% ± 7.8% by 30nM S17777 (n=4, p>0.05 versus 10nM PD128907 alone, p<0.05 versus PD128907 + S14297). Applied alone, S14297 had no significant affect on c-fos mRNA levels at concentrations up to 100nM. These data suggest that neuronal D3 receptors are coupled to IEG stimulation, and hence may be important in modulating the long-term function of the striatum.

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280P ROPINIROLE AND BROMOCRIPTINE PREVENT MPP$^+$-EVOKED HYDROXYL RADICAL FORMATION IN THE RAT STRIATUM *IN VIVO*

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When infused directly into the striatum, 1-methyl-4-phenylpyridinium (MPP$^+$) increases hydroxyl radical (OH$^\cdot$) production by mechanisms thought to involve autoxidation of dopamine (DA) (Chiueh *et al*, 1994). DA agonists have been shown to have antioxidant properties possibly due to their ability to control DA release by their action on presynaptic DA autoreceptors. For this reason, we have investigated whether bromocriptine, the D-2 DA agonist, and ropinirole, novel D-2/D-3 DA agonist can alter MPP$^+$-evoked OH$^\cdot$ production in the rat striatum using *in vivo* microdialysis.

Microdialysis probes were inserted into the left striatum of male Wistar rats (280-320g) using standard stereotaxic techniques under chloral hydrate (500mg/kg ip) anaesthesia. Probes were perfused at a flow rate of 2µl/min with artificial extracellular fluid (aECF) containing sodium salicylate (5mM) to trap OH$^\cdot$, forming 2,3-dihydroxybenzoic acid (2,3-DHBA) (Chiueh *et al*, 1994). One hour after implantation, dialysate samples were collected every 10 mins throughout the experimental period and analysed for levels of 2,3-DHBA, DA and DOPAC by HPLC with electrochemical detection. After a period of 1h, probes were perfused with ropinirole (1-1000µM (n=8-9) or bromocriptine (0.1-10µM, n=8-9), or vehicle (aECF, n=10-11) for 1h. MPP$^+$ (10mM, in the presence of the drug/vehicle) was then perfused for a period of 10 min followed by a further 2h perfusion with drug or vehicle. Data (expressed mean ± s.e. mean) was analysed by one-way ANOVA followed by Dunnet's test.

MPP$^+$ increased 2,3-DHBA formation by between 12.4 and 19.8 pmol/120 min). Ropinirole concentration-dependently reduced the MPP$^+$-evoked increase in 2,3-DHBA formation (Figure) without altering MPP$^+$-evoked DA efflux (total DA efflux (pmol/130 min): control 82.6±9.0; 1µM 54.0±8.6; 10µM 58.1±11.5; 100µM, 54.6±6.5; 1mM, 73.3±12.1; p>0.05) or decrease in DOPAC efflux (total DOPAC efflux (pmol/130 min): control 68.1±5.73; 1µM 71.9±9.4; 10µM 53.16±11.3; 100µM, 61.1±7.0; 1mM, 82.8±9.6; p>0.05). Similarly bromocriptine concentration-dependently reduced the MPP$^+$-evoked increase in 2,3-DHBA formation (Figure) without altering MPP$^+$-evoked DA efflux (total DA efflux (pmol/130min): control 78.1±6.5; 0.1µM 101.5±13.2; 1µM 70.6±11.5; 10µM, 60.3±5.4; p>0.05or decrease in DOPAC efflux (total DOPAC efflux (pmol/130min): control 72.3±10.1; 0.1µM, 101.7±10.2; 1µM, 42.79±8.43; 10µM, 62.24±6.5).

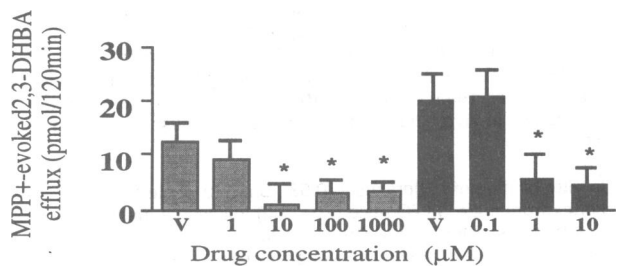

Figure. The effect of ropinirole ■ and bromocriptine ■ on MPP$^+$-evoked 2,3-DHBA efflux. V=vehicle. * p<0.05 compared to vehicle.

Both ropinirole and bromocriptine concentration-dependently prevented MPP$^+$-evoked OH$^\cdot$ efflux at concentrations that do not alter MPP$^+$-evoked dopamine efflux. These data suggest that these dopamine agonists protect against MPP$^+$-evoked OH$^\cdot$ production by mechanisms not involving dopamine receptor stimulation. This may be due to a direct antioxidant action as has been described *in vitro* by Yoshikawa et al. (1994) and Gassen et al. (1996) Thus, these dopamine agonists may have a twofold beneficial effect in Parkinson's disease, acting as dopaminergic agents and as a free radical scavenger which may slow the progress of the disease.

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281P THREE NEW D_3 DOPAMINE RECEPTOR ANTAGONISTS: SYSTEMATIC COMPARISON OF BEHAVIOURAL PROFILES

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Among the family of D_2-like dopamine receptors, clarification of the functional role of the D_3 receptor has been hindered by a lack of selective agents, particularly antagonists. At the level of behaviour, there endure arguments as to whether low doses of putative D_3 agonists reduce and D_3 antagonists increase spontaneous activity, and whether these effects involve pre- or postsynaptic sites (Daly & Waddington, 1993; Waters et al., 1993). We describe here a systematic, ethologically-based behavioural comparison of three new selective D_3 antagonists, GR103691, nafadotride and U99194A in comparison with the non-selective D_2-like antagonist haloperidol.

Young male Sprague-Dawley rats were habituated to observation cages for 3h before being challenged s.c. with drug or vehicle and then assessed over two 1h periods using a rapid time-sampling behavioural check-list technique to resolve all behaviours evident (Deveney & Waddington, 1995). Data were then analysed using analysis of variance (ANOVA) or the Kruskal-Wallis non-parametric ANOVA, followed by Student's t-test or Mann-Whitney U-test. Haloperidol [0.0008 - 0.1 mg/kg] failed to stimulate any behaviour and reduced [P<0.05] baseline levels of grooming. GR103691 [0.008 - 1.0 mg/kg; D_3-D_2 selectivity ratio: 100, Murray et al., 1995] and nafadotride [0.025 - 1.6 mg/kg; D_3-D_2 selectivity ratio: 10, Sautel et al., 1995] were without any overall stimulatory or other effects. Conversely, U99194A [1.67 - 45 mg/kg; D_3-D_2 selectivity ratio: 25, Waters et al., 1993] over the first hour readily induced non-stereotyped locomotion [+597%, P<0.001] and sniffing [+172%, P<0.001] with eating [+624%, P<0.01] and chewing[+350%, P<0.05] and some episodes of rearing [+183%, at 15.0 mg/kg, P<0.05], but reduced baseline levels of grooming [-78%, P<0.05]; over the second hour, U99194A continued to stimulate locomotion, sniffing and chewing but now induced grooming also [+366%, P<0.05]. These actions of U99194A were antagonised significantly [P<0.05] by 0.02 - 0.5mg/kg haloperidol.

The findings indicate that the behavioural effects of these D_3 antagonists are distinct and bear no apparent relationship to their relative selectivities for D_3 over D_2 receptors. U99194A has a characteristic profile of behavioural activation, with its ethogram distinct from that of known D_2-like and D_1-like agonists; however, sensitivity to haloperidol suggests some form of dopaminergic mechanism. The extent to which the differences observed here are D_3 mediated or else involve other dopaminergic or non-dopaminergic mechanisms remains to be determined. Such ethologically based evaluation appears useful for full characterisation of drug profiles.

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282P A MOUSE *IN VIVO* BINDING ASSAY TO MEASURE CENTRAL α_1-ADRENOCEPTORS USING [^{3}H]PRAZOSIN

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Extensive in vitro evaluation of central α-adrenoceptors (AdR) using, radioligands such as the α1-AdR subtype antagonist [^{3}H]prazosin suggests a widespread CNS distribution with highest densities in the cerebral cortex, hippocampus and thalamus. In vivo measurement of cortical α1-AdRs in rodent is more problematical, with negligible specific binding seen in rat cortex (Ohkura et al, 1995; Dyve et al, 1987). In this study, we report the in vitro and in vivo binding of [^{3}H]prazosin in mouse cortex and whole brain.

In vitro binding of [^{3}H]prazosin (0.03 - 3nM) to whole brain or cortex from male BKTO mice (20 - 30g) followed a method modified from Morrow and Creese (1986). For in vivo binding studies, mice were dosed intravenously with [^{3}H]prazosin (N.E.N., 77μCi/mmol, 20μCi/mouse), 40 mins after systemic administration of test compound. Following euthanasia by decapitation 20 mins later, brains were removed, homogenates prepared (0.4g/10ml wet wt./vol) and aliquots filtered through GF/B filters to determine the bound fraction. Non - specific binding was determined using 10mg/kg i.p. prazosin.

[^{3}H]Prazosin bound specifically and saturably to mouse cortex [K_D = 0.14 (0.14;0.14) nM, B_{max} = 170 ± 48 fmol/mg, n = 3] and whole brain [K_D = 0.16 (0.12;0.23) nM, B_{max} = 100 ± 8.2 fmol/mg, n = 3]. In vitro, the rank order of affinity of compounds to displace 0.3nM [^{3}H]prazosin was suggestive of binding to α1 AdRs in both preparations. In vivo, prazosin (0.01 - 10mg/kg i.p.), haloperidol (0.3 - 10mg/kg s.c.) and clozapine (0.03 - 30mg/kg s.c.), inhibited [^{3}H]prazosin binding with a rank order that followed their in vitro profile at α-1AdR (table 1, prazosin > clozapine ~ haloperidol).

Table 1. In vitro and in vivo binding of [^{3}H]prazosin in mouse.

[^{3}H]Prazosin In vitro	Cortex K_i(nM)	Whole brain K_i (nM)
Prazosin	0.12 (0.095;0.15)	0.042 (0.013;0.14)
WB4101	0.97 (0.47;2.0)	0.79 (0.45;1.1)
Benzoxathian	2.2 (1.31;3.7)	1.5 (0.97;2.4)
Phentolamine	5.6 (4.3;7.6)	1.3 (0.66;2.4)
Clozapine	5.2 (4.3;6.2)	4.3 (3.3;5.6)
Haloperidol	11 (9.4;13)	6.4 (2.4.;17)
MDL-100,907	250 (200;320)	300 (160;570)
Cirazoline	260 (180;370)	260 (190;370)
Clonidine	280 (230;330)	220 (170;290)
In vivo		ED_{50}(mg/kg)
Prazosin		0.15 (0.11;0.20) i.p.
Haloperidol		0.83 (0.76;0.90) s.c.
Clozapine		2.2 (1.5;3.5) s.c.

K_D, K_i and ED_{50} 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 = 3 - 5).

These studies confirm that in vitro, [^{3}H]prazosin binds with high affinity to α1-AdRs in mouse brain, and that in mice, [^{3}H]prazosin can be utilised to characterise α1-AdR in vivo.

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Morrow, A.L.. and I. Creese, 1986, *Mol. Pharmacol.* 29, 321.
Ohkura, T., Yamada, S., *et al*, 1995, *Jpn. J. Pharmacol.* (Suppl. 1), 217P.

283P INDUCTION OF PROSTATE APOPTOSIS BY DOXAZOSIN: α_1-ADRENOCEPTOR-DEPENDENT AND -INDEPENDENT ACTIONS?

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Doxazosin is used to provide acute relief of the symptoms of benign prostatic hyperplasia (BPH) produced as a consequence of bladder outlet obstruction. This acute effect is undoubtedly due to an action on the peri-urethral tone of the prostate via an action on alpha1-adrenoceptors. However, as this effect is maintained for up to six years in the face of a perceived ongoing hyperplasia, which would be expected to counteract the benefit achieved, additional actions of the drug may contribute to the longevity of the response. This paper examines the action of doxazosin on tissue proliferation and hyperplasia.

Doxazosin in vitro inhibited the growth of hormone dependent and independent human prostatic cancer cell lines (DU-145 and LNCaP) in a concentration dependent manner. The threshold for effect was approximately 100nM with full inhibition of growth achieved in the range 1 to 10uM, irrespective of whether thymidine incorporation or cell number was used as the primary endpoint. The lower concentrations are within the range of plasma drug levels achieved at therapeutic doses of doxazosin. It has been hypothesised (Yang et al., 1996) that this effect is independent of the alpha1-adrenoceptor and is mediated via induction of apoptosis.

In the clinical study presented here, the incidence of apoptosis has been evaluated in situ using the TUNEL assay in biopsies from 30 BPH patients before and after treatment with doxazosin.

The drug was used in the normal therapeutic dose range (2-6mg) for up to 20 months, with apoptosis and symptoms (IPSS) being assessed at baseline and time of surgery.

The baseline apoptosis levels for stromal and epithelial components were between 1.0 and 1.8%. A significant increase in apoptotic indices for both stromal (6 fold) and glandular epithelial (>10 fold) was observed after three months of doxazosin treatment. By 12 months the apoptotic indices were returning towards baseline values. This enhanced apoptosis correlated temporarly with stromal degeneration and symptom improvement (r = 0.79).

These findings indicate that a pro-apoptotic effect may contribute to the overall clinical profile of doxazosin and that a component of this may be independent of the alpha1-adrenoceptor.

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284P IDENTIFICATION OF THE α_{1L}-ADRENOCEPTOR SUBTYPE IN HUMAN PROSTATE AND RAT SALIVARY GLAND USING RADIOLIGAND BINDING WITH [^{3}H]TAMSULOSIN

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Three subtypes of the α_1-adrenoceptor (α_{1A}, α_{1B}, and α_{1D}) have been cloned and in functional studies, a further subtype (α_{1L}) with a low affinity for prazosin and RS17053 has been proposed to exist (Michel et al., 1995). Tamsulosin (YM617), an antagonist with high affinity for α_{1A}, α_{1D} and α_{1L}-adrenoceptors (Noble et al., 1997), is now available in a tritiated form and has been used in radioligand binding experiments to investigate the presence of the putative α_{1L}-adrenoceptor in human prostate and rat tissues.

Membranes from human prostate (HP, from patients undergoing transurethral resection of the prostate), rat salivary gland (rSG) and liver (rL) were prepared and incubated with appropriate concentrations of [^{3}H]tamsulosin and displacing drugs. Non-specific binding was defined in the presence of 10μM phentolamine. Displacement curves (using 12 concentrations) were carried out using [^{3}H]tamsulosin concentrations of 0.5nM (HP), 0.3nM (rSG) and 2nM (rL). At these radioligand concentrations, specific binding was >85% in all three tissues.

Prazosin	**pK_i (1)**	**pK_i (2)**	**% (2)**	**nH**
human prostate	10.3±0.3	8.9±0.1	77±7	0.81±0.05*
rat salivary gland	10.2±0.4	9.0±0.1	57±10	0.84±0.05*
rat liver	9.9±0.1			1.11±0.06
RS17053				
human prostate	8.9±0.1	7.5±0.1	57±2	0.66±0.05**
rat salivary gland	8.9±0.2	7.7±0.2	56±1	0.70±0.01**
rat liver	7.0±0.1			1.01±0.05

Table 1. Mean affinities (±sem; n=3-6) from biphasic/one-site curve-fits for prazosin and RS17053 displacement of [^{3}H]tamsulosin. Hill coefficients (nH) significantly less than unity, $p<0.05$*; $p<0.01$**.

Saturation experiments showed [^{3}H]tamsulosin to bind with an affinity some 10-fold higher in HP (pK_d=9.9±0.1) and rSG (pK_d= 10.1±0.1) than in rL (pK_d=8.9±0.1), consistent with its selectivity for α_{1A} over α_{1B}-adrenoceptors.

In competition experiments, prazosin and RS17053 displacement curves were best described by a two-site fit for both HP and rSG in contrast to a single-site fit for rL. These analyses were confirmed by Hill coefficients, which were significantly less than unity only in tissues taken from HP and rSG. In HP, prazosin identified a receptor population consisting of 77% with a low affinity, whilst RS17053 identified 57% as low affinity sites (Table 1). Results were similar in rSG, where prazosin and RS17053 identified 57% and 56% low affinity sites respectively. The high and low affinity values obtained correspond closely to values previously reported for both drugs at α_{1A} and α_{1L}-adrenoceptors respectively (Ford et al., 1996).
The α_{1D}-adrenoceptor cannot account for the low affinity sites in HP and rSG since displacements performed with BMY 7378 generated single low pK_i values (HP=6.0; rSG=6.1) equivalent to its affinity at the α_{1A}-adrenoceptor subtype. In rat liver, both prazosin and RS17053 defined a single population of α_1-adrenoceptors with affinities that are consistent with the presence of the α_{1B}-adrenoceptor subtype in this tissue.

In conclusion, this study has shown prazosin and RS17053 to define an α_1-adrenoceptor population consisting of over 50% of the α_{1L}-adrenoceptor subtype in human prostate and rat salivary gland.

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Michel, M *et al.*, (1995) Naunyn-Schmiedeb. Arch. Pharmacol., **352**, 1-10.
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285P THE EFFECT OF P2-RECEPTOR ANTAGONISTS AND ATPASE INHIBITION ON SYMPATHETIC PURINERGIC NEUROTRANSMISSION IN GUINEA-PIG ISOLATED VAS DEFERENS

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In guinea-pig vas deferens stimulation of the sympathetic nerves releases ATP as a cotransmitter with noradrenaline (NA). ATP activates P2X receptors, inducing a rapid inward current, excitatory junction potentials (e.j.p.s), action potentials and a phasic contraction. Intracellular microelectrode recordings have been used to investigate the effects of two P2X receptor antagonists, pyridoxal 5'-phosphate (P-5-P) and the suramin analogue NF023 (Ziyal *et al.*, 1997). The effect of the ATPase inhibitor 6-N,N,diethyl-D-β,γ-dibromo methylene ATP (ARL 67156) was estimated electrophysiologically and by measurement of purine and NA overflow using HPLC combined with fluorometric and electrochemical detection respectively. (For methods see Todorov et al. 1996).

P-5-P produced a concentration-dependent reduction in the magnitude of fully facilitated e.j.p.s evoked at 1Hz (0.5 msec pulse-width, subthreshold voltage). For example, 10^{-4}M P-5-P reduced e.j.p.s from a mean control level of 18.3 ± 1.1 mV (n=16) to 3.5 ± 0.2 mV (n=16) within ten minutes. P-5-P also reduced the resting membrane potential of the cells from -71.2 ± 0.5 mV (n=16) to -65.8 ± 0.9 mV (n=16). NF023 also produced a concentration-dependent reduction in e.j.p. magnitude and was more potent than P-5-P. For example 3 x 10^{-5}M NF023 reduced e.j.p.s from a control mean value of 18.1 ± 1.0 mV (n=14) to 3.4 ± 0.2 mV (n=10) within ten minutes. NF023 did not produce significant depolarization of the smooth muscle cells. In the presence of P-5-P or NF023 (10^{-5} - 10^{-4}M) addition of ARL 67156 (100 μM) enhanced e.j.p. magnitude, partially reversing the effect of the antagonists. The amount of ATP in samples of superfusate collected every 10 seconds during nerve stimulation at 8 Hz for 60 seconds was greatly enhanced by 100 μM ARL 67156 (Figure 1. Pooled data from 9-12 tissues). Overflow of ADP, AMP adenosine and NA were also increased. In conclusion, P-5-P and NF 023 are fast acting, effective inhibitors of e.j.p.s, and thier inhibitory effect is reversed by ARL 67156. The ATPase inhibitor also enhances ATP overflow produced by sympathetic nerve stimulation in this tissue.

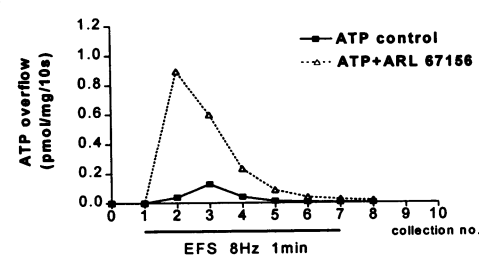

Figure 1. The effect of ARL 67156 on nerve stimulation-evoked release of ATP in guinea-pig isolated vas deferens.

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286P ACTIVATION BY P2X PURINOCEPTOR AGONISTS OF SENSORY NERVES INNERVATING THE RAT KNEE JOINT

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Six different types of ATP-gated ion-channel receptors ($P2X_{1-6}$) are expressed in dorsal root ganglia (DRG) and ATP evokes a depolarising current when applied to isolated DRG neurones (Jahr & Jessell, 1983). These findings have led investigators to believe that P2X purinoceptors, in particular the $P2X_3$ subtype, which is found only in sensory ganglia, may be involved in peripheral nociception (see Burnstock & Wood, 1996). The aim of this study was to determine whether αβ methylene ATP (αβmeATP), a P2X agonist, can activate nociceptive DRG afferents innervating the rat knee joint *in vivo*.

Male Wistar rats (mean weight 335g, range 250-420g) were anaesthetised with pentobarbitone (60mg.kg^{-1} i.p., supplemented hourly i.v.) and the trachea, right carotid artery, right femoral artery and vein were cannulated. Extracellular recordings were made from filaments of the medial articular nerve (MAN) innervating the left knee joint using techniques similar to those described previously for the rat ankle joint (Birrell *et al.* 1991). Drugs were applied by close intra-arterial injection into the lower abdominal aorta via the femoral cannula. Data are expressed as the mean change in action potential frequency ± s.e.mean.

Recordings were made from 60 mechanosensitive fibres with receptive fields in the knee joint. Conduction velocities were mainly in the C-fibre range (<2 m.s^{-1}) but some Ad fibres (2-5 m.s^{-1}) were also recorded. 49% of these afferents were excited by αβmeATP (6-600nmol, Table 1). The responses had a very short delay to onset (200nmol: 0.68±0.2s) and were of short duration (200nmol: 1.6±0.3s). Half of the αβmeATP-sensitive afferents also responded to capsaicin (3-20nmol) with a similar duration of response (10nmol: 1.6±0.4s) but with a longer onset latency (10nmol: 1.9±0.3s).

Table 1. Change in action potential frequency evoked by saline, capsaicin and αβmeATP injected i.a. Mean±s.e.mean, n = 6-12.

Basal discharge (impulses.s^{-1})	saline (0.3ml)	capsaicin (10nmol)	αβmeATP (200nmol)
0.07±0.02	0.06±0.15	28.7±8.8	7.3±2.3

Preliminary data suggests that P2X receptor antagonists may inhibit the αβmeATP-evoked discharge (suramin 60mg.kg^{-1} i.a., apparent mean agonist ED_{50} before: 24±8 nmol; after: 267±168 nmol n=3; PPADS 10mg.kg^{-1} i.a., apparent agonist ED_{50} before: 13±3 nmol; after: 81±14 nmol n=4).

Our results shows that nociceptors in the rat knee joint can be excited by a P2X purinoceptor agonist. This suggests that αβmeATP-sensitive P2X receptors are involved in the initiation of nociceptive input to the central nervous system which is consistent with recent finding of algesic responses to αβmeATP in the conscious rat (Bland-Ward & Humphrey, 1997).

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287P INTERACTION BETWEEN ADENOSINE A_1 AND A_2 RECEPTOR-MEDIATED RESPONSES ON RAT HIPPOCAMPAL NEURONES

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Previous work has been carried out on the effects of adenosine on transmitter release and on the excitability of postsynaptic neurones, but little is known about the effects of adenosine on the coupling between the two. The effect of adenosine receptor agonists and antagonists have now been studied on the population excitatory postsynaptic potential (popEPSP) slope, the population spike (PS) amplitude, and the relationship between the two (E-S coupling) in the CA1 area of rat hippocampus. The popEPSP gives primarily a measure of presynaptic function while the PS reflects the excitability of the postsynaptic neurone. E-S coupling gives an indication of the ability of a given level of synaptic depolarisation to induce the postsynaptic cell to fire an action potential.

Slices (450μm thick) of rat hippocampus were prepared as described by Higgins and Stone (1996). Test stimulation was given at 20s intervals via a concentric bipolar electrode placed in the stratum radiatum. The preparation was allowed to stabilise for at least 45mins before recordings of orthodromic extracellular popEPSPs and PSs were made from the stratum radiatum and stratum pyramidale respectively using glass microelectrodes of tip diameter ~2μm filled with 0.9% NaCl. Input/output (I/O) curves were obtained by varying the stimulus intensity and measuring the responses using a CED interface. Five stimuli were delivered at each stimulation intensity and the average computed. From these I/O curves, E-S curves were constructed by expressing popEPSP slopes and PS amplitudes as a percentage of maximum control values and plotting PS amplitude as a function of pop EPSP slope. To quantify shifts in the E-S slope the PS amplitude seen at a popEPSP slope of 70% of maximum control value was measured before and after drug addition. This was measured for each E-S curve and the values expressed as mean ± s.e. mean.

Adenosine 50μM (n=5) significantly reduced both the popEPSP slope (40.9%±4.1; $p<0.01$) and PS amplitude (65.5%±12.7; $p<0.001$) and shifted the E-S curve to the right (43.5%±15.1; $p<0.05$) compared with control values, showing that the PS amplitude had decreased more than could be accounted for by the decrease in popEPSP. The A_1 agonist N^6 cyclopentyladenosine (CPA) 50nM (n=5) also caused a decrease in popEPSP slope (28.9%±7.9; $p<0.01$) and reduction of the PS amplitude (83.9%±4.3; $p<0.001$) resulting in a shift to the right of the E-S curve (81.8%±5.2; $p<0.001$). The selective A_{2a} agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS21680; 30nM n=5, 100nM n=7) and the A_{2a} antagonist 4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385; 50nM n=6) had no significant effect. However, activation of the A_{2a} receptors with CGS 21680 significantly attenuated the ability of CPA to shift the E-S curve. Shifts in the E-S curve caused by CPA 50nM in the presence of CGS 21680 30nM or 100nM were 32.5%±9.8 and 48.3%±8.1 respectively, significantly lower than those seen with CPA 50nM alone ($p<0.01$, n=5 and $p<0.05$ n=7 respectively).

These results indicate an inhibitory effect of A_1 receptors on E-S coupling and the existence of functionally important A_{2a} receptors in the CA1 area of rat hippocampus which are able to interact with A_1 receptors .

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288P CHARACTERISATION OF A K_{ATP} CHANNEL CURRENT IN RAT STRIATAL CHOLINERGIC INTERNEURONES

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Within the striatum, medium spiny neurones are particularly vulnerable to anoxia whilst interneurones are more resistant to such insults (Francis & Pulsinelli, 1982). Since unidentified interneurones within the striatum have been shown to hyperpolarise in response to aglycaemia (Calabresi *et al.*, 1997), we have investigated whether striatal cholinergic interneurones exhibit K_{ATP} channel activity using patch clamp recordings in brain slices from 14-28 day-old male Sprague Dawley rats

Experiments were performed at 32-35°C in ACSF which contained (mM) 125.0 NaCl, 25.0 $NaHCO_3$, 10.0 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, bubbled with a 95%, 5% O_2/CO_2 gas mixture, whilst the pipette solution comprised (mM) 120.0 Kgluconate, 10.0 NaCl, 2.0 $MgCl_2$, 0.5 K_2EGTA, 10.0 HEPES, 0.1 Na_2GTP, pH 7.2. To this, 2mM Na_2ATP was added when required.

Striatal cholinergic interneurones were identified morphologically (soma in excess of 30μm using infrared videomicroscopy), electrophysiologically (e.g. resting membrane potential of -55.6 ± 1.0mV) and pharmacologically (the NK_1 receptor agonist [Sar^9, $Met(O_2)^{11}$]Substance P (100nM) produced a 15.6 ± 2.8mV (n=20) depolarisation). In the absence of ATP in the electrode, neurones slowly hyperpolarised upon membrane breakthrough from their initial resting potential to -83.6 ± 4.2mV with cessation of action potential firing and decrease in apparent input resistance (from 263.9 ± 27.2MΩ to 123.5 ± 22.3MΩ). This hyperpolarisation was reversed by 100μM tolbutamide which produced a rapid (within 2min) and reversible depolarisation (of 17.5 ± 1.9mV (n=15)) with concomittant increase in apparent input resistance (to 301.9 ± 3.1MΩ (n=15)) and return of action potential firing. The ability of tolbutamide to depolarise these cells and to increase input resistance persisted after treatment of the slice with 1μM TTX (n=5). Similarly 200nM glibenclamide produced a 19.1 ± 3.5mV (n=8) depolarisation which was associated with a 267.2 ± 56.8% (n=8) increase in apparent input resistance.

In contrast, when 2mM Na_2ATP was added to the electrode solution, this characteristic hyperpolarisation was not observed (n=25). However, under these conditions, bath application of 500μM diazoxide hyperpolarised these neurones (by 10.1 ± 1.9mV (n=12)) in a manner that was unaffected by treatment of the slice with 1μM TTX (n=5).

To examine this further, neurones were voltage clamped at -60mV. In the absence of ATP, an outward current was seen to slowly develop (103.2 ± 19.2 pA in amplitude with reversal potential of -97.2 ± 6.9mV (n=16)) which could be completely blocked by bath application of tolbutamide (100μM, n=9) or glibenclamide (200nM, n=7). Once again, the addition of 2mM Na_2ATP to the electrode solution prevented the development of this current.

Finally, to confirm the identity of these neurones, in three recordings, the cytosolic contents were aspirated and subjected to RT-PCR using primers specific for choline acetyl transferase (ChAT) and the K_{ATP} channel subunits Kir6.1 and Kir6.2. In each cell, ChAT was detected together with Kir6.1 but not Kir6.2.

In conclusion, we have demonstrated that cholinergic interneurones within the rat striatum exhibit K_{ATP} channel activity and that the channel responsible for this current contains the Kir6.1 K_{ATP} channel subunit.

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289P DIFFERENTIAL SENSITIVITY TO ENGLITAZONE OF K_{ATP} CURRENTS, ACTIVATED BY DIAZOXIDE OR LEPTIN, IN THE INSULIN SECRETING CELL LINE CRI-G1

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The hyperglycaemic agent diazoxide (Trube, Rorsman & Ohno-Shosaku, 1986) and the ob gene product leptin (Harvey, et al., 1997) both hyperpolarise CRI-G1 insulin secreting cells via activation of ATP-sensitive K^+ channels (K_{ATP}). In this study whole cell patch clamp recording was used to examine the effects of englitazone, an inhibitor of K_{ATP} channels (Rowe,et al., 1997) on the activation of K_{ATP} channels by diazoxide and leptin.

Recording pipettes contained (mM): 140 KCl, 0.6 $MgCl_2$, 2.73 $CaCl_2$, 10 EGTA, 5 ATP and 10 HEPES pH 7.2 (free Ca^{2+} of 0.1 μM) and the bath solution comprised (mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES pH 7.4. Under current clamp conditions with 5 mM ATP in the pipette to maintain K_{ATP} channels in the closed state, the resting membrane potential of CRI-G1 cells was -38 ± 1.1 mV (mean ± sem n=24). Application of diazoxide (200 μM) hyperpolarised CRI-G1 cells to -71 ± 1.4 mV with a concomitant increase in slope conductance from 0.54 ± 0.07 to 11.4 ± 1.41 nS (n=14). The sulphonylurea tolbutamide (100 μM) completely reversed the membrane hyperpolarisation and increase in conductance by diazoxide, with a reversal potential of -79 ± 0.7 mV (n=4). In addition englitazone (0.3 - 100 μM) caused a concentration-dependent and reversible inhibition of K_{ATP} channel activation by diazoxide (n=20). From the concentration inhibition curve englitazone had a half maximal inhibitory concentration (IC_{50}) of 7.7 μM and a Hill coefficient of 0.87. These values correlate well with those reported for the inhibitory action of englitazone on K_{ATP} channels activated following dialysis with a solution with no added ATP (Rowe et al 1997).

Application of leptin (10 nM) hyperpolarised CRI-G1 cells to -72 ± 1.3 mV (n=10). Examination of the voltage clamped currents indicate that leptin increased the slope conductance from 0.53 ± 0.09 nS to 8.56 ± 1.38 nS and the reversal potential associated with this conductance increase was -78 ± 0.8 mV (n=10). Tolbutamide (100 μM) completely reversed the leptin-induced hyperpolarisation and increased slope conductance to pre-leptin levels in all 5 cells tested and the reversal potential associated with this action of tolbutamide was -79 ± 0.4 mV (n=5). In contrast to the actions of diazoxide, englitazone (1-100 μM) was less potent at inhibiting the leptin-induced membrane hyperpolarisation and increase in conductance. Thus at low concentrations (1-30 μM) englitazone failed to inhibit K_{ATP} channels activated following leptin addition (n=15), however higher concentrations of englitazone (>30 μM) did inhibit the actions of leptin in a reversible manner (n=8). The calculated IC_{50} value and Hill coefficient were 52 μM and 3.2, respectively.

In conclusion, englitazone was approximately ten fold less potent at inhibiting the actions of leptin compared to diazoxide. Differences in the mechanism of activation of K_{ATP} channels by diazoxide and leptin may explain this shift in the englitazone concentration inhibition curve .

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290P GLUTATHIONE INHIBITS A β-NAD⁺-ACTIVATED NON-SELECTIVE CATION CHANNEL IN THE CRI-G1 INSULIN-SECRETING CELL LINE

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We have recently shown that a ß-NAD⁺-activated non-selective cation channel (NS_{NAD}) activated by oxidative stress causes irreversible depolarization in the CRI-G1 insulin secreting cell-line (Herson & Ashford, 1997). The redox state of pyridine nucleotides and glutathione are altered during oxidative stress (Cochrane, 1991). Consequently we have investigated the effects of glutathione on the NS_{NAD} channel in excised inside-out patches. Results are expressed as mean ± SEM.

The patch pipette contained (mM): 140 NaCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 HEPES (pH 7.2) and the bath solution contained (mM): 140 NaCl, 0.6 $MgCl_2$, 5.02 $CaCl_2$, 5 EGTA, 10 HEPES (pH7.2) 50 µM free Ca^{2+}. The addition of 1 mM ß-NADH in the presence of 1 mM ß-NAD⁺ caused an 82.1 ± 4.8% inhibition of channel activity (n=6) compared to control (ß-NAD⁺ alone). The addition of reduced glutathione (GSH) in the continued presence of 0.5 mM ß-NAD⁺ caused a concentration dependent decrease in NS_{NAD} channel activity. 10 mM causing complete inhibition (n=12), 5 mM causing 90.5 ± 4.5% inhibition (n=7), 3 mM causing 17.6 ± 11% inhibition (n=6) and 1 mM having no effect (n=3). The concentration-response curve, Fig. 1, shows that GSH inhibition is characterized by a Hill coefficient of >7 and an IC_{50} of 3.7 mM. Conversely, 2 mM oxidized glutathione (GSSG) had no significant effect on channel activity (n=3) and was unable to stimulate channel activity (50 µM-5 mM, n=7) in the absence of ß-NAD⁺. We used dithiothreitol (DTT), another strong reducing agent, in order to show that inhibition was not due to glutathione reducing potential. 10 mM DTT had no significant effect on channel activity in the presence of 0.5 mM ß-NAD⁺ (n=5).

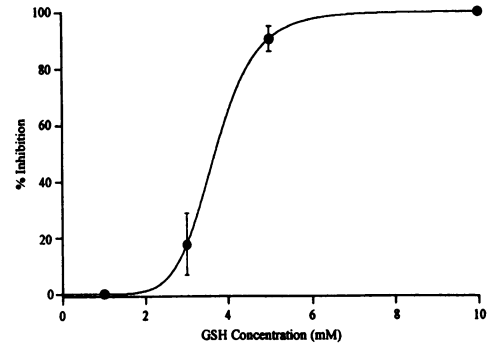

Fig 1. Reduced glutathione concentration-response curve. Patches were perfused with varying concentrations of GSH in the continued presence of 0.5 mM ß-NAD⁺. Each point represents the mean of at least 3 experiments.

These results indicate that the NS_{NAD} channel, which has been shown to sense oxidative stress, is regulated by the redox status of both the pyridine nucleotides and glutathione.

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291P STIMULATION OF NERVE GROWTH FACTOR LEVELS IN CONTROL AND DIABETIC RATS BY TREATMENT WITH CLENBUTEROL

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Reduced nerve growth factor (NGF) content in peripheral tissues is implicated in the pathogenesis of diabetic neuropathy (Fernyhough *et al.*, 1995). This may be secondary to deficits in calcitonin-gene related peptide (CGRP) and substance P (Diemel *et al.*, 1994).

Clenbuterol is reported to induce NGF synthesis *in vitro* (DeBernardi *et al.*, 1991) and in the CNS (Semkova *et al.*, 1996). Previously we demonstrated that clenbuterol can induce NGF protein synthesis in the diabetic rat, although this synthesis may have been secondary to increased hindlimb muscle mass. In this study we compared different doses of clenbuterol. Animals were made diabetic with a single injection of streptozotocin (50 mg kg^{-1}, i.p.). Control and diabetic rats were injected daily with three different doses (namely 30 µg kg^{-1}, 100 µg kg^{-1} and 300 µg kg^{-1} s.c.) of clenbuterol for the duration of the 8 week study. Untreated control and diabetic animals were maintained for comparison. The levels of NGF-like immunoreactivity (NGF-LI, measured by ELISA) were compared to those of substance P-like immunoreactivity (SP-LI) and CGRP-like immunoreactivity (CGRP-LI, measured by radioimmunoassay) in the right sciatic nerve. A marked increase in NGF protein was seen in control animals at all three doses. However, the 47% reduction in NGF protein in the sciatic nerve of diabetic rats was completely restored to control values by treatment with the lowest dose of clenbuterol. Reductions in sciatic nerve NGF-LI and SP-LI were concomitant with a 20% decrease in g-PPT mRNA (substance P precursor) in the dorsal root ganglia of diabetic animals. The NGF protein levels in soleus and extensor digitorum longus (EDL) muscles - target tissues for the production of NGF - were also determined. Diabetes was associated with reduced NGF protein in these hindlimb muscles (soleus, 72% of control, $p<0.05$; EDL, 73% of control). Treatment with clenbuterol (30 µg kg^{-1}) normalised the soleus muscle NGF content in treated diabetic animals but was without effect on EDL NGF content.

In the same study, the retrograde transport of NGF was also examined after clenbuterol treatment. The left sciatic nerve was ligated under halothane anaesthesia. Twelve hours later, untreated diabetic rats showed a significant reduction in the accumulation of NGF distal to the ligation (31% of control, $p<0.01$). Treatment with 30 µg kg^{-1} clenbuterol reversed this deficit; the levels of NGF accumulation in treated control animals was also raised above control values. Local synthesis of NGF at the point of ligation remained unaltered by clenbuterol treatment. Thus, the reduced trophic support from NGF in diabetic rats was prevented by 30 µg kg^{-1} clenbuterol as was deficient expression of NGF target genes. This dose of clenbuterol was without effect on hindlimb muscle mass.

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292P INTRODUCED BY DISTINCT K⁺-CHANNELS UNDERLIE EARLY GLUCOCORTICOID INHIBITION IN AtT20 CELLS AND RAT ANTERIOR PITUITARY CELLS

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The AtT20 mouse pituitary corticotroph tumour cell line displays the basic hallmarks of early glucocorticoid inhibition of stimulated ACTH secretion: action through Type II glucocorticoid receptors and requirement for mRNA and protein biosynthesis (Woods et al.,1992). Previous work in AtT20 cells has pointed to BK-type K^+-channels as prime mediators of this effect of glucocorticoids (Shipston et al.,1996). The objective of the present study was to test whether BK channels have a role in corticosteroid inhibition in rat anterior pituitary cells. Studies were carried out on AtT20 cells or rat anterior pituitary cells (150-200 g male Wistar rats) in primary culture 96h after plating. Cells were pretreated with steroids for 2h, K^+- and Ca^{2+}-channel drugs were applied between 90 and 120 min of preincubation after which corticotrophin releasing factor (CRF), or 8-(4-chlorophenylthio)-3':5'adenosine monophosphate (CPT-cAMP) was applied in fresh medium containing channel drugs for 30-60 min. CPT-cAMP was used in these studies in order to obviate inhibition of cAMP formation by intracellular Ca^{2+} (Antoni et al.,1995). The release of ACTH into the medium was monitored by radioimmunoassay. As previously reported (Lim et al.,1997), the L-channel agonist compound Bay-K8644 (BayK, 5μM) in combination with tetraethylammonium (TEA, 5-10mM) stimulated ACTH release by AtT20 cells up to 5-fold, and displayed synergistic stimulation of ACTH when given together with 0.1 CPT-cAMP. The effect of 0.1mM CPT-CAMP on ACTH release was reduced by dexamethasone (maximal inhibition 80±8%, all data are means±s.e.mean, n=3 each in quadruplicate, IC_{50}=3 nM), However, in the presence of BayK/TEA the maximal inhibition was 18.3±3% (n=3) at 100nM dexamethasone. In primary cultures of rat anterior pituitary cells, the inhibition of CPT-cAMP- and 0.1nM CRF-stimulated ACTH release by 10nM dexamethasone or 100nM corticosterone was abolished by inhibitors of mRNA or protein synthesis. Combination of 50mM KCl, 5μM BayK and 0.1mM CPT-cAMP synergistically stimulated ACTH release and this was much less effectively inhibited by 100nM corticosterone (27±3%, n=3) than a response of similar magnitude elicited by 1mM CPT-cAMP (59 and 53 %). Similar data were obtained for 10nM dexamethasone. Combination of 5μM BayK and 5 mM TEA had only a minor effect on glucocorticoid inhibition and up to 20mM TEA was also without effect on secretagogue-induced ACTH release either in the presence or in the absence of glucocorticoid. In order to probe for the involvement of TEA resistant K^+ channels we used astemizole (1-30μM) and clofilium (0.3-10μM) that block IsK and HERG currents (Suessbrich et al.,1996). Both compounds enhanced ACTH release in the presence of 0.1nM CRF and 100nM corticosterone: at 10μM clofilium corticosterone inhibition was 32±12 % of control, n=4, at 10μM astemizole 46 and 33%, whereas in the absence of blockers it was 79±3%, n=4. Basal and 0.1nM CRF-stimulated ACTH release were not consistently enhanced by these drugs. The IC_{50}s of cortico-sterone inhibition of CRF-evoked ACTH release were 9 and 65nM in the presence and absence of 10μM clofilium, respectively. These data show that early corticosteroid inhibition in both AtT20 cells and rat anterior pituitary cultures is mediated by Type II glucocorticoid receptors and induction of mRNA and protein. While in AtT20 cells BK-type K^+-channels are a prime target of glucocorticoid action, in primary cultures such a role is not apparent. Instead, the pharmacological analysis reported here suggests the involvement of HERG-type K^+-channels in the inhibitory action of corticosterone in normal rat anterior pituitary cells.

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293P EXCITATION OF RAT ISOLATED VAGUS NERVE BY PROSTACYCLIN MIMETICS

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We have recently shown that prostacyclin analogues such as cicaprost inhibit the motility of the rat isolated colon by releasing inhibitory NANC neurotransmitters (Qian & Jones, 1995). We have also shown that BMY 45778, a highly potent non-prostanoid prostacyclin mimetic (see Meanwell *et al.*, 1994), has only weak inhibitory activity on the colon preparation and on prolonged exposure blocks the action of cicaprost (Jones *et al.*, 1995). To further investigate rat IP-receptors mediating neuronal excitation, we have selected a simpler preparation, the rat isolated vagus nerve.

Extracellular recording of depolarisation of the desheathed cervical vagus nerve (2 - 3 cm) from male Sprague-Dawley rats (330-350 g) was carried out using a "grease-gap" technique (Ireland & Tyers, 1987). Both halves of the chamber were perfused with Krebs-Henseleit solution (95% O_2/5% CO_2) at 27°C, and the potential difference between the chambers was recorded. Drugs were applied to one side of the nerve (only preparations responding to 20 mM KCl with depolarisations >2mV were used).

5-HT (0.1 - 30 μM) and four prostacyclin mimetics, cicaprost (0.03 - 3 nM), iloprost (0.1 - 100 nM), taprostene (3 - 300 nM) and BMY 45778 (3 - 300 nM) consistently depolarised the nerve, with similar maximum responses of about 0.5 mV. 5-HT responses were rapid in onset (3 min contact) and offset (15 min between doses), and the EC_{50} value of 1080 ± 120 nM (s.e. mean, n = 4) agrees with previous studies (Ireland & Tyers, 1987). In contrast, responses to prostacyclin mimetics were slower in onset and extremely persistent following wash-out, necessitating cumulative dosing with 20 min contact times. Cicaprost was a highly potent agonist (EC_{50} = 0.65 ± 0.41 nM). Responses to 10 nM cicaprost were unaffected by either 1 μM tetrodotoxin or removal of calcium from the bathing fluid.

Equi-effective molar ratios (EMR, cicaprost = 1.0) for iloprost and taprostene were 4.9 and 50 respectively (n = 4), in agreement with EMR of 2.4 and 23 for relaxation of human pulmonary artery rings (cicaprost IC_{50} = 0.60 nM) (Jones *et al.*, 1997). Depolarisation responses to BMY 45778 were sustained and an established depolarisation to 10 nM cicaprost was not inhibited by the addition of 300 nM BMY 45778 (n = 4). However, BMY 45778 had a much lower potency on the vagus nerve (EMR = 120) compared to human pulmonary artery (EMR = 3.0). Other non-prostanoid prostacyclin mimetics, such as BMY 42393 which was inactive on the rat colon, are being investigated on the vagus nerve.

In conclusion, the rat cervical vagus nerve preparation is highly sensitive to IP-receptor agonists and is also depolarised by the non-prostanoid prostacyclin mimetic BMY 45778.

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