1P ADENOVIRALLY-OVEREXPRESSED BETA 2-ADRENOCEPTORS ENHANCE THE CONTRACTILE RESPONSE TO CGP 12177A IN ADULT RAT CARDIOMYOCYTES

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A propranolol resistant state of the beta 1-adrenoceptor (β1AR) has been shown to mediate the effects of the 'putative' beta 4-adrenoceptor (Kaumann *et al.*, 2001). Adenovirally overexpressed β1ARs increase the responsiveness to isoproterenol (ISO) and CGP 12177A (CGP) in adult rat cardiomyocytes (Lewis *et al.*, 2002) CGP has also been shown to activate adenylyl cyclase in CHO cells overexpressing β2AR (Pak and Fishman, 1996). We have examined the effect of adenovirally-overexpressed β2ARs on the inotropic responses to CGP in adult rat cardiomyocytes.

Methods: Single rat ventricular cardiomyocytes were isolated using methods previously described and transfected with adenovirus containing sequence for the human β2AR (as previously published). β2AR density was measured by [125I]-iodocyanopindolol binding to ventricular myocyte membranes. Inotropic responses to ISO were studied 24 hours after transfection by measuring cell shortening in electrically stimulated ventricular myocytes. Inotropic responses to CGP (in the presence of 1μM propranolol) were studied in cells with greater than 50% increase in basal amplitude of contraction to 0.3nM ISO.

Results: Binding confirmed an approximate 200-fold increase in ventricular β2AR density. There was a significant upward shift of the concentration response curves (CRC) to CGP in β2AR transfected cells (Repeated measures ANOVA, $p<0.02$)and a doubling of the maximum response (Uninfected 2.09 ± 1.21% shortening (mean ± SD), n=9; β2AR transfected 4.53 ± 2.01%, n=9, $p<0.05$)). However, there was no significant change in the EC50 (Uninfected EC50 434nM (95% CI 196 to 1072nM, n=9); β2AR transfected EC50 259nM (158 to 602nM, n=9, $p<0.2$)) (Fig 1). 50nM ICI 118,551 (β2AR selective antagonist) had no effect on the maximum response to CGP in the β2AR transfected cells (Maximum 4.53 ±2.01%, following ICI 4.45 ±0.93%, $p>0.05$).

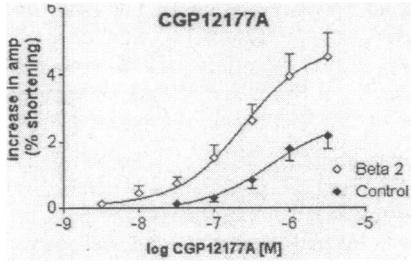

Fig 1. CRCs to CGP in control and β2AR infected myocytes

Conclusions: The lack of shift in the CRC to CGP following over-expression of β2ARs compares with a 10-fold left shift following 18-fold overexpression of β1ARs (Lewis *et al.*). This suggests that β2ARs do not contribute significantly to the 'putative' β4AR effect. The modest upward shift in the CRCs may be due to a small (possibly local) increase in basal intracellular cAMP.

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2P PROTECTION AGAINST REPERFUSION-INDUCED BUT NOT ISCHAEMIA-INDUCED ARRHYTHMIAS WITH CHRONIC ADMINSTRATION OF 17β-OESTRADIOL

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Menopause has been associated with a 3-fold increase in the risk of coronary heart disease which may be due to loss of oestrogen (Welty, 2001). Recently, we have shown that 17β-oestradiol (100 μg kg^{-1} day^{-1}) abolished reperfusion-induced ventricular fibrillation (VF), compared to a 54% incidence in controls (Byrne *et al.*, 2002). The aim of this study was to determine if withdrawal of endogenous oestrogen, by ovariectomy, and replacement by chronic administration of 17β-oestradiol would alter ischaemia-induced arrhythmias.

Female Wistar rats (180-250g) were ovariectomized, or sham-operated, under Hypnorm/diazepam anaesthesia. After 12 to 16 days, rats (240-320g) were treated with 17β-oestradiol (Study 1 - 3, 10 & 30 μg kg^{-1}; Study 2 – 30, 100 & 300 μg kg^{-1}) or vehicle (sesame oil) s.c. daily for 7 days. Rats were then anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.). The trachea was canulated to permit artificial ventilation. Carotid arterial blood pressure and a Lead I ECG were recorded. A left thoracotomy was performed and a ligature was placed around the left coronary artery. After 10 min stabilisation the artery was occluded and resulting arrhythmias monitored for 25 min.

In Study 1, 17β-oestradiol (3, 10 and 30 μg kg^{-1}) tended to reduce the total number of ventricular premature beats (VPBs) and the incidence of VF but none of these apparent differences were statistically significant (Table 1). Study 2 was therefore carried out using higher doses.

With the higher doses of 17β-oestradiol (30, 100 and 300 μg kg^{-1} no further reductions in arrhythmias were seen and the numbers of VPBs and the incidence of VF tended to increase again (Table 1). Combination of the data from the control and the 30 mg kg^{-1} 17β-oestradiol groups still failed to reveal any significant difference in either the number of VPBs (565±138 and 283±78; P=0.091) or the incidence of VF (35% and 10%; P=0.072) respectively.

Table 1: The total number of ventricular premature beats (VPBs; mean ± s.e. mean) and the incidences of ventricular tachycardia (VT) and ventricular fibrillation (VF).

	n	VPBs	%VT	%VF
Sham	12	332 ± 123	85	23
Control-Study 1	13	457 ± 180	92	38
3 μg kg^{-1}	13	360 ± 74	77	15
10 μg kg^{-1}	12	302 ± 88	92	8
30 μg kg^{-1}	10	154 ± 51	70	10
Control-Study2	10	695 ± 218	100	30
30 μg kg^{-1}	11	396 ± 121	91	9
100 μg kg^{-1}	11	643 ± 61	64	18
300 μg kg^{-1}	11	791 ± 491	91	27

These results indicate that chronic administration of 17β-oestradiol does not significantly reduce ischaemia-induced arrhythmias. The dose-dependent reduction in reperfusion-induced VF observed previously is therefore likely to depend on a mechanism that is unique to reperfusion.

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3P 17ß-OESTRADIOL HAS A GREATER INHIBITORY EFFECT ON THE L-TYPE CALCIUM CURRENT IN FEMALE THAN MALE CARDIAC VENTRICULAR MYOCYTES

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Ischaemia-induced ventricular arrhythmias are suppressed by 17ß-oestradiol to a greater extent in female vs. male rats (Philp *et al.*, 2002). Oestrogen has been shown to inhibit the L-type Ca^{2+} current in female ventricular myocytes (Meyer *et al.*, 1998; Tanabe *et al.*, 1999) but its action in males is unknown. If 17ß-oestradiol possesses gender specific L-type Ca^{2+} channel blocking activity, this might explain its gender specific antiarrhythmic action, since Ca^{2+} channel blockers are antiarrhythmic in rats (Curtis *et al.*, 1987). Studies were therefore carried out to examine the effects of 17ß-oestradiol on the L-type Ca^{2+} current in male and female ventricular myocytes.

Male and female Wistar rats (250 – 320g) were killed by stunning followed by cervical dislocation. Ventricular myocytes were isolated by enzymatic digestion. The peak L-type Ca^{2+} current was measured using the whole-cell patch clamp technique in K^+ free solutions at 35°C. Cells were held at –40 mV to inactivate Na^+ current and depolarised to +10 mV at 0.5 Hz to elicit the peak Ca^{2+} current. Cells were patched in normal Tyrode then, switched to vehicle (0.2% ethanol) for 3 min and vehicle recordings were then taken. Cells were then exposed to 17ß-oestradiol (0.1, 1, 10, 30 or 100 µM) for a further 3 min and recordings taken between 3-4 min of exposure. Experiments were conducted until $n \geq 9$.

The vehicle had no effect in either sex on the peak L-type Ca^{2+} current (pA/pF in males –6.84 ± 0.66 vs. –6.81 ± 0.69 and in females –5.02 ± 1.05 vs. –4.71 ± 0.75). 17ß-Oestradiol dose dependently inhibited the L-type Ca^{2+} current with significantly greater inhibition in female vs. male cells (Figure 1).

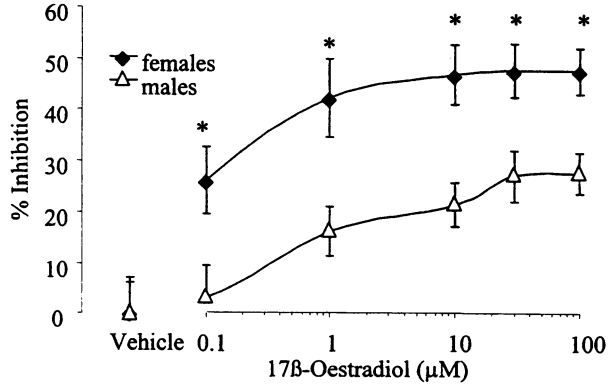

Figure 1. Effects of 17ß-oestradiol on the L-type Ca^{2+} current. *P < 0.05 vs. male (Student's unpaired t test).

In conclusion, the greater sensitivity of L-type Ca^{2+} currents in female myocytes to 17ß-oestradiol may explain the gender difference in the antiarrhythmic effects of 17ß-oestradiol.

Supported by the British Heart Foundation FS/99060

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4P OESTROGEN WORSENS OUTCOME FOLLOWING MYOCARDIAL INFARCTION IN MICE

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There is evidence that the risk of death following myocardial infarct (MI) is higher in young compared to post-menopausal women (Vaccarino *et al.*, 1999). The aim of this study was to investigate the effect of oestrogen on the outcome of MI using a mouse model following coronary artery ligation (CAL). Female mice (C57Bl6/129SvJ, 11-14 wks old) were ovariectomised and pellets containing 17 beta-estradiol (0.05mg) or vehicle (placebo) were implanted subcutaneously. 7 days later CAL was performed under anaesthesia (ketamine/ xylazine/atropine). In shams the ligature was passed under the artery. 15 days later, mice were cannulated for measurement of blood pressure (MABP) and heart rate (HR).

Hearts were weighed, fixed and processed for measurement of infarct size using Van Gieson stained thin sections.

A significant proportion of CAL mice died due to cardiac rupture during the 7 days following surgery (Table 1). The incidence of cardiac rupture was doubled in oestrogen-treated mice relative placebo. In surviving mice, CAL caused significant infarction and a reduction in MABP. Oestrogen treatment did not modify infarct size in survivors but was linked to a relative increase in heart weight. These studies suggest that oestrogen may be detrimental to compensatory remodelling post-myocardial infarction.

Reference: Vaccarino V., Parsons L., Every N.R., *et al.*, (1999) *N. Engl. J. Med.* 341, 217-225.

This work is supported by British Heart Foundation (PG/99192)

Table 1. The influence of oestrogen on the response to CAL.

	placebo		oestrogen	
CAL or sham	Sham (n)	CAL (n)	Sham (n)	CAL (n)
Mortality **(%)**	-	**29 (17)**	-	**61 (18)$^{\delta}$**
IS **(% LV free wall)**	-	**56 ± 3 (8)**	-	**53 ± 3 (6)**
HW **(% BW)**	**0.61± 0.04 (11)**	**0.70 ± 0.05 (12)**	**0.58 ± 0.03 (11)**	**0.77 ± 0.09 *(7)**
MABP **(mmHg)**	**86 ± 1 (9)**	**78 ± 3* (9)**	**100 ± 6 (9)**	**71 ± 6 **(6)**
HR **(beats/min)**	**229 ± 19 (9)**	**244 ± 18 (9)**	**309 ± 29 (9)**	**238 ± 13 (5)**

IS (infarct size), HW (heart weight expressed as % of body weight), MABP (mean arterial blood pressure), HR (heart rate). Results are mean ± SEM. * P<0.05, ** P <0.01, unpaired t-test vs matched sham group; δ P<0.05 chi-squared test.

5P MODIFICATION OF VASCULAR FUNCTION BY THE OESTROGEN RECEPTOR β: THE EFFECT OF AGEING

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Oestrogen receptor beta (ERβ) is the principle ER in human vascular smooth muscle (Hodges *et al.*1998). Recent studies have suggested that ERβ may mediate the vasculoprotective effects of oestrogen e.g. in vascular injury (Lindner *et al.*, 2000) and age-associated hypertension (Zhu *et al.*, 2002).
The present study aimed to determine 1) the distribution of ERβ in mouse aorta and 2) the effect of aging on smooth muscle and endothelial cell function in wild-type (WT) mice and mice without a functional ERβ (βERKO).
Binding of a polyclonal ERβ antibody to paraffin sections of aorta was detected using peroxidase conjugated Strep-Avidin. Intact aortic rings from young (12-18wk) and aged (52- 55wk) male mice were mounted on a small vessel myograph. Cumulative concentration response curves were constructed for phenylephrine (Phe, 10^{-9}- 10^{-6}M) and for acetylcholine (ACh, 10^{-10}-10^{-5}M) and sodium nitroprusside (SNP, 10^{-11}-10^{-6}M) in tissues pre-constricted with an 80% maximal concentration of Phe. Responses to Phe were also obtained in the presence of NO synthase inhibitor, L-NAME (100μM) and/or the cyclooxygenase inhibitor, indomethacin (10μM).
Immunoreactive ERβ was associated with the nuclei of aortic endothelial and smooth muscle cells. Phe produced concentration dependent contractions in all tissues. The Phe E_{max} was greater in aorta from young WT than young βERKO mice. This difference was not seen in aorta from ageing mice (Table 1).
Endothelium-dependent and independent relaxations induced by ACh and SNP respectively were not different between experimental groups.

Table 1- Phe contractile response characteristics in mouse aorta

	Wild type		βERKO	
	Young (4)	Ageing (8)	Young (4)	Ageing (9)
EC_{50} (M)	$4.3x10^{-8}$ ± $0.9x10^{-8}$	$11.0x10^{-8}$± $4.5x10^{-8}$	$6.3x10^{-8}$± $1.8x10^{-8}$	$5.5x10^{-8}$± $1.0x10^{-8}$
Emax	160± 12 %	130± 5 % *	128 ± 9 % *	143 ± 9 %

The Emax data is expressed as a % of the maximum response obtained to 60mM KCl . All results expressed as mean ± SEM * $p<0.05$, Tukey following one-way ANOVA, vs young WT

L- NAME increased the contractile response to Phe in all tissues although this increase was significantly greater in young βERKO (40 ± 19%) compared to young WT (6.1% ± 6.7, $p<0.05$, one way ANOVA with post hoc Tukey test). Indomethacin inhibited the contraction to Phe equally in all experimental groups.

These data suggest that ERβ regulates vascular function in young mice, perhaps through control of basal NO synthesis, although endothelial function was not modified. The influence of ERβ on vascular function appears to be lost during aging.

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This research was funded by the Wellcome Trust (060322) and British Heart Foundation (PG/99192).

6P LOCAL INFUSION OF PACLITAXEL DOES NOT INFLUENCE NEOINTIMAL FORMATION IN THE PORCINE CORONARY ARTERY AFTER BALLOON INJURY

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Paclitaxel is a promising drug for the treatment of restenosis after balloon angioplasty. Its mechanism of action is thought to be through stabilising microtubules, thereby preventing cellular proliferation and migration. Local delivery of paclitaxel at the time of injury has been shown to reduce restenosis in normal (Herdeg *et al* 2000) and hypercholesterolaemic (Oberhoff *et al* 2001) rabbit carotid artery, an effect which was associated with marked vessel enlargement and impaired vessel function. In this study we have examined the effect of local paclitaxel delivery on neointimal development in the pig coronary artery. Also, since locally delivered paclitaxel may exert long-term deleterious effects on vascular function, we have measured vascular contractility and relaxation in vessels exposed to local paclitaxel infusion at the time of injury.

In vivo study: Pigs (18-20Kg) were sedated with azaperone and anaesthetised with a mixture of halothane and nitrous oxide (1% halothane for maintenance). The coronary artery was accessed via a femoral cutdown using a 7F guide catheter under fluoroscopic guidance. A 3.5mm balloon catheter was advanced into the left anterior descending coronary artery (LAD) and inflated three times to 10atm pressure for 30secs. A 3.5mm SCIMED™ dispatch catheter was then advanced to the same position and inflated to 6atm. Paclitaxel (5mls of 50μM solution) was delivered over 3 infusions of 5mins duration. Control pigs were infused an equivalent volume of drug vehicle (saline containing 50μl of dimethyl sulphoxide). All animals were allowed to recover from anaesthetic and euthanised 28 days later. The coronary arteries were removed for functional studies and planimetric analysis of neointima.

In vitro study: The injured LAD and control left circumflex (LCx) arteries were dissected from porcine hearts used in the *in vivo* study. 3-4mm rings were set-up in 10ml baths (37°C) containing gassed Krebs solution (95%O_2/5%CO_2). Rings were set to optimum resting tension and allowed to equilibrate for 1 hour. Dose response curves to KCl (5-80mM) and 5-HT (1nM-10μM) were constructed. After washout, rings were then contracted with 0.5μM U46619 and once stabilised, relaxation to cumulative addition of calcimycin (1nM-1μM) and SIN-1 (1nM-1μM) was measured.

Nine pigs infused with saline had no procedural complications while four of the nine pigs infused with paclitaxel developed severe bradycardia proceeding to fatal asystole after approximately 5 minutes of drug infusion. Twenty-eight days later, there was no significant difference in neointima, expressed as a percentage of medial+neointimal area in control (22.5±4.1%; n=9) vs. paclitaxel-infused animals (19.7±2.5%; n=5). Similarly, there was no vessel enlargement in paclitaxel-treated animals (EEL circumference was 6.6±0.4mm in control vs. 6.2±0.2mm, n=9/5). Paclitaxel did not significantly affect contractility of the injured vessel to KCl or 5-HT or relaxation to calcimycin or SIN-1 (Table 1). Higher concentrations of calcimycin induced a contraction in pigs which had received local paclitaxel infusion 28 days previously.

	KCl	5-HT	Calcimycin	SIN-1
Control	12.3±1.1	3.0±0.7	96.5±4.9	101.3±4.0
+Paclitaxel	15.5±1.9	2.6±1.5	77.4±17.9	73.0±16.8

Table 1: Values are mean±SEM of maximum contractions (g) or % relaxation of U46619-induced tone in porcine LAD 28 days after injury. (n=9 for control and n=5 for paclitaxel-treated group).

In this study, local delivery of paclitaxel did not influence development of neointima in the porcine coronary artery and promoted serious and potentially fatal cardiac side effects. From our *in vitro* data, these effects of paclitaxel do not appear to be related to dysfunction of coronary artery smooth muscle or endothelium.

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7P THE EFFECT OF NO-ASPIRIN AND ASPIRIN ON ARRHYTHMIAS AND INFARCT SIZE RESULTING FROM CORONARY ARTERY OCCLUSION IN A RAT MODEL OF DIABETES

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The use of aspirin in the secondary prevention of acute myocardial infarction is limited by its gastric side effects. NCX4016 is a member of a new class of NO-NSAIDs that release nitric oxide (NO) and has reduced gastric side effects, whilst maintaining the pharmacological profile of aspirin (Del Soldato *et al.* 1999). The release of NO may contribute to the cardioprotective effect of NCX4016 observed in normal animals (Rossoni *et al.* 2001, Wainwright *et al.* 2002). Since diabetes is a major risk factor for CV disease, the aim of this study was to assess the effect of chronic NO-aspirin (NCX4016), on the consequences of coronary artery occlusion in streptozotocin induced diabetic rats.

Male SD rats (300-350g) were given a single injection of either streptozotocin (STZ, 60mg kg^{-1}, i.p.) to induce diabetes, or saline (0.1ml $100g^{-1}$, i.p.) for time matched control animals. Diabetic rats were given insulin daily (2.5U kg^{-1}, s.c.) to partially control the diabetes; control animals received saline daily (1ml Kg^{-1}, s.c.). Rats were housed in pairs and maintained for 4 weeks. For the last 5 days before coronary artery occlusion (CAO) animals received orally once daily either (i) Vehicle (PEG_{400} 1ml kg^{-1}, n= 10 for both control & diabetic rats), (ii) aspirin (65.2mg kg^{-1}, n=9 control, n=7 diabetic), (iii) NCX4016 (60mg kg^{-1}, n=8 control & diabetic), or (iv) NCX4016 (120mg kg^{-1}, n=9 control, n=8 diabetic). One hour following the last oral dose, rats were anaesthetised with sodium pentobarbitone (60mg kg^{-1}, i.p.) and prepared for CAO as described previously (Clark *et al.*1980). Following 15-min stabilisation hearts were subjected to 30-min regional ischaemia, and 120-min reperfusion. Mean arterial blood pressure (MABP), heart rate (HR) and the number and incidence of arrhythmias (VPB) were recorded via an ECG from standard (lead II) limb electrodes. At the end of reperfusion hearts were stained to determine area at risk (AAR) and infarct size. Data are expressed as mean ± s.e.mean and compared using ANOVA with Tukey's post-hoc test. The percentage incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and mortality was compared using Fischer's exact test.

None of the drug treatments significantly modified initial MABP, HR or plasma glucose levels compared to vehicle treated in either non-diabetic (111±10 mmHg, 373±17 bpm, 13±1 mM) or diabetic rats (105±7 mmHg, 378±18 bpm, 45±3 mM). In non-diabetic animals NCX4016 significantly reduced mortality from VF at both doses (30% vs. 13% (60mg kg^{-1}) and 11% (120mgkg^{-1}); $P<0.05$) but aspirin had no effect (33%). Neither drug treatment had any effect on total VPB count in non-diabetics. NCX4016 at both doses significantly reduced the incidence of VT in diabetic animals (100% vs. 86% (60mg kg^{-1}) and 50% (120 mg kg^{-1}); $P<0.05$). In addition, the total VPB count was significantly reduced by high dose NCX4016 (1438±501 vs. 177±82 (120mg kg^{-1}); $P<0.05$), low dose NCX4016 showed a tendency to reduce VPB's (378±135 (60mg kg^{-1})) whereas aspirin had no effect (1368±646). In contrast to non-diabetic rats, NCX4016 did not reduce the incidence of or mortality from VF in diabetic rats. In non-diabetic animals infarct size was reduced by both aspirin (41±4 % Vs 22±6% of AAR, $P<0.05$) and NCX4016 (60mg kg^{-1}, 10±2%, $P<0.05$). However, while aspirin had no effect on infarct size in diabetic rats (36±8% vs. 33±10%) NCX4016 caused a modest reduction in infarct size (20±4% of AAR (120mg kg^{-1}) $P<0.05$).

These results demonstrate that NCX4016 has the potential to protect the diabetic heart in ischaemia and reperfusion. However further studies are required to investigate the mechanisms underlying this effect.

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8P ADRENOMEDULLIN-INDUCED CARDIOPROTECTION: THE ROLE OF NITRIC OXIDE

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Adrenomedullin (AM), a novel 52-amino acid hypotensive peptide, possesses multifunctional biological properties, of which its effect in the regulation of cardiorenal homeostasis is the most important. The nitric oxide (NO) pathway has been widely implicated in the actions of AM e.g. its hypotensive effects (Hinson et al, 2000). We have previously demonstrated that AM exhibits cardioprotective effects against ischaemia-induced arrhythmias and myocardial injury. This is accompanied by a concomitant fall in arterial blood pressure and an increase in reactive oxygen species (ROS) generation from activated leukocytes. (Looi *et al*, 2002). However it is unknown whether or not these effects are NO dependent. Thus the aim of this study was to investigate the role of NO in these observed effects exerted by AM.

Pentobarbitone (60mg/kg)-anaesthetised male Sprague-Dawley rats (290-400g) were subjected to ligation of left main coronary artery for 30 min followed by 120 min of reperfusion. The number and incidence of cardiac arrhythmias, mean arterial blood pressure (MABP) and heart rate were monitored throughout the experiments. Ex vivo ROS generation in response to zymosan was determined in whole blood by luminol chemiluminescence (CL). Hearts were stained with Evan's blue and triphenyltetrazolium chloride at the end of the experiments for area at risk and infarct size measurements respectively. The NO synthase (NOS) inhibitor N(G)-nitro-L-arginine (L-NNA) was infused (0.5mg/kg/min) from 30 min prior to i.v administration of AM (1.0 nmol/kg; n=7) or saline (n=8). Both AM and saline were administered 5 min prior to occlusion. Saline-treated controls devoid of L-NNA infusion were also performed (n=7). Data is expressed as mean±s.e.m. Comparison within group was performed using paired t-test. The percentage incidence of ventricular fibrillation (VF) was compared using Fisher's exact test. Statistical significance was taken if $P<0.05$.

L-NNA resulted in a marked and significant increase in MABP 25 min post-infusion and neither subsequent AM nor saline treatment affected MABP (98±4 to 149±5 mmHg and 95±5 to 148±5 mmHg respectively; $P<0.05$). The incidences of VF in AM and saline-treated animals in the presence of L-NNA were 57% and 50% respectively, which was not significantly different from the control (43%). However, L-NNA infusion resulted in a significantly higher incidence of mortality during ischaemia in both AM and saline treatment compared to control (71% and 50% vs. 14%; $P<0.05$). The total number of ventricular ectopic beats that occurred during ischaemia in rats that survived the whole period of ischaemia for AM (n=2) and saline (n=4) treatment and control (n=6) were 1510±571, 1508±293 and 1444±336 respectively while infarct sizes were 47±4%, 48±6% and 42±3% of area at risk respectively. L-NNA infusion resulted in a reduction in CL generation 15 min post-infusion in both groups (14±2% and 20±7% reduction). Neither AM nor saline treatment reversed the reduction in CL generation. Statistical analysis could not be performed due to the low sample size resulting from the high mortality in the AM treatment group. However, this is in line with our previous findings, in sham operated animals, the increase in CL following AM treatment was also abolished by L-NNA (n=3).

This study demonstrates that inhibition of NOS increases mortality, and abolishes the cardioprotective effects of AM. The abolition of the AM-induced hypotensive effect and increase in CL generation by L-NNA suggests that these effects are NO dependent.

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Y. H. L is supported by the ORS and University of Strathclyde studentship.

9P SUPEROXIDE ANION CONTRIBUTES SIGNIFICANTLY TO THE IMPAIRED FUNCTION OF RAT MESENTERIC ARTERIES TREATED WITH LIPOPOLYSACCHARIDE

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Expression of inducible nitric oxide synthase (iNOS) leads to overproduction of nitric oxide (NO) and impaired vascular function. With use of an NO-sensitive probe, we aimed to make a quantitative determination of the effect of lipopolysaccharide (LPS)-generated iNOS on the ability of carbachol (CCh) to release endothelial NO and produce relaxation, and how this was modified by L-arginine (L-A).

Ring segments of superior mesenteric artery of male Sprague Dawley rats were mounted in a wire myograph for tension recording. The NO-sensitive probe (WPI) was calibrated with NO dissolved in water and then introduced to the lumen of the vessel. The probe was unresponsive to CCh (10μM), phenylephrine (PE; 1μM) or nitrite (3mM) and its sensitivity was unaltered in the presence of L-A (100μM) or Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin (MnTMPyP; 100μM). Following control responses, LPS (20μg/ml) was added to the bathing medium for 2 hr. Following washout the tissue was left quiescent for a further 2 hr before responses to CCh were determined. In some experiments the effect of L-A (100μM) or MnTMPyP (100μM) on LPS-treated tissue was determined. Statistical comparison were determined by ANOVA.

Following PE (1μM)-mediated contraction, CCh (10nM-3μM) induced relaxation and simultaneously increased luminal [NO] (maximum of 96.0±0.9% and 14.9±0.7nM, respectively. n=5). In LPS treated tissues, both relaxation and luminal [NO] were dramatically impaired ($P<0.001$, n=5) at low concentrations of CCh (30nM-300nM) although values at maximal CCh were unaltered. Co-incubation with the highly specific inhibitor of iNOS, 1400W (10μM), prevented the inhibitory effect of LPS ($P>0.05$, n=3). Addition of L-A, the substrate of iNOS, to pre-contracted vessels produced little change in tension and [NO] in controls (1.2±0.5% and 0.4±0.1nM, respectively) but a substantial change in LPS-treated tissue (35.5±5.6% and 13.6±0.9nM, respectively. $P<0.001$, n=4). Co-incubation with L-A entirely prevented the inhibitory effect of LPS, CCh stimulated relaxation and NO output were not different from controls ($P>0.05$, n=4). Since iNOS can generate the free radical and inactivator of NO, superoxide anion, in L-A deficient cells we proposed that iNOS may mediate some of its inhibitory effects via superoxide. Indeed addition of the membrane-permeant superoxide scavenger MnTMPyP, which had no effect on control responses, substantially reduced the inhibitory effect of LPS on CCh (10μM)-mediated responses (relaxation increased from 7.2±3.4 to 46.3±11.7% and [NO] from 0.3±0.1 to 3.2±0.4nM. $P<0.001$, n=3), although both relaxation and [NO] were still significantly impaired ($P<0.01$, n=3) from control levels.

Our findings demonstrate that the impairment of CCh-mediated relaxation of superior mesenteric arteries treated with LPS is due to the formation of iNOS. By use of the NO-sensor we can show that this impairment to relaxation is due to a decrease in CCh-stimulated NO output. It appears that the inhibition of CCh-mediated NO production is due in large part to the production of superoxide anion from iNOS, which can be prevented by application of L-arginine.

Supported by the British Heart Foundation (PG/1999177)

10P MODULATION OF NITRERGIC NERVE MEDIATED VASODILATION BY INDUCIBLE NITRIC OXIDE SYNTHASE

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The excessive production of NO has been demonstrated in a variety of cerebrovascular diseases including subarachnoid haemorrhage, cerebral ischaemia and meningitis as a result of iNOS expression. Excessive NO production has a negative feedback effect on endothelial NOS activity (Rengasamy & Johns, 1993). In addition, high NO concentrations have been shown to inhibit soluble guanylate cyclase activity (Scott & Nakayama, 1998). In vivo studies have highlighted the importance of maintaining constitutive NOS function in diseases involving excessive NO production, as this improves the survival rate. NO synthesised by neuronal NOS, located in nitrergic nerves, plays a major role in the control of cerebrovascular tone (Iadecola *et al.*, 1994). Therefore an inhibitory effect of NO of iNOS origin on neuronal NOS could play a major part in the progression of any of the aforementioned diseases. The effect of iNOS induction on porcine basilar artery nitrergic vasodilation was investigated.

The basilar artery was removed from pig heads of either sex, obtained from the local abattoir. The vessels were cleaned of any connective tissue and placed in Krebs-Henseleit solution gassed with 95% oxygen and 5% carbon dioxide. Artery rings (2mm) were treated with lipopolysaccharide 100μg/ml (LPS) and incubated at 37°C for 18 hours. The vessels were then mounted in a 4 channel, small vessel myograph and stretched, with a force of 15mN, for isometric recordings. Functional iNOS expression was confirmed by assessing the vasodilator response to L-arginine 30μM. Transmural nitrergic nerves were stimulated with 20 square pulses at 2Hz with a voltage between 10 and 35 volts and pulse duration of 0.2 msec in vessels preconstricted with U46619 (9, 11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$, 100nM). The relaxations were expressed as a percentage of forskolin (1μM) induced relaxation. Responses to the vasodilator SIN-1 (3- morpholino -sydnonimine) were performed in a cumulative manner in U46619 (100nM) preconstricted vessels.

LPS treatment increased vascular sensitivity to L-arginine (control 78.4±4.9% maximum contraction vs LPS 33.6±6.9%; $p<0.05$; N=6), which was significantly reversed by the administration of 1400W 10μM (N-[3-(aminomethyl) benzyl] acetamidine dihydrochloride), an iNOS-specific inhibitor (LPS+1400W 63.9±4.1%; $p<0.05$; N=6), restoring the responses to control levels. LPS treatment significantly reduced the size of the responses to nitrergic nerve stimulation (control 22.9±3.7% vs LPS 10.8±1.1%; $p<0.05$; N=6). The administration of 1400W to these LPS treated vessels, rapidly restored the responses to nitrergic nerve stimulation to control levels (LPS+1400W 25.09±1.81%, vs control+1400W 24.86±3.90%). LPS treatment significantly reduced vascular sensitivity to SIN-1-induced vasodilation (control 6.08±10.94% maximum contraction vs LPS 61.00±7.39%; $p<0.05$; N=5-6; maximum SIN-1 response). The effect of LPS on responses to SIN-1 were rapidly reversed by the administration of 1400W (LPS+1400W 1.35±6.96%; $p<0.05$; N=5-6).

This data is the first to show a significant inhibition of nitrergic vasodilation following LPS treatment. Responses to SIN-1 were similarly reduced suggesting a reduction in vascular sensitivity to NO following iNOS expression. The inhibition of iNOS activity rapidly restored the NO-dependent responses suggesting that reduced enzyme activity and not changes in enzyme expression was responsible for the reduced vascular response to nitrergic and SIN-1 mediated relaxation.

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Supported by the British Heart Foundation (FS99011)

11P ALTERED NEUROGENIC VASODILATION IN RABBIT BASILAR ARTERY FOLLOWING EXPERIMENTA SUBARACHNOID HAEMORRHAGE

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Delayed cerebral vasospasm is a condition that can occur secondary to intracranial bleeding, resulting in significant morbidity and mortality. Superoxide dismutase (SOD) has been shown to be beneficial in both animal and clinical studies of this condition, suggesting a role for superoxide in its pathophysiology. Endothelium-dependent vasodilation is impaired in vasospasm, possibly due to destruction of endothelium-derived nitric oxide by superoxide. This study sought to determine if nitrergic vasodilation of cerebral arteries is similarly modified in delayed cerebral vasospasm, utilising a rabbit model of subarachnoid haemorrhage (SAH).

Male, New Zealand White rabbits (2.9 to 4.2kg) received two intracisternal injections of autologous blood (0.75ml.kg^{-1}) two days apart, as described previously (Nelson *et al.*, 1991). Sham-operated rabbits received saline instead of blood. Animals were euthanased five days after the first intracisternal injection and their basilar arteries removed. 2mm basilar rings underwent isometric tension recording in a wire myograph, containing Krebs solution at 37°C, and guanethidine (10μM), gassed with 95% O_2 / 5% CO_2. Rings were contracted by the addition of histamine (10μM). Electrical field stimulation (EFS, 20-80V, 0.4ms width, 1-2Hz, 5 seconds) of the intramural nerves was delivered via parallel platinum electrodes. EFS produced relaxation of histamine pre-contracted basilar rings. Control responses to EFS were obtained in each ring before evaluation of test agents.

The superoxide anion-generating agent LY83583 (10μM) significantly reduced EFS-induced relaxation in rings obtained from non-operated (from 46 ± 5 to 20 ± 10 %, n = 6, $P<0.05$) and sham-operated (from 49 ± 5 to 21 ± 6 %, n = 13, $P<0.05$) rabbits. In contrast, EFS-induced relaxation was resistant to the effects of LY83583 in rings obtained from SAH animals (54 ± 4 v 47 ± 8 %, n = 14). Furthermore, it was found that the nitric oxide synthase inhibitor L-NAME (300μM) significantly inhibited EFS-induced relaxation of rings from both non-operated (from 46 ± 5 to 24 ± 7 %, n = 21, $P<0.05$) and sham-operated (from 60 ± 5 to 43 ± 8 %, n = 9, $P<0.05$) rabbits, but had no significant effect on EFS in rings obtained from SAH animals (57 ± 5 v 63 ± 9 %, n = 9).

Taken together, the findings of this study suggest that following experimental SAH neurogenic vasodilation in cerebral arteries may change from being mediated predominantly by nitric oxide to a mediator less susceptible to the oxidative stress associated with delayed cerebral vasospasm (e.g. calcitonin gene related peptide or vasoactive intestinal peptide). This novel putative mechanism warrants further study and may potentially offer a new avenue of investigation in the treatment or prophylaxis of this condition.

This work was supported by the British Heart Foundation (grant no. FS/97046).

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12P CHARACTERISATION OF THE NOVEL P2 RECEPTOR ANTAGONIST MRS2179 IN THE PORCINE ISOLATED CORONARY ARTERY

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Until recently, characterization of P2 receptors in native tissues has been hampered by the dearth of selective ligands. With the recent availability of MRS2179 (2'-deoxy-N^6-methyladenosine 3',5'-bisphosphate), however, it is now possible to distinguish $P2Y_1$ receptors (as opposed to $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptors) with some confidence (Boyer et al., 1996). This study aimed to assess the ability of MRS2179 to inhibit relaxatory responses to P2Y-like and A_{2A} adenosine receptors and contractile responses to P2X receptors in the porcine isolated coronary artery (PCA).

Segments of PCA (dissected from whole hearts transported on ice from the abattoir) from pigs (either sex) were mounted for isometric tension recordings, as described previously (Alexander *et al.*, 2000). Tissue viability was assessed by eliciting contractions in the presence of 60 mM KCl. The thromboxane A_2 analogue U46619 (11α,9α-epoxymethano-PGH_2) was utilised (up to 100 nM) to elicit a contraction to about 60% of that to KCl. Thereafter, concentration-relaxation curves were constructed in the presence of increasing cumulative concentrations of ADP or the stable adenosine analogue NECA (5'-*N*-ethylcarboxamidoadenosine) in the absence or presence of MRS2179. Contraction responses to bolus applications of α,β-methyleneATP (α,β-meATP) were constructed in the presence of 1 nM U46619. Data reported are means ± s.e.m. of results from at least six separate experiments and were initially compared for statistical significance by ANOVA.

In pre-constricted arteries, ADP and NECA caused concentration-dependent relaxations with pD_2 values of 4.79 ± 0.20 and 7.47 ± 0.15, respectively, and maximal relaxations (R_{max}) of 98 ± 6 and 86 ± 5 %, respectively. Pre-incubation with 1 μM MRS2179 failed to alter relaxatory responses to NECA (pD_2 values 7.68 ± 0.13; R_{max} 82 ± 7 %). The response to ADP, however, was shifted significantly ($P<0.01$, Student's unpaired t-test) to the right (3.75 ± 0.22) without altering the maximal response (95 ± 7 %), allowing calculation of an apparent pA_2 value for MRS2179 of 6.99. Contractions elicited by α,β-meATP at 1 and 10 μM (20 ± 6 and 30 ± 7 % KCl-induced tone, respectively) were unaffected by pre-incubation in the presence of 10 μM MRS2179 (17 ± 5 and 40 ± 7% KCl-induced tone, respectively).

The affinity of MRS2179 at human recombinant $P2Y_1$ receptors (6.75, Moro et al., 1996) and the $P2Y_1$ receptor of turkey erythrocytes (6.99, Boyer et al., 1996) is consistent with the relaxatory P2Y receptor described here being a $P2Y_1$ receptor. The lack of effect of MRS2179 at the relaxatory A_{2A} receptor in this same tissue underlines it's selectivity. Although MRS2179 has been reported to display some activity as an antagonist of rat recombinant $P2X_1$ and $P2X_3$ receptors with IC_{50} values of 1 and 13 μM, respectively (Brown et al., 2000), it showed no activity against the P2X response in the PCA. Thus, it appears likely that either MRS2179 exhibits species differences as a $P2X_1$ antagonist or that the contractile P2X receptor in the PCA is not a $P2X_1$ receptor.

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13P INHIBITION OF BOTH NITRIC OXIDE AND GUANYLYL CYCLASE EXPOSES AN EDHF IN LARGE BOVINE PULMONARY ARTERIES

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Bradykinin-induced relaxation of small bovine pulmonary (supernumerary) arteries is mediated by an EDHF when nitric oxide is removed or following inhibition of guanylyl cyclase. Removal of nitric oxide results in a reduction in tissue sensitivity where as guanylyl cyclase inhibition does not (Tracey *et al.*, 2002). One explanation for this is that inhibition of nitric oxide/guanylyl cyclase upregulates the EDHF and that inhibition of guanylyl cyclase can upregulate this mechanism to a greater extent than removing nitric oxide. The present study examined the ability of large pulmonary arteries to utilise an EDHF following removal of nitric oxide and/or inhibition of guanylyl cyclase.

Bovine lungs were obtained fresh from the local abattoir. Segments of 4th generation conventional arteries (diameter approx. 5 mm) were dissected from the lungs and freed of surrounding connective tissue. The vessels were then weighed and suspended between stainless steel hooks in Krebs-Henseleit buffer (37^{o}C) under a tension of 2 g and gassed with a mixture of O_2:CO_2 95%/5% v/v). In some rings the endothelium was removed by abrading the luminal surface with forceps. The tissues were allowed to equilibrate for 1 hour then contracted with U46619 (0.3 μM). Concentration response curves for bradykinin-induced relaxation were constructed. Paired tissues acted as time controls. Relaxations are expressed as % decrease of the U46619-induced tone. Results are means ± s.e. mean. The significance of differences was determined using Student's t-test.

In endothelium-intact but not denuded artery rings contracted with U46619, bradykinin (0.1nM-1μM) evoked a concentration-dependent relaxation (pEC_{50} 9.46 ± 0.64; maximum relaxation (Rmax), 100 %, n=6) which was markedly reduced by the nitric oxide scavenger hydroxocobalamin (200 μM) (pEC_{50}, 7.61±0.37; Rmax, 40±16.7, n=4). In the presence of the guanylyl cyclase inhibitor ODQ (10μM) relaxation to bradykinin was not concentration-dependent; high concentrations of bradykinin produced a rapid fall in tone. In the presence of hydroxocobalamin and ODQ bradykinin induced full relaxation (pEC_{50}, 8.14±0.34; Rmax, 125.8±16%, n=7).

The concentration response curve for bradykinin-induced relaxation was not significantly changed by the potassiun channel blockers apamin and charybdotoxin (both 100 nM) or the gap junction inhibitor carbenoxolone (100μM) however the bradykinin response in the presence of hydroxocobalamin and ODQ was abolished by these potassium channel blockers and by carbenoxolone.

In large pulmonary arteries *in vitro* bradykinin normally mediates relaxation via nitric oxide however, following both removal of nitric oxide and inhibition of guanylyl cyclase bradykinin induces relaxation, which appears to be mediated by an EDHF–like mechanism involving apamin and charybdotoxin-sensitive potassium channels and gap junctions.

Tracey, A., Irvine, J., Bunton, D., MacDonald, A., Shaw, A.M.

(2002) *Br. J. Pharmacol.* **135**, 79P

14P POTASSIUM ION AS THE EDHF IN BOVINE CORONARY ARTERY

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The identity of endothelium-derived hyperpolarizing factor (EDHF) remains unresolved. The K^+ ion is a potential candidate since it is released from the endothelium and promotes smooth muscle hyperpolarization and relaxation through activation of Na^+/K^+ ATPase and the inward rectifier (K_{IR}; Edwards *et al.*, 1998). Although not all agree with this concept (Lacy *et al.*, 2000), we wished to determine if the K^+ ion could account for the activity of EDHF in the bovine coronary artery.

Sections of myocardium containing the left anterior descending coronary artery were cut from bovine hearts at a local abattoir and transported to the laboratory. Coronary arterial rings (2.5 mm wide) were mounted for isometric tension recording on stainless steel hooks in 10 ml organ baths containing Krebs solution at 37°C. In some rings, the endothelium was removed by gentle abrasion. All experiments were conducted in the presence of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (100 μM), and the cyclooxygenase inhibitor, indomethacin (3 μM). Tone was raised using the thromboxane A_2-mimetic, U46619 (0.01-0.1 μM), and vasodilator responses were elicited using bradykinin (0.1-300 nM) or KCl (final bath concentration of K^+ raised from 5.9 to 13 mM). Vasodilator responses are given as % reduction (mean ± s.e.mean, n≥6 observations) of tone and differences were determined using one-way ANOVA with the Bonferroni post-test.

Bradykinin (0.1-300 nM) induced powerful vasodilatation (max. 99.7 ± 0.2 %), which was mediated by EDHF (Zygmunt & Högestätt, 1996) since it was inhibited by the combination of apamin and charybdotoxin (both 100 nM; max. 16.3 ± 2.9 %, P<0.001). The K_{IR} inhibitor, Ba^{2+} (30 μM), had no effect on bradykinin-induced vasodilatation (max. 89.4 ± 2.3 %), but the Na^+/K^+ ATPase inhibitor, ouabain (1 μM), produced powerful blockade (max. 35.2 ± 5.7 %, P<0.001). KCl (final bath concentration of K^+: 13 mM) also induced vasodilatation, which was similar in endothelium-denuded and endothelium-containing vessels (39.4 ± 7.9 % and 35.6 ± 6.4 %, respectively). The KCl-induced vasodilatation of endothelium-denuded rings was unaffected by the combination of apamin and charybdotoxin (both 100 nM; 36.7 ± 8.0 %) or by Ba^{2+} (30 μM; 42.3 ± 5.6 %), but was abolished by ouabain (1 μM; -5.8 ± 1.5 %, P<0.001).

The blockade of bradykinin-induced vasodilatation by ouabain, but not by Ba^{2+}, suggests a role for Na^+/K^+ ATPase but not K_{IR} in the EDHF response in the bovine coronary artery. Moreover, the ability of K^+ to produce ouabain- but not Ba^{2+}-sensitive, endothelium-independent vasodilatation is consistent with this ion functioning as the EDHF in this tissue.

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15P ASCORBATE INHIBITS EDHF IN THE BOVINE EYE BUT NOT IN THE PORCINE CORONARY ARTERY

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Endothelium-derived hyperpolarising factor (EDHF)-mediated vasodilatation in the ciliary vascular bed of the bovine eye is powerfully inhibited by ascorbate (McNeish *et al.*, 2002). In this study we have sought to determine the selectivity of the blockade in the ciliary vascular bed and establish whether similar blockade occurs in a large conduit blood vessel, the porcine left anterior descending coronary artery.

Bovine eyes and pig hearts were obtained from a local abattoir. Eyes were perfused according to the method of McNeish *et al.* (2002). In the eye, vasodilator responses were elicited by injecting bolus doses of acetylcholine (ACh; 0.1-100 nmol) or levcromakalim (0.1-30 nmol). Coronary arteries were cut into 2.5mm wide rings, mounted between steel hooks and placed in 10 ml organ baths at 37°C filled with Krebs containing indomethacin (3 μM) and L-NAME (100 μM). Tone was raised using U46619 (0.1-1 μM) and EDHF-dependent vasodilator responses were elicited with bradykinin (0.1-300 nM). Blocking drugs were present for at least 20 min except ascorbate which was present for > 120 min. Vasodilator responses are given as % reduction of U46619-induced tone. Data are mean ± s.e.mean, of n≥5 observations. Differences were determined using one-way ANOVA with Bonferroni's post-test.

As previously reported (McNeish *et al.*, 2002) ACh produced dose-dependent vasodilatation (max 59.5 ± 4.5%) in the bovine ciliary bed and ascorbate (50 μM) powerfully blocked these responses (max 28 ± 2.4%, P<0.001). The dose-dependent vasodilatation induced by levcromakalim (max 80.3 ± 3.4%) was unaffected by ascorbate (max 80.3 ± 3.2%). In rings of porcine coronary artery, bradykinin induced concentration-dependent, EDHF-mediated vasodilatation (max 80.3 ± 6.5%), which was blocked significantly (max 11.3 ± 3.3%, P<0.001) by the combination of apamin (100 nM) and charybdotoxin (100 nM), almost significantly by apamin alone (max 62.2 ± 5.2%), but not by charybdotoxin alone (max 71.3 ± 9.2%). Ascorbate (150 μM), either alone (max 76.7 ± 4.7%), or in combination with charybdotoxin (max 76.1 ± 5.9%) failed affect vasodilatation. Ascorbate also failed to enhance the small inhibitory action of apamin (max 56.2 ± 5.0%).

Ascorbate-induced blockade of EDHF-mediated vasodilatation in the bovine ciliary circulation (McNeish *et al.*, 2002) appears to be selective, as vasodilator responses to the K_{ATP} channel opener, levcromakalim, are unaffected. In the porcine coronary artery EDHF-mediated vasodilatation was abolished by the combination of charybdotoxin and apamin, but ascorbate alone in combination with charybdotoxin or apamin had no effect. Thus, it appears that ascorbate does not block either small (apamin-sensitive) or intermediate (charybdotoxin-sensitive) conductance calcium-activated K^+ channels in the porcine coronary artery. Whether the ability of ascorbate to block EDHF-mediated vasodilatation is related to vessel size, flow or some other factor remains to be determined.

McNeish A.J., Wilson W.S., Martin W. (2002). *Br. J. Pharmacol.* **135**, 1801-1809.

16P PHARMACOLOGICAL CHARACTERISATION OF RAMP2/CL AND RAMP3/CL ADRENOMEDULLIN RECEPTORS WITH ADRENOMEDULLIN$_{22-52}$ AND CGRP$_{8-37}$

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Adrenomedullin (AM) is a multifunctional regulatory peptide, with two known receptors formed by the calcitonin receptor-like receptor (CL) and receptor activity-modifying protein (RAMP) 2 or RAMP3. RAMP2 and RAMP3 have low amino acid homology yet pharmacological differences between these AM receptors have not been demonstrated, nor have the functional effects of antagonists been examined (Poyner *et al.*, 2002). Therefore, we have examined the abilities of the antagonist fragments of human adrenomedullin and CGRP, AM$_{22-52}$ and CGRP$_{8-37}$, to inhibit the effects of AM at humanRAMP2/ratCL and human RAMP3/ratCL receptors transiently expressed in Cos-7 cells.

Cos-7 cells, cultured in Dulbecco's Modified Eagle Medium/10% foetal calf serum, were transiently transfected using calcium phosphate (Clontech). After 48-72 hours, the cells were pre-incubated with serum-free medium containing 500μM isobutylmethylxanthine for 30 min. The cells were treated with antagonist for 15 min and then exposed to rat AM (1pM to 1μM) for a further 15 min. Reactions were terminated with 95% ethanol and cAMP measured as described previously (Poyner *et al.*, 1998). Means are quoted ± s.e.mean and were analysed using Graphpad Prism 3 software. Statistical analysis was performed with a two-tailed, unpaired Student's t-test with significance at $P < 0.05$. Apparent pA_2 values were calculated as (dose ratio-1)/[Antagonist].

In RAMP3/CL transfected cells, AM elevated cAMP with a pEC_{50} of 8.95 ± 0.21 (n=3). There was no significant change in pEC_{50} in the presence of 10μM AM$_{22-52}$ (8.27 ± 0.36, n=3). 10μM CGRP$_{8-37}$ caused a parallel rightward shift in the concentration response curve to AM with a significant change in pEC_{50} (AM 8.56 ± 0.25; AM /CGRP$_{8-37}$ 7.06 ± 0.14, both n=3, P= 0.006). The apparent pA_2 was 6.48 ± 0.20. In RAMP2/CL transfected cells, 10μM AM$_{22-52}$ caused a significant, parallel rightward shift in the concentration response curve to AM (pEC_{50} values: AM 7.98 ± 0.12; AM and AM$_{22-52}$ 6.71 ± 0.07, both n=3, P =0.0009). The apparent pA_2 value was 6.25 ± 0.17. 10μM CGRP$_{8-37}$ had no significant effect (AM 8.02 ± 0.29; AM /CGRP$_{8-37}$ 7.25 ± 0.28, both n=3).

Thus, in this study, 10μM AM$_{22-52}$ significantly antagonised cAMP responses to AM at the RAMP2/CL complex but not the RAMP3/CL complex. The opposite was seen with CGRP$_{8-37}$, which was able to antagonise the effects of AM at RAMP3/CL receptors with a pA_2 in line with estimations for its effectiveness at putative CGRP$_2$ receptors.

This work was supported by the Wellcome Trust and an MRC studentship (DLH).

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17P QUANTIFYING LIGAND BINDING TO THE ADENOSINE-A_1 RECEPTOR AT THE SINGLE CELL LEVEL USING FLUORESCENCE CORRELATION SPECTROSCOPY (FCS)

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FCS is a non-invasive technique which measures fluctuations in fluorescence intensity in a confocal volume of $<10^{-15}$l. Statistical analysis of these fluctuations gives information about the speed of diffusion (i.e. mass) and concentration of the fluorescent molecules present (Schwille, 2001). Thus free ligand (fast diffusing) and bound ligand (slow diffusing) can be quantified simultaneously on a single cell. We have used FCS to measure binding of the fluorescent ligand, xanthine amine cogener-BODIPY®630/650 (XAC-BY630; Briddon *et al.*, in press) to the human adenosine A_1 receptor (A_1-AR).

CHO cells expressing either the human A_1-AR or an A_1-AR-Topaz fusion were cultured on glass-bottomed 8-well plates and prepared for live cell measurement as described previously (Briddon *et al.*, in press). FCS measurements were made using a Zeiss Confocor 2, fitted with an Axiocam CCD camera for x-y positioning. Cells were incubated with ligands at 22°C for the times indicated and the confocal volume was positioned on the upper membrane. Data were collected for 2x30s, following a 15s pre-bleach and analysed using a multi-parameter equation using Zeiss AIM software.

Initially, the diffusion characteristics of the A_1-AR-Topaz fusion protein (A_1-AR-Tpz) were determined in CHO-A1Tpz cells. Autocorrelation analysis showed the diffusion time (τ_D) for the A_1-AR was 15.0±0.9ms (mean±s.e.mean, n=84). A second component (τ_D=118±14μs) was also seen, probably caused by an optical event within the fluorophore (Widengren *et al.*, 1999). FCS analysis of XAC-BY630 in buffer showed a single component diffusion (τ_D=60±2μs, n=10). On the upper membrane of CHO-A1 cells incubated with XAC-BY630 (1-40nM, 10-60 min, n=71), two further slow-diffusing species were detected in addition to free ligand. The first component had a similar diffusion time (τ_{D1}=17.4±1.1ms; 69/71 cells) to that seen for A_1-AR-Tpz, suggesting that it is receptor-bound ligand. The second was a very slow diffusing component (τ_{D2}=345±41ms, 61/71 cells). Following preincubation with 8-cyclopentyl-1,3-dipropyl xanthine (DPCPX) (1μM, 30 min), t_{D2} was present in 30/31 cells, suggesting this component is non-specific binding. However, the t_{D1} component was present in only 17/31 cells. In addition, in cells exposed to 15nM XAC-BY630 for 30 min the amount of τ_{D1} component was reduced from 51.8±14.9 to 13.6±5.4 receptors/μm^2 by DPCPX (n=8 and 4, respectively, Student's t-test, $P<0.05$), further suggesting this component is A_1-AR bound ligand.

We have used FCS to quantify binding to the A_1-AR and measure receptor diffusion in single live cells. Further development will allow quantitative receptor-ligand binding of the endogenous A_1-AR in acutely dispersed cells.

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We thank The Wellcome Trust for financial support.

18P CERTAIN CLASSICAL "BETA –BLOCKERS" STIMULATE GENE TRANSCRIPTION IN CHO-K1 CELLS EXPRESSING THE HUMAN BETA-1 ADRENOCEPTOR

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The human beta-1 receptor has recently been proposed to have two agonist conformations; a 'catecholamine conformation' where classical agonists act, and a 'CGP 12177 conformation' where CGP 12177 is an agonist (Konkar et al., 2000). Beta-antagonists inhibit the catecholamine responses whereas the CGP 12177 agonist responses are relatively resistant to beta-blockers (Konkar et al., 2000; Lowe et al., 2002). As CGP 12177 was originally developed as a beta-blocker, the aim of this study was to determine whether other beta-blockers are able to activate this second agonist conformation.

CHO-K1 cells were stably transfected with both the human beta-1 adrenoceptor and a secreted placental alkaline phosphate (SPAP) reporter gene under the transcriptional control of six cyclic AMP response elements (CRE). SPAP secretion was measured as described previously (McDonnell et al., 1998).

The selective beta-1 antagonist CGP 20712A inhibited responses to isoprenaline and CGP 12177 to yield log K_D values of –8.91 and –7.93 respectively consistent with the presence of the two agonist conformations of the human beta-1 receptor (Baker et al., this meeting).

Acebutolol (log EC_{50} –7.16 ± 0.07, 43.1 ± 5.4% of the maximal response to isoprenaline, n=8), carvedilol (-8.51 ± 0.13, 39.7 ± 3.0%, n=9) and propranolol (-7.19 ± 0.35, 13.7 ± 3.5%, n=7) all stimulated a gene transcription response. The acebutolol and carvedilol responses were antagonised by CGP 20712A yielding log K_D values of –9.94 ± 0.09 (n=5) and –8.27 ± 0.13 (n=9) respectively. Atenolol, bisoprolol, CGP 20712A, ICI 118551, metoprolol and sotalol had no effect on gene transcription at concentrations up to 10μM (n=3-4). Concentration response curves to alprenolol (47.9 ± 4.0% of isoprenaline max) and pindolol (66.4 ± 4.2%) were fitted best by a two component analysis (alprenolol: site 1 log EC_{50} = –8.66 ± 0.14; site 2 log EC_{50} = -6.13 ± 0.19; site 1 = 52.9 ± 4.0%, n=9; pindolol: site 1 log EC_{50} = –8.51 ± 0.20; site 2 log EC_{50} = –5.30 ± 0.20; site 1 = 43.6 ± 2.8% n=8). In the presence of 100nM CGP20712A, these responses appeared to contain only a single component with log EC_{50} values of –6.03 ± 0.03 (n=5) and –6.20 ± 0.12 (n=5) for alprenolol and pindolol respectively. No response was seen to any of the ligands used in CHO-K1 cells transfected only with the CRE-SPAP reported gene and not the beta-1 receptor.

These data show that several beta-1 antagonists can stimulate gene transcription in CHO-K1 cells expressing the human beta-1 receptor at 1.1pmol/mg protein. These compounds show a differential sensitivity to antagonism by CGP 20712A. Furthermore alprenolol and pindolol appear to activate gene transcription via two separate components which also differ in their sensitivity to CGP 20712A. This study suggests that the secondary conformation of the human beta-1 receptor first identified by CGP 12177 may also be a recognised by other clinically used beta-blockers.

JGB holds a Wellcome Trust Clinical Training Fellowship.

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19P DIFFERENTIAL ANTAGONIST AFFINITIES FOR ISOPRENALINE AND CGP 12177 STIMULATED GENE TRANSCRIPTION IN CHO-K1 CELLS EXPRESSING THE HUMAN β-1 ADRENOCEPTOR AT LOW LEVELS

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Previous studies of isoprenaline-stimulated cyclic AMP accumulation in CHO-K1 cells expressing the human β_1 adrenoceptor (β_1-AR) have suggested that CGP12177 can interact with a low affinity conformation of the β_1-AR that is relatively resistant to antagonism by β_1-AR antagonists (Konkar et al., 2000). Here we have evaluated the characteristics of isoprenaline and CGP 12177 stimulated gene transcription in CHO-K1 cells stably transfected with the human β_1-AR and a cyclic AMP response element-regulated luciferase reporter gene (CHO-β1 cells).

^{3}H-CGP 12177 binding to β_1-ARs in intact cells was measured essentially as described previously, with non-specific binding determined with 100nM CGP 20712A (Baker et al., 2002). Luciferase activity was measured in CHO-β1 cells, grown in 96-well plates, after a 5 hour incubation with agonists at 37°C in DMEM/F12 media. Antagonists, when used, were added 30 minutes prior to the addition of the agonists. After 5 hours, the cells were washed with 200μl phosphate buffered saline and luciferase activity was then measured using a Luclite kit (Packard) as per the manufacturers instructions.

Binding of ^{3}H-CGP 12177 to intact CHO-β1 cells showed that the transfected receptor was expressed at 51.7 ± 1.3 fmol/mg protein (n=3). The K_D for ^{3}H-CGP 12177 obtained from these binding studies was 0.12 ± 0.02 nM (n=3). Isoprenaline stimulated an increase in CRE-mediated luciferase production ($logEC_{50}$ = -6.98 ± 0.09; 7.6 ± 0.7 fold over basal; n=10). This response was antagonised by CGP 20712A to yield a log K_D value of -8.62 ± 0.12 (n=4). Propranolol and atenolol also antagonised this response yielding log K_D values of -7.48 ± 0.10 (n=4) and –6.25 ± 0.22 (n=3) respectively. CGP 12177 behaved as a low efficacy agonist in this system (log EC_{50} = -7.55 ± 0.05; n=10). producing a maximal luciferase response that represented 55.2 ± 3.4% of that produced by isoprenaline. The response to CGP 12177 was less sensitive to antagonism by CGP 20712A (log K_D = -7.63 ± 0.11; n=4) and propranolol (log K_D= -6.46 ± 0.20; n=3) than when isoprenaline was used as the agonist. 10 μM atenolol did not cause any shift of the concentration-response curve to CGP 12177 suggesting that the K_D was greater than 10μM (n=3).

In this low receptor expressing system, where the CGP 12177 maximal response was only half that of isoprenaline, the K_D values obtained for CGP 20712A and propranolol as antagonists of this response differed by an order of magnitude from those obtained when isoprenaline was used as the agonist. These data, using gene transcription as a measure of receptor activation, support previous with suggestions based on other functional readouts that isoprenaline and CGP 12177 may be acting at different conformations of the β_1-AR, (Konkar et al., 2000; Lowe et al., 2002).

We thank the Wellcome Trust for financial support and S. Rees and GlaxoSmithKline for providing the CHO-β1 cells.

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20P THE EXPRESSION LEVELS OF HEPATIC α_1-ADRENOCEPTORS CHANGE WITH AGE IN α_{1B}-ADRENOCEPTOR KNOCKOUT MICE

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Binding studies, carried out previously on wildtype (WT) and α_{1B}-adrenoceptor (AR) knockout (KO) mice livers (age not specified) found that although the WT liver expressed α_1-ARs, the α_{1B}-AR KO liver had no detectable levels of α_1- AR. This led to the conclusion that WT murine liver expressed a pure population of α_{1B}-ARs (Cavalli *et al*, 1997). In contrast to this study we found that both the WT and α_{1B}-AR KO liver expressed α_1- ARs and that while we agree with Cavalli *et al* that the WT liver expresses α_{1B}-ARs, the α_{1B}-AR KO liver expresses α_{1A}-ARs (Deighan *et al*, 1999). We now present data to demonstrate that this discrepancy between the two studies may be dependent on the age of the mice used.

Saturation binding studies were performed with liver membrane preparations isolated from 129/Sv/C57-Black 6J (male, 30-45g) WT and α_{1B}-AR KO mice. Membrane preparations were incubated with ^{3}H-prazosin (0.025-5nM) in a Tris-HCl buffer (pH 7.4) for 30 minutes at 22°C. Reactions were terminated by rapid filtration using a Brandell cell harvester. Non-specific binding was measured in the presence of 10μM phentolamine. Statistical analysis was carried out using either a one-way or a two-way ANOVA and a p value of less than 0.05 was considered significant.

^{3}H-prazosin identified specific saturable sites of high affinity in WT livers at all three ages with no significant differences in affinity. At 3 months of age the B_{max} was significantly greater than at 4 and 16 months. Expression levels of α_1-ARs were similar at 4 and 16 months. When compared with age matched WT mice, the B_{max} in 3 month KO livers was significantly reduced. This was accompanied by a decrease in affinity ($p<0.05$). At 4 months of age, the density of α_1-ARs and their affinity for ^{3}H-prazosin has significantly increased ($p<0.05$), compared with 3 month KO livers. By 16 months α_1-ARs had dropped to undetectable levels ($p<0.05$).

	WT		KO	
	B_{max} (fmol/mg)	K_D (nM)	B_{max} (fmol/mg)	K_D (nM)
3 months	76±3.3	0.3±0.05	7.4±0.73†	1.0±0.27†
4 months	50±3.1*	0.3±0.08	30±2.0*†	0.15±0.04*
16 months	52±0.07 *	0.2±0.07	N.D	N.D.

Table 1:- B_{max} and K_D values obtained from saturation curves using ^{3}H-prazosin in WT and α_{1B}-AR KO livers at 3, 4 and 16 months ($n\geq3$). N.D. – not detectable. $p<0.05$, * vs 3m, † vs WT

These results demonstrate that the discrepancy between Cavalli *et al* and Deighan *et al* may have arisen as a result of mice of different ages being used in the studies. Unfortunately Cavalli *et al* do not state the age of the mice used for their binding studies. Our own earlier study used mice that were 4 months of age, allowing for detection of α_1-ARs and subsequent subtyping. In addition, it would seem from this data that the appearance of the α_{1A}-AR in the α_{1B}-AR KO liver occurs mainly between 3 and 4 months of age and has disappeared again by 16 months.

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We are grateful to Professor S. Cotecchia for kindly providing the knockout and wild-type mice. Supported by BHF, MRC and the EC (VASCAN 2000, QLG1-1999-00084).

21P ADVENTITIAL CELLS ARE IDENTIFIED AS THE MAJOR LOCATION OF VASCULAR α_{1B}-ADRENOCEPTORS AND MAY DRIVE VASCULAR REMODELLING

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Of the three α_1-adrenoceptor (AR) subtypes, α_{1B}-AR have the minor role in arterial vasoconstriction: α_{1A}-are dominant in resistance and α_{1D}-dominant in conducting arteries (Daly et al, 2002). We now show, using fluorescent ligands for α_1-AR and a mouse harbouring an α_{1B}-AR-GFP construct that the major vascular location for α_{1B}-AR is adventitial cells.

Mice: C57-Black 6J (male, 30-45g) WT, α_{1B}-AR KO or a new line, the α_{1B}-AR-GFP, harbouring an α_{1B}-AR-GFP construct (Awaji et al, 1998) under its own promoter (Zuscik, 2001). Laser-scanning confocal microscopy or triple wavelength fluorescence microscopy located α_1-AR binding sites in the arterial wall using the fluorescent antagonist ligand QAPB (MacKenzie et al, 2000): the red version allowed separation from GFP. Cells were isolated from aortic adventitia, cultured overnight under aseptic conditions, loaded with Fura-2-AM and calcium signals to phenylephrine and antagonist ligand binding were monitored simultaneously.

In intact first order mesenteric resistance arteries QAPB bound to adventitial cells and medial smooth muscle cells, both being attenuated by phenoxybenzamine. Adventitial cell binding was punctate and intracellular. As in other species smooth muscle binding occurred diffusely over the cell surface and at punctate intracellular sites.(MacKenzie et al, 2000). In similar arteries from the α_{1B}-AR-GFP mouse GFP-derived fluorescence was found in adventitial cells with a subcellular distribution similar to that found with QAPB. Cultured aortic adventitial cells showed GFP-derived fluorescence colocalised with QAPB. In these cells Phenylephrine produced rises in intracellular calcium that were attenuated by QAPB.

Elimination of this receptor in the α_{1B}-adrenoceptor KO reduced adventitial cell number in the carotid artery.

Conclusions: 1. the site of adrenoceptor-mediated remodelling is the vascular adventitia. This points to the adventitia as the source of remodelling induced by other factors and explains the common denominator of adventitial hyperplasia in models of hypertension (Kantachuvesiri et al, 2001) and loss of noradrenaline-driven remodelling in the α_{1B}-AR-KO mouse (Vecchione et al, 2002). 2. adventitial cells are subject to adrenergic influence, making sense of the location of adrenergic nerves at the medial-adventitial border. 3. receptors labelled with GFP can be expressed in a mouse, a technique that, generalised, should be of great utility.

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Research supported by MRC and EC FP5 project 'Vascan' *(QLG1-CT-1999-00084)*

22P EVIDENCE THAT PROSTAGLANDIN E_2 OPENS CARDIAC INWARD RECTIFIER POTASSIUM CHANNELS: AN EXPLANATION FOR PREVENTION OF TORSADE DE POINTES

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Previously, it has been reported that prostaglandin E_2 (PGE_2) dose-dependently shortened cardiac action potential duration and reduced the incidence of torsade de pointes in an anaesthetized rabbit model (Farkas & Coker, 2001). The aim of the present experiments was to investigate possible explanations for this antiarrhythmic action of PGE_2.

Male Wistar rats (250 to 350g) were killed humanely by stunning and exsanguination. The heart was removed rapidly and left atrial and left papillary muscle preparations were set up in a Krebs bicarbonate solution at 37°C gassed with 95% O_2/5%CO_2 and resting tension set at 10mN. Each preparation was stimulated at 1 Hz with square wave pulses of 5 ms duration at twice threshold voltage (1-5V). After equilibration for 1hr, the effective refractory period (ERP) was measured by interpolation of extra stimuli (Shaw *et al.*, 1997). The thoracic aorta was also removed from each rat, cut into rings, stored in Krebs solution at 4°C for 2 to 3 hr, and then set up in organ baths as above and allowed to equilibrate for 1 hr.

In aortic ring preparations PGE_2 alone (0.1 to 100nM) caused either concentration-dependent vasoconstriction (n=6), variable, non-sustained vasoconstriction (n=2) or no response (n=11). No vasodilation was observed in response to PGE_2 in either phenylephrine or KCl preconstricted aortic rings and PGE_2 did not alter the concentration-dependent vasodilator responses to pinacidil (10nM to 10μM).

In cardiac preparations PGE_2 (100nM) reduced the ERP, terikalant (100nM, teri), glibenclamide (10μM, glib) and 4-aminopyridine (100μM, 4-AP) increased the ERP and almokalant (1μM, almo) had no effect. Only terikalant prevented PGE_2–induced shortening of the ERP (Table 1).

Table 1. Effect of K^+ channel blockers and PGE_2 on effective refractory period (ms) in rat isolated cardiac muscle.

	Control	Teri	Glib	4-AP	Almo
Baseline	40±3	38±2	32±4	31±4	31±1
+ blocker	40±3	56±3$^{\#}$	41±5$^{\#}$	39±4$^{\#}$	32±1
+ PGE_2	32±3*	64±4*	32±4*	29±5*	26±2*

Values are mean ± s.e. mean, n=6-10. $^{\#}P<0.05$ compared to baseline, *$P<0.05$ compared to + blocker, paired *t* test.

These results indicate that PGE_2 did not alter vasodilation mediated via activation of ATP-dependent K^+ channels in vascular smooth muscle. In addition, the effects of PGE_2 on the ERP of cardiac muscle were not altered by blockade of delayed rectifier K^+ channels with almokalant, transient outward K^+ channels with 4-aminopyridine, or ATP-dependent K^+ channels with glibenclamide, whereas blockade of inward rectifier K^+ channels with terikalant completely prevented the effect of PGE_2. Thus, opening of inward rectifier K^+ channels by PGE_2 is a plausible explanation for its ability to reduce the incidence of torsade de pointes.

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23P ACTIVATION OF A MEMBRANE CURRENT AND CONTRACTION BY THE PROSTANOID EP_3-RECEPTOR AGONIST SULPROSTONE IN GUINEA-PIG AORTA

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The prostanoid EP_3-receptor agonist, sulprostone, potently contracts human pulmonary artery and guinea-pig aorta preparations, but its maximum effect is lower than observed with strong agonists, such as phenylephrine and U-46619 (Jones *et al*, 1998). Further investigations on guinea-pig aorta have suggested that Rho-kinase-induced Ca^{2+}-sensitization may be the major mechanism (Shum *et al*, 2002). In some preparations, removal of extracellular Ca^{2+} reveals that Ca^{2+} influx contributes to sulprostone (>10 nM)-induced contraction and that L-type (nifedipine-sensitive) and non-L-type (nifedipine-insensitive) Ca^{2+}-permeable channels are involved. We now report experiments designed to characterise the Ca^{2+} entry pathway(s) in smooth muscle cells of guinea-pig aorta that contribute to sulprostone action.

Tension was recorded from endothelium-denuded rings of thoracic aorta from 400-550 g male guinea-pigs (Jones *et al*, 1998). Smooth muscle cells were isolated by papain digestion (4°C, ~17 hours) as described by Clapp and Gurney (1991). For whole-cell, patch-clamp experiments, cells were superfused at 1 ml/min with physiological salt solution containing (mM) NaCl 135, KCl 5, NaH_2PO_4 1, $MgCl_2$ 1, $CaCl_2$ 2.5, glucose 10, HEPES 10 (pH 7.4). Pipettes contained (mM) 140 KCl, 1 $MgCl_2$, 3 MgATP, 0.1 EGTA, 10 HEPES (pH 7.2). Cells were clamped at -80mV and the current response to a -10mV step used to measure cell capacitance, against which current amplitudes were normalised. Data are expressed as mean ± s.e.mean. Statistical differences were analysed by one- or two-factor ANOVA, with or without repeated-measures.

In the presence of nifedipine (1 μM), the contraction induced by 300 nM sulprostone was inhibited by 19 ± 3% (n=18, $p<0.001$) when 2.5 mM EGTA was added. It was also inhibited by 29 ± 9% (n=4, $p<0.001$) by 10 μM SK&F 96365 and 35 ± 9% (n=4, $p=0.08$) by 300 μM mefenamic acid, both blockers of non-selective cation channels (NSCC). In cells clamped at -80 mV, 300 nM sulprostone activated a sustained inward current (amplitude -0.8 ± 0.2 pA/pF) in 67% of cells studied (16 of 24; Figure 1). The NSCC blocker $NiCl_2$ (200 μM) consistently and reversibly inhibited the current (86 ± 16%, n=9, $p<0.05$). Also, the current persisted in the presence of the Cl^--channel blocker, niflumic acid (NFA, 10 μM), in 53% of the cells, but its magnitude was reduced by 56 ± 24% (n=8, $p<0.05$).

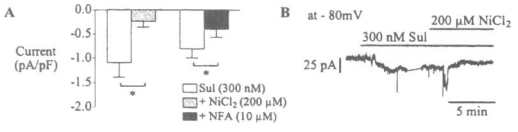

Figure 1. A: Histogram showing the effects of $NiCl_2$ and NFA on the membrane current activated by sulprostone (Sul). *$p<0.05$. B: Tracing shows the sustained sulprostone current and the inhibitory effect of $NiCl_2$. The solid bars indicate the presence of drugs and the dotted line denotes the zero-current level.

Thus the EP_3-receptor agonist sulprostone may therefore activate Ca^{2+}-permeable NSCC, as well as Cl^- channels, in guinea-pig aorta smooth muscle cells. The resulting depolarisation could open L-type Ca^{2+}-channels. The variable nifedipine sensitivity of contraction may be related to variation in the relative contributions of different Ca^{2+} influx pathways and the channels modulating membrane potential.

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24P MUSCARINIC RECEPTORS AND CALCIUM SIGNALLING IN RABBIT ISOLATED PULMONARY ARTERY SMOOTH MUSCLE CELLS

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Acetylcholine (ACh) is an endothelium-dependent vasodilator, but when pulmonary artery endothelium is damaged it causes vasoconstriction through a direct action on smooth muscle muscarinic receptors (Dinh-Xuan *et al.*, 1989). In rabbit main pulmonary artery this action involves M_2 and M_3 receptors (O'Neill *et al*, 2002). The muscarinic agonist, oxotremorine sesquifumarate (Oxo-S), also contracts this preparation, but the effects appear to be mediated solely through M_2 receptors (O'Neill *et al*, 2002). This study characterised responses to ACh and Oxo-S in isolated pulmonary artery smooth muscle cells (PASMC) by monitoring changes in the fluorescence of the Ca^{2+} indicator, fluo-4. Arteries were obtained from male New Zealand rabbits (2-2.5 kg) killed by lethal injection of sodium pentobarbitone (140 mg kg^{-1} i.v.). PASMC were isolated by enzymatic digestion, loaded with 1 μM fluo-4 at 22°C for 45 min, washed and bathed in physiological salt solution (PSS). Fluorescence was excited at 488 nm and measured between 503 and 553 nm using a confocal microscope. Calcium signals were quantified as the change in fluorescence (ΔF) relative to the background fluorescence (F_o). Ca^{2+}-free solution was prepared by replacing $CaCl_2$ in the PSS with equimolar $MgCl_2$ and adding 5mM EGTA. To study G_i involvement, PASMC were incubated with pertussis toxin (PTX; 5μg ml^{-1}) for 3-4 hr at 22°C and compared with control cells treated in the same way, but without exposure to PTX. Experiments were performed 22°C. Results are expressed as mean ± s.e.m.

Elevating the extracellular K^+ concentration from 5 to 50 mM evoked a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which peaked at $\Delta F/F_o = 3.4 \pm 0.6$ (n=54) and was followed by contraction in 67 ± 5 % of PASMC. ACh (100μM) induced a rise in $[Ca^{2+}]_i$ in 65% of the K^+-responsive cells, with $\Delta F/F_o = 1.4 \pm 0.1$ (n=35). In the same preparations Oxo-S (10 μM) induced responses in only 21% of the K^+-responsive cells, although the rise in $[Ca^{2+}]_i$ ($\Delta F/F_o = 2.2 \pm 0.5$, n=11) was significantly greater than evoked by ACh ($p<0.05$). Upon removal of extracellular Ca^{2+}, a similar proportion (62 ± 8 %) of 38 PASMC responded to K^+, but with a significantly ($p<0.05$) reduced rise in $[Ca^{2+}]_i$ ($\Delta F/F_o = 1.0 \pm 0.2$, n=22). In these conditions, 64% (n=14) of the K^+-responsive cells showed a rise in $[Ca^{2+}]_i$ when challenged with ACh ($\Delta F/F_o = 0.8 \pm 0.1$), whereas the response to Oxo-S was abolished. After incubation of cells with PTX, responses to ACh were not significantly altered ($\Delta F/F_o = 0.7 \pm 0.2$, n=26; 50% of K^+-responsive PASMC) compared to time-matched controls ($\Delta F/F_o = 0.7 \pm 0.1$, n=19; 58% of K^+-responsive PASMC). In contrast, responses to Oxo-S were abolished. These results were obtained in cells isolated from main pulmonary artery, but results were similar in cells from intrapulmonary artery.

The results indicate that Oxo-S evoked Ca^{2+} signals depend on G_i and Ca^{2+} influx, consistent with M_2 receptors. Although responses to ACh probably also involve M_2 receptors, they depended primarily upon Ca^{2+} mobilisation from intracellular stores and a PTX-insensitive G-protein, consistent with M_3 receptors. Since only 32% of ACh-sensitive cells responded to Oxo-S, M_2 and M_3 receptors may be expressed on different subpopulations of PASMC.

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25P THE RHO KINASE INHIBITOR, Y-27632, INHIBITS CONTRACTILE RESPONSES TO CARBACHOL, THAPSIGARGIN AND POTASSIUM IN THE MOUSE ANOCOCCYGEUS

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Calcium sensitisation in smooth muscle results in increased tension at a constant intracellular calcium concentration. The main mechanism underlying calcium sensitisation seems to involve inhibition of myosin light chain phosphatase (MLCP) by Rho kinase (ROK). In order to investigate whether this pathway might operate in the mouse anococcygeus we have investigated the effects of Y-27632, a ROK inhibitor (Uehata *et al*, 1997), on contractile responses to carbachol (CCh; 50 μM), thapsigargin (Tg; 100 nM), and high potassium Krebs' (high K^+; 65 mM) in both intact and permeabilised muscle.

Anococcygeus muscles were dissected from male mice (LACA; 25-35g) and set up for the recording of isometric tension (Ayman *et al*, 2001). The Krebs solution contained phentolamine (1 μM), L-N^G-nitroarginine (L-NOARG; 50 μM) and verapamil (10 μM); in addition, muscles were preincubated with guanethidine (30 μM; 10 mins). Verapamil was omitted when responses to high K^+ were investigated. The protocol used for β-escin (50 μM; 10 min) permeabilisation of muscle strips is described in detail elsewhere (Ayman *et al*, 2001). The bathing medium contained EGTA (2 mM) and the concentration of free calcium was adjusted by the addition of $CaCl_2$. Tension in permeabilised strips was normalised with respect to that in the presence of 1 μM calcium. Results are presented as mean ± s.e.m. for at least 4 observations. Statistical analysis was by Student's t-test or ANOVA with Student-Newman-Keuls *post hoc* test. $P<0.05$ was considered significant.

In intact tissues, CCh and Tg produced sustained contractions of similar magnitude (316 ± 63 mg and 247 ± 38 mg respectively). Cumulative addition of Y-27632 produced concentration-dependent relaxations of muscles pre-contracted using either CCh (pD_2 5.2 ± 0.2) or Tg (pD_2 5.4 ± 0.5). In both cases, 100% inhibition occurred at 200 μM Y-27632. Contractions produced by either CCh or high K^+ were inhibited following a 10 min pre-exposure to 10 μM Y-27632 (56 ± 7% and 69 ± 7% inhibition respectively). In permeabilised muscle strips pre-exposed to calcium (1 μM), addition of GTP (100 μM) increased tension to 171 ± 13% (n = 19). In 6 tissues, addition of CCh further increased tension from 185 ± 17% to 329 ± 45% ($P<0.05$). This response was inhibited by Y-27632 (10 μM), the tension dropping to 195 ± 30%. In 4 tissues tested, the addition of Tg increased tension from 181 ± 49% to 334 ± 42% ($P<0.05$) and this fell to 150 ± 17% with the addition of Y-27632. In the presence of GTP alone, Y-27632 had no significant effect on tension (168 ± 22% to 139 ± 23%; n = 6).

These results support a role for ROK in calcium sensitisation in the mouse anococcygeus. However the finding that Y27632 is equally effective at inhibiting contractions produced by CCh, Tg and high K^+ suggests that there may be significant tonic activation of the ROK pathway in this tissue.

SA is the recipient of a BBSRC CASE award to Pfizer UK.

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26P CHOLINERGIC, ADRENERGIC, NITRERGIC AND PURINERGIC NEUROTRANSMISSION IN THE PIG BLADDER DOME

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The bladder dome and neck have very distinct and varying functions in the maintenance of normal bladder control. The dome of the bladder is thought to receive a predominant cholinergic innervation and is involved in normal emptying, whilst the bladder outlet region has an additional sympathetic component (Fletcher and Bradley 1978; de Groat 1997) important in maintaining normal continence. The majority of work investigating this innervation has relied on imaging techniques to locate and identify nerve populations; functional data in support of this is relatively scarce. The aim of this study was to investigate the innervation of the bladder dome of the pig and determine the relative importance of the parasympathetic, sympathetic and NANC innervation in mediating contractile responses to electrical field stimulation.

The bladders from female pigs (70-90Kg) were obtained from a local abattoir. Paired longitudinal strips of pig detrusor (approx. 20 x 5mm) were isolated from the pig bladder dome. The urothelium and serosa were removed before suspension in 5ml organ baths. The tissues were bathed in Krebs-bicarbonate solution (gassed with 95% O_2/ 5% CO_2), placed under 1g of tension and maintained at 37°C. Responses to electrical field stimulation (40V, 10Hz, 0.1msec pulse width, 5-second duration, 100 second repetition) were recorded in the absence and presence of atropine (1mM), guanethidine (10μM) or αβ-methyleneATP (αβ-meATP) (10μM) to determine the proportion of cholinergic, adrenergic and purinergic mediated contraction respectively. L-NNA (100μM), an inhibitor of nitric oxide synthase was also used to determine whether nitric oxide (NO) is released in the tissues. The results are presented as mean ± s.e.m and comparisons made using a paired Student's t-test.

In the bladder dome, adrenergic neurone blockade with guanethidine (10μM) had no significant effect on the nerve-induced responses of the detrusor (n=8). Atropine significantly inhibited responses, with contraction reduced to 26.8 ± 5.7% of control ($p<0.05$, n=8). L-NNA caused a small but significant potentiation of responses to field stimulation (13.9 ± 2.59 %, $p< 0.05$, n = 8). Desensitisation of the P2X receptors with αβ-meATP (10μM) also caused a significant increase (75.66 ± 33.87%) in muscle contraction ($p<0.05$, n = 8). αβ-meATP in the presence of L-NNA failed to enhance contraction and significantly reduced responses to field stimulation (81.78 ± 2.96%, $p<0.05$, n = 8).

These data suggest that nerve transmission in the pig bladder dome is predominantly cholinergic, with an inhibitory mechanism mediated via nitric oxide. Purinergic mechanisms postjunctionally contribute a minor contractile component whilst prejunctionally ATP appears to cause the release of NO and the inhibition of muscle contraction.

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27P Ca^{2+} IMAGING AND ELECTROPHYSIOLOGICAL STUDIES OF EPIBATIDINE–INDUCED TRANSMITTER RELEASE FROM POSTGANGLIONIC SYMPATHETIC NERVE TERMINALS IN MOUSE ISOLATED VAS DEFERENS

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Previous studies have shown that ATP is released from postganglionic sympathetic nerve terminals in the aganglionic mouse vas deferens when neuronal nicotinic cholinergic receptors are activated (nAChRs; Brain *et al.*, 2001) but these studies lacked the spatial and temporal resolution to determine the kinetics of transmitter release. Here, the nAChR agonist epibatidine was used to characterise the kinetics of transmitter release when nAChRs on varicosities are activated.

Mice (Balb/C) were humanely killed. For the confocal experiments, the Ca^{2+} indicator Oregon Green 488 BAPTA-1 10 kDa dextran was loaded into nerve and smooth muscle cells of the vas deferens as previously described (Brain *et al.*, 2002).

During the slow, bath application of 100 nM epibatidine, the frequency of spontaneous Neuroeffector Ca^{2+} Transients (sNCTs), which are focal smooth muscle Ca^{2+} transients generated by the release of single packets of the transmitter ATP (Brain *et al.*, 2002), increased from 0.023 ± 0.003 Hz per cell (mean ± s.e.m; average over 10 minutes prior to drug addition) to 0.10 ± 0.03 Hz (average from 1 to 8 minutes time period following drug application; $P < 0.05$; Student's *t*-test; $n_c = 5$ cells; $n_v = 5$ vasa deferentia). The maximum frequency of spontaneous activity occurred at 4 ± 1 minutes ($n_c = 5$).

In separate experiments, the membrane potentials of smooth muscle cells were monitored using conventional intracellular recording techniques. Local, rapid application (picospritzing) of 1 μM epibatidine increased the frequency of occurrence of spontaneous excitatory junction potentials (sEJPs) in 19% of cells investigated ($n_c = 68$; $n_v = 18$) from 0.50 ± 0.05 Hz (during the 20 s prior to drug application) to 2.7 ± 0.2 Hz (during the 20 s following drug application; $P < 0.05$; $n_c = 13$; $n_v = 7$). The maximum frequency of sEJPs occurred at 6 ± 1 s ($n_c = 13$). In the presence of 100 nM saxitoxin (a Na^+ channel blocker), local application of 1 μM epibatidine also increased the frequency of sEJPs from 0.050 ± 0.09 Hz to 2.4 ± 0.3 Hz ($P < 0.05$; $n_c = 3$ cells; $n_v = 1$).

Epibatidine-induced NCTs occurred at a similar frequency to epibatidine-induced spontaneous Ca^{2+} transients in nerve terminals (0.09 ± 0.02 Hz per varicosity; Brain *et al.*, 2001). Given that several varicosities innervate each smooth muscle cell, it is clear that each spontaneous Ca^{2+} transient in a varicosity does not elicit transmitter release. The 6 s delay between the application of epibatidine and the maximum sEJP frequency may be due to the time taken for the drug to diffuse to its site of action or due to a delay between the activation of nAChRs and transmitter release. Given that sEJPs were elicited by epibatidine in the presence of saxitoxin, it is likely that nAChR–induced transmitter release does not depend on the generation of spontaneous nerve terminal action potentials.

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28P ACUTE HEMODYNAMIC EFFECTS OF CISAPRIDE AND ERYTHROMYCIN IN HEALTHY SUBJECTS

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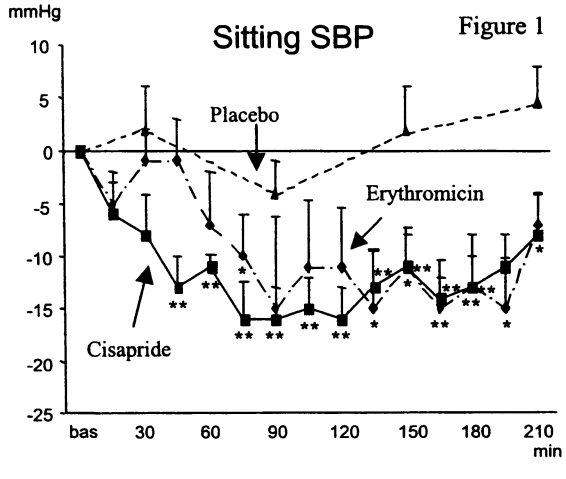
Aims. Although the pro-kinetic effects of erythromycin (E) and cisapride (C) are well characterised (Tonini, 1999), few data are available on their acute hemodynamic effects in humans. We studied the acute blood pressure (BP), heart rate (HR), and gastric emptying effects of oral E and C in healthy subjects in a randomised, double-blinded, crossover study with subsequent comparisons with a placebo (P) group.

Methods. Ten healthy subjects (age 54.9±7.5 years, mean±SEM) receiving E 250mg and C 10mg and 26 age- and sex-matched healthy subjects (age 48.5±5.0 years) receiving P were studied. Sitting and standing BP and HR were measured (at 15min intervals in the E/C group and at 60min intervals in the P group) before and after dosing. The pro-kinetic activity was assessed by the paracetamol absorption kinetics method (Heading *et al.*, 1973).

Results. Compared to P, both E and C caused a profound and sustained reduction in systolic BP (mean sitting ΔSBP for E -8±4 mmHg, $p<0.05$; mean sitting ΔSBP for C -10±4 mmHg, $p<0.01$, Figure 1, * $p<0.05$, ** $p<0.01$; mean standing ΔSBP for C -10±4 mmHg, $p<0.05$, ANOVA for repeated measurements). No significant differences were detected in diastolic BP and HR. The hypotensive effects of E and C were similar and independent of age.



There was no significant difference between E and C in paracetamol lag time (tlag 0.15±0.02 vs. 0.13±0.02 hours, respectively), absorption half-life (t½ abs 0.09±0.02 vs. 0.06±0.02 hours), and tmax (0.42±0.10 vs. 0.29±0.05 hours).

Conclusions. A single oral dose of E and C induces significant acute hypotensive effects, which are not age-related. E and C have similar effects on gastric emptying.

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29P REGIONAL HAEMODYNAMIC EFFECTS OF THE PPAR γ ACTIVATOR, G1262570, IN CONSCIOUS RATS

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Thiazolidinediones (TDZ) are high affinity ligands for PPARγ which, in addition to their anti-diabetic effects, have cardiovascular actions (see Bishop-Bailey (2000) for review). G1262570, is a novel, N-aryl tyrosine, non-TDZ, activator of human PPARγ, and we have now assessed its regional haemodynamic actions in conscious male, Sprague-Dawley rats (300-350g), using a dosing regime maximally efficacious for glucose lowering in diabetic rodents, but which is significantly above that required for treatment of patients with type 2 diabetes mellitus (unpublished data, GSK).

Animals were chronically instrumented with renal, mesenteric and hindquarters pulsed Doppler probes, at least 14 days before placement of i.a. and i.v. catheters. All surgery was carried out under anaesthesia (fentanyl and medetomidine, 300 $\mu g\ kg^{-1}$ of each i.p., supplemented as required). Post-surgery, anaesthesia was reversed and analgesia was provided with atipamezole and nalbuphine, respectively (1 mg kg^{-1} of each s.c.). In the first experiment, animals (n = 9) were infused with G1262570 (2 mg ml^{-1}, 0.4 ml h^{-1}) continuously for 2h, twice daily (between 07.15 and 09.45 h and between 13.30 and 15.30 h) for 4 days. Another group (n = 9) was infused with glycine-based vehicle over the same periods. In the second experiment, rats were given G1262570 (as above) together with the β_2-adrenoceptor antagonist, ICI 118551 (0.2 mg kg^{-1}, 0.1 mg kg^{-1} h^{-1}; n = 8), or its vehicle (saline; n = 8).

In experiment 1, prior to the first administration of G1262570, resting values (mean ± s.e.mean) were: heart rate (HR): 338 ± 7 beats min^{-1}; mean arterial blood pressure (MAP): 103 ± 2 mm Hg; renal, mesenteric, hindquarters Doppler shift (RDS, MDS, HDS, respectively): 8.9 ± 0.8, 10.0 ± 0.8 and 4.1 ± 0.3 kHz; renal, mesenteric, hindquarters vascular conductance (RVC, MVC, HVC, respectively): 86 ± 7, 98 ± 9 and 40 ± 3 (kHz mm Hg^{-1} 10^3). Over the 4 days, there were progressive cardiovascular effects of G1262570, such that, after the last infusion on day 4, there was significant ($P \leq 0.05$, Friedman's test) tachycardia (+46 ± 10 beats min^{-1}), fall in MAP (-14 ± 3 mm Hg), and hyperaemic vasodilatation in the hindquarters (HDS, +28 ± 7%; HVC, +49 ± 9%). All these effects were significantly (Mann Whitney U test) different from vehicle, which caused no consistent changes. The haemodynamic effects of G1262570 were not influenced by ICI 118551. After the last infusion on Day 4, there were similar increases in HR (+23 ± 14, 18 ± 14 beats min^{-1}; falls in MAP (-19 ± 4, -21 ± 4 mm Hg) and increases in HVC (60 ± 8, 69 ± 15%) in animals given G1262570 in the absence and presence of ICI 118551 respectively. Thus, the fall in MAP observed with G1262570 was associated with marked hindquarters vasodilatation that did not involve β_2-adrenoceptors. This effect may contribute to the increase in whole body glucose disposal produced by treatment with PPARγ ligands. Any putative contribution from insulin sensitisation to the haemodynamic effects of G1262570 remains to be investigated.

This work was supported by GSK

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30P THE CARDIOVASCULAR ACTIONS OF AMPHOTERICIN B, A POLYENE ANTIFUNGAL ANTIBIOTIC, IN ANESTHETIZED RATS

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Amphotericin B (AmpB) is a polyene antifungal antibiotic that preferentially binds to ergosterol, a fungal cell membrane constituent. AmpB disrupts the osmotic integrity of the fungal cell membrane leading to cellular death but also may interact with cholesterol moieties on mammalian cells (Andreoli, 1974). While clinical doses of AmpB (0.3-1.5 mg/kg) have a broad range of activity against most pathogenic fungi, AmpB may be associated with adverse cardiovascular events including hypotension and irregular heart rhythms (Cleary *et al.*, 1993).

These studies characterised the actions of cumulative AmpB doses (0.3 - 3 mg/kg; n, 5) to a single dose (0.7 mg/kg; n, 5) on the blood pressure (BP), heart rate (HR) and electrocardiogram (ECG) of pentobarbital anaesthetised, ventilated male CD-1 rats (300-350 g). AmpB was administered at 10 min intervals as a 45 sec slow bolus dose via the jugular vein. Mean arterial blood pressure and a Lead II ECG were digitally recorded online.

AmpB reduced BP dose-dependently. At a dose of 1 mg/kg, BP was reduced from 101 ± 13 to 68 ± 17 mmHg ($p<0.05$). AmpB also produced a dose-related prolongation of the PR, QRS and Q-aT intervals of the ECG suggesting that it may block cardiac ion channels. The Q-aT interval was most sensitive measure to AmpB. At the 1 mg/kg dose, the Q-aT interval was prolonged to 103 ± 11 msec from a pre-drug value of 57 ± 6 msec ($p<0.05$). When AmpB was administered as a single dose of 0.7 mg/kg, all animals experienced arrhythmias. The difference in response between single and cumulative dosing suggests that AmpB produces marked cumulative cardiovascular actions consistent with a narrow therapeutic window. Analysis of animal ECG's showed that all animals administered AmpB at doses greater than 0.3 mg/kg experienced premature ventricular contractions (PVC). The log-normalised incidence of PVC's was 1.3 ± 0.1 and 1.2 ± 0.1 for 0.7 and 3.0 mg/kg AmpB, respectively. Animals given a single 0.7 mg/kg dose had an arrhythmic incidence of 0.8 ± 0.1; thus, PVC arrhythmia incidence was not dose-related in this species.

These studies show that AmpB produces a dose-related effect on the heart and that the cardiovascular depressant actions may result from direct actions on the heart. The changes in ECG measures suggest an interaction of AmpB with ion channels that modulate the cardiac action potential. Thus AmpB may potentially block cardiac ion channels. These studies provide an indication as to the potential mechanism for adverse event development (proarrhythmia) associated with the administration of AmpB during antifungal therapy in the clinic.

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31P INHIBITION OF HUMAN PLATELET ACTIVATION BY A SHORT-ACTING NITRIC OXIDE DONOR DRUG IS SUBSTANTIALLY PROLONGED BY ALBUMIN AND PLASMA THIOLS

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Nitric oxide (NO) is a labile free radical messenger with potent antiplatelet activity (Radomski *et al*, 1987). Under physiological conditions, the half-life of NO is short, suggesting that its bioactivity is rapid, and only significant to tissues within close diffusable range of the site of production. However, recent evidence indicates that the activity of NO may be prolonged through formation of an S-nitrosothiol 'reservoir' (Stamler *et al*, 1992). Here, we tested the ability of plasma thiols to prolong the antiplatelet action of a short acting NO-donor drug, diethylamine diazeniumdiolate (DEA/NO), via the formation of an S-nitrosothiol reservoir.

Venous blood was obtained from healthy volunteers aged 20-40 (14 male; 8 female). Platelet rich plasma (PRP) was derived by centrifugation of whole blood (120 *g*; 10 min), and washed platelets (WP) prepared by centrifugation of PRP (1200 *g*; 10 min) in the presence of PGI_2 (300 ng/ml) prior to resuspension in Tyrode buffer.

Pre-incubation of PRP and WP with DEA/NO for 1 min inhibited collagen (2.5μg/ml)-induced platelet aggregation by 82 ± 5% (n=8) and 91 ± 2% (n=8) respectively (mean ± s.e.mean). However, after a 30 min pre-incubation with DEA/NO, collagen-induced aggregation in WP was almost completely restored (5 ± 3% inhibition; n=8), but aggregation in PRP remained markedly inhibited (72 ± 7%; P<0.001; unpaired *t* test; n=8). At this timepoint, DEA/NO-derived NO was no longer detectable (< 10 nM) with an NO-electrode in either PRP or WP.

Reconstitution of WP with physiologically relevant concentrations of human serum albumin (HSA; 1%), but not low molecular weight (LMW) thiols, glutathione (5 μM), cysteinyl-glycine (10 μM) or cysteine (10 μM), partially restored DEA/NO-mediated inhibition of platelet aggregation (39 ± 10%; n=8) after 30 min. However, inhibition of platelet aggregation at this time was fully restored by co-incubation of HSA with any of the LMW thiols. Furthermore, this inhibition was reversed by the addition of the NO-scavenger, oxy-haemoglobin (10 μM).

S-nitrosothiol concentration was determined using an established method (Marley *et al*, 2000). In PRP, incubation of DEA/NO (2 μM) caused a time-dependent increase in S-nitrosothiol concentration to a maximum of 74 ± 15 nM (n=6) after 10 min. In HSA reconstituted WP, maximal S-nitrosothiols were detected after 30 min (46 ± 9 nM; n=6), while addition of glutathione (5 μM) increased S-nitrosothiol formation by approximately two-fold (105 ± 19 nM; P<0.001; n=6).

These results clearly demonstrate that LMW thiols play an important role in both the formation and activation of an S-nitrosothiol reservoir that significantly prolongs the action of drug-derived NO. Furthermore, these data may have important implications for the antiplatelet action of endothelium-derived NO.

This work was funded by the British Heart Foundation.

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32P ALTERED DEGREE OF ACTIVATION AND STEROID INHIBITION IN LEUKOCYTES FROM ANNEXIN 1 NULL MICE

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Using conventional gene targeting technology we have recently raised a mouse strain that lacks the annexin 1(Anx-A1) gene (Hannon *et al.*, 2002*)*. We have used this tool to investigate the role of this protein in mediating neutrophil activation as well as the effect glucocorticoid action has on phagocytosis of IgG and reactive oxygen species released in macrophages.

Groups (n=4-5) of male or female wild type or Anx-A1 null mice weighing 25-30g were anaesthetised with halothane and blood collected by cardiac puncture. Other mice were killed and peritoneal cavities washed with sterile saline buffer. Blood aliquots (200 μl) were incubated with platelet activating factor (PAF; 0.3-3 μM) or formyl-peptide (fMLP; 1-10 μM) and stained with rat anti-mouse CD11b mAb (clone 5C6; 5 μg/ml) for 1 h at 4°C. Second antibody staining and flow cytometry analysis was done as described (Harris *et al.* 1995). Peritoneal cells (>80% macrophages, MØ) were suspended at $5x10^6$ per ml for the experiments of phagocytosis (Getting *et al.*, 1997), whereas $2.5x10^6$ per ml cells loaded with di-hydrorodhamine123 (Molecular Probes, Eugene, OR) prior to flow cytometry analysis for reactive oxygen species generation (ROS; Euzger *et al.*, 1999). Hydrocortisone (10 μM) was incubated for 1 h prior to experimentation. Data are mean ± SEM of >3 experiments performed in duplicate or triplicate observations, and statistical differences were analysed by Mann-Whitney U test (P<0.05 was taken as significant). Blood neutrophils prepared from Anx-A1 null mice had lower CD11b levels on their cells surface: 68 ± 4 and 99 ± 7 fluorescence units for -/- and +/+ mice, respectively (n=6, P<0.05). Both PAF and fMLP caused a significant increase in CD11b expression in both phenotypes, however there was a more pronounced effect in the Anx-A1 null mice. For example, fMLP increased CD11b levels by 16±1%, 49±3% and 66±7% in wild type mice, at 1, 3 and 10 μM respectively. The corresponding values for Anx-A1 null mice were 35±3%, 99±10% and 92±7% (n=4, P<0.05 at each fMLP concentration). Similar findings were obtained with PAF. No differences were observed between peritoneal MØ of wild type and Anx-A1 null mice with respect to IgG phagocytosis and ROS production, however the latter cell type was insensitive to the inhibitory effect of hydrocortisone. For instance, the steroid suppressed IgG phagocytosis from maximum by 19±3% in control cells whilst it was inactive in Anx-A1 null MØ (n=4, P<0.05). For ROS production, hydrocortisone produced 32±2% and 7±1% of inhibition in +/+ and -/- cells (n=3 experiments).

In conclusion, this data indicates the existence of a tonic inhibitory role for endogenous Anx-A1 in controlling neutrophil and MØ activation.

This work was supported by a PhD studentship of the Nuffield Foundation (Oliver Bird Fund).

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33P FURTHER STUDIES ON THE MECHANISM OF BRADYKININ-INDUCED BRONCHOCONSTRICTION IN ACTIVELY SENSITISED BROWN NORWAY RATS

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We have shown that the bronchoconstrictor response to bradykinin (BK) is maximally potentiated 24 h after ovalbumin (OA) challenge in actively sensitised (AS) Brown Norway (BN) rats. The enhanced response, which is entirely B_2 receptor mediated, is a consequence of a non-cholinergic component which does not involve mast cells or prostanoids (Ellis *et al.*, 2002). The aim of the present study was to characterise the non-cholinergic component of this response by investigating the involvement of leukotrienes, tachykinins and the peptidases, angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP).

Male BN rats (250-300g) were sensitised to OA, anaesthetised using sodium pentothal (70 $mgkg^{-1}$, i.p.) and prepared for recording of lung function, blood pressure and heart rate as previously described (Hannon *et al.*, 2001). Bronchoconstrictor responses to bolus i.v. injections of BK and methacholine (MCh) were established in AS animals 24 h after i.t. challenge with saline (0.2 ml) or OA (0.3 $mgkg^{-1}$). Two doses of BK were administered (100 & 300 μgkg^{-1}) with 15 min between doses, followed 10 min later by two doses of MCh (10 & 20 μgkg^{-1}) with 5 min between doses. Data (mean ± s.e.mean) were analysed by Student's *t* test taking a *P* value <0.05 as significant.

Neither the cysteinyl-leukotriene receptor antagonist, iralukast, (5 $mgkg^{-1}$ i.v., 15 min before BK, n=4) nor the 5-lipoxygenase inhibitor, CGS8515, (25 $mgkg^{-1}$ p.o., 2 h before BK, n=4) inhibited the potentiated response to BK 24 h after OA challenge, thus excluding a leukotriene role in this response.

Substance P did not cause bronchoconstriction in this animal model. However, a neurokinin A (NKA) analogue, [β-Ala^8]NKA(4-10) (100 μgkg^{-1}), caused bronchoconstriction which was also augmented 24 h after OA challenge mirroring that of BK. A NK_2 receptor antagonist, SR48968, (1 $mgkg^{-1}$ i.v., 15 min before BK, n=4), inhibited the response to [β-Ala^8]NKA(4-10) by 82.7 ± 1.3% (P<0.01, n=4) but had no significant effect on the response to BK 24 h after OA challenge, thus eliminating a role for tachykinins in this response.

Both NKA and BK are inactivated by ACE and NEP, which are present in lung tissue (Schilero *et al.*, 1994). In the presence of captopril (ACE inhibitor; 2.5 $mgkg^{-1}$ i.v.) and thiorphan (NEP inhibitor; 2.5 $mgkg^{-1}$ i.v.), ~5 and ~100-fold decreases of the [β-Ala^8]NKA(4-10) and BK doses respectively, were required in both saline- and OA-challenged animals (n=4) to achieve the same bronchoconstrictor response as seen in the absence of inhibitors. Thus, potentiation of BK is still seen after peptidase inhibition and downregulation of peptidase activity after allergen challenge does not account for the potentiation of the BK response.

The present data show that the bronchoconstrictor response to BK augmented 24 h following allergen challenge is not mediated by leukotrienes or tachykinins, and is not potentiated as a consequence of decreased peptidase activity in the lungs. A direct bronchoconstrictor effect on the airway smooth muscle mediated by B_2 receptors cannot be excluded.

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34P EFFECTS OF FOLIC ACID SUPPLEMENTATION ON INFLAMMATORY AND THROMBOGENIC MARKERS IN CHRONIC SMOKERS. A RANDOMISED CONTROLLED TRIAL

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Aims. Cigarette smoking may induce pro-inflammatory and pro-thrombotic changes (Wallenfeldt *et al.*, 2001; Miller *et al.*, 1998). It is not known whether these abnormalities are caused by increased homocysteine levels. We investigated whether lowering homocysteine by folic acid supplementation might reduce the plasma concentration of inflammatory and thrombogenic markers in chronic smokers.

Methods. Twenty-four healthy cigarette smokers (age 37.8±2.5 years, mean±SEM) were randomly assigned to four weeks of folic acid 5 mg/d or placebo. The following parameters were measured before and after treatment: 1) markers of inflammation (C-reactive protein, CRP; white cell count, WCC); 2) blood coagulation screen (Activated Partial Thromboplastin time Ratio, APTR; International Normalized Ratio, INR); 3) pro-thrombotic markers (fibrinogen; factor VIII coagulant activity, VIII:C; von Willebrand factor, vWF; D-dimer).

Results. A significant reduction in homocysteine concentrations was observed in the group receiving folic acid (10.8±0.6 vs. 8.2±0.5 μmol/L, p<0.001). This was associated with a significant reduction (Mann Whitney U test) in plasma fibrinogen (3.1±0.3 vs. 2.9±0.1 g/L, p<0.05) and D-dimer (102±44 vs. 80±26 μg/L, p<0.05) concentrations. By contrast, no significant changes were observed in CRP (2.2±0.7 vs. 1.7±0.7 mg/L), WCC (7.2±0.5 vs. 6.8±0.5 10^9 cells/L), APTR (0.91±0.02 vs. 0.93±0.02), INR (0.92±0.01 vs. 0.91±0.01), vWF (103±8 vs. 102±9 U/dL), and VIII:C (120±8 vs. 107±8 U/dL, p<0.01) levels. Changes in folic acid plasma concentrations were significantly and negatively correlated with fibrinogen (r=-0.48, p=0.01) but not D-dimer (r=-0.15, p=0.5) changes. Changes in plasma homocysteine concentrations did not correlate with changes in either fibrinogen or D-dimer. No significant changes in homocysteine, inflammatory and thrombogenic markers were observed in the placebo group.

Conclusions. Short-term folic acid supplementation had no significant effects on inflammatory markers but induced a significant reduction in plasma fibrinogen and D-dimer concentrations in healthy young chronic smokers. Thus, folic acid might have an anti-thrombotic effect in this high-risk group independent of the homocysteine lowering effect.

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35P CANNABIDIOL DOES NOT AFFECT VASORESPIRATORY RESPONSES TO CAPSAICIN AND ANANDAMIDE IN ANAESTHETISED RATS

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Cannabidiol (CBD) is a non-psychoactive constituent of *cannabis sativa*. Capsaicin, the pungent chemical in chilli peppers, is an agonist at vanilloid VR-1 receptors which have been implicated in nociceptive/cardiovascular-respiratory reflexes (Smith *et al.*, 2001). Anandamide, an endogenous cannabinoid, has been reported to activate VR-1 receptors *in vitro* and *in vivo*. (Gauldie *et al.*, 2001; Smart *et al.*, 2000). The aim of the study was to investigate whether CBD affects the vasorespiratory actions of capsaicin or anandamide in anaesthetised rats.

Male Wistar rats (353g: 264-455g, mean weight : range, n=29) were anaesthetised with pentobarbitone 60mg kg^{-1} i.p.; anaesthesia was maintained by either injecting or continuously infusing 6mg pentobarbitone hourly (i.v., jugular vein); rectal temperature was maintained at 36-37°C by a blanket. A tracheal cannula was inserted and airflow measured using an electrospirometer (MacLab). The right carotid and femoral arteries were cannulated for measuring mean arterial blood pressure (BP; MacLab) and drug administration respectively.

Neither CBD (0.32 - 320 nmoles) nor vehicle (10% methanol) affected basal cardio-respiratory parameters. Anandamide (n=11) and capsaicin (n=19) induced dose-dependent hypotensive hyperventilatory responses (Figure 1) that were unaffected (P>0.05 pre-post CBD) by pre-treatment with CBD (32 nmoles i.a., 60-180s before agonist).

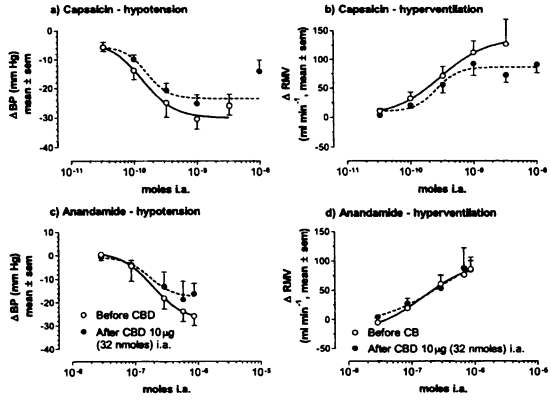

Figure 1. Changes in mean blood pressure (BP) and respiration (respiratory minute volume; RMV) evoked by capsaicin (a,b – n = 5-19) or anandamide (c,d n = 3-11) before and after CBD 10μg (32 nmoles) given i.a. 1-3 mins before each dose of agonist. Basal values: BP 120 ± 3 mm Hg; RMV 147 ± 8 $ml.min^{-1}$ - mean ± SEM, n = 29.

The data indicate that CBD has no action on blood pressure or respiration in rats anaesthetised with pentobarbitone, nor does it significantly affect the vasorespiratory responses evoked by anandamide and capsaicin, acting at VR-1 receptors.

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36P DIFFERENTIAL MODULATION OF IMMEDIATE EARLY GENE EXPRESSION AND GABAERGIC NEURONE ACTIVITY IN THE NUCLEUS ACCUMBENS IN RESPONSE TO Δ9- TETRAHYDROCANNABINOL ADMINISTRATION

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Administration of Δ9-Tetrahydrocannabinol (THC), the primary psychoactive cannabis constituent, has been shown to produce effects indicative of both aversion and reinforcement in rodents, depending on the dose employed (Valjent and Maldonado, 2000). We hypothesised that these effects on behavioural output may be associated with differential patterns of activation of the nucleus accumbens (NAc), a neural substrate implicated in these effects. We therefore employed several complementary immediate early genes (IEGs) to map regional neural activation following acute administration of a range of doses of THC in the rat. In addition, parvalbumin mRNA expression was used as a marker of GABAergic interneuron activity.

Rats (n = 8 / group) were administered THC (0.01 – 5.0 mg/kg i.p.) or vehicle as control, and were euthanased 75 minutes post-injection. The regional expression of mRNA encoding the IEGs *c-fos, zif-268, nur77, fosB, c-jun,* and *junB* and parvalbumin were quantified using *in situ* hybridisation of radiolabelled oligonucleotide probes to coronal brain sections. Following subsequent densitometric analysis, data was analysed by one –way ANOVA, followed when appropriate by the Newman Keuls post-hoc test. In all cases statistical significance was defined as $p < 0.05$.

Results showed that THC – induced increases in IEG mRNA expression followed complex pattern of dose – dependent regulation. For example, in the shell portion of the NAc, the expression of *FosB* mRNA was increased in a linear fashion according to dose of THC administered, reaching significance at 1 and 5 mg/kg compared to control values (Control: 0.029 ± 0.005; 1 mg/kg THC: 0.058 ± 0.007; 5 mg/kg THC: 0.062 ± 0.009), whereas increases in *zif-268* mRNA were significantat 1.0 mg/kg THC but not at 5 mg/kg THC when compared to control values (Control: 0.147 ± 0.025; 1 mg/kg THC: 0.165 ± 0.021; 5.0 mg/kg THC: 0.146 ± 0.008). Expression of parvalbumin mRNA showed a contrasting pattern of regulation, as 0.01 mg/kg THC produced a small non-significant increase compared to control values, whereas the larger dose of 5 mg/kg THC decreased expression compared to 0.01 mg/kg THC but not to controls (Control: 0.030 ± 0.003; 0.01 mg/kg THC: 0.038 ± 0.006; 5.0 mg/kg THC: 0.024 ± 0.005).

These results suggest that activity in distinct accumbal neuronal populations may be differentially affected according to the dose of THC administered. These effects may relate to dose-dependent changes in activity of innervating structures (prefrontal cortex and hippocampus) also detected in this investigation, coupled with local effects of THC on control of presynaptic glutamate (Shen *et al.*,1996) and GABA (Katona *et al.*, 1999) release. These processes may underlie the reported biphasic dose effects of THC on reinforcement and aversion related behaviour.

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37P CORRELATION OF CONVULSANT THRESHOLDS TO INFUSED BICUCULLINE AND 4-AMINOPYRIDINE WITH EXTENT OF PENTYLENETETRAZOLE (PTZ) KINDLING IN MICE

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Certain newer anticonvulsant drugs, e.g. levetiracetam, have a greater action in kindled (or epileptic) animals than control animals. In order to explore this phenomenon further we examined the model of PTZ kindling in greater detail by measuring sensitivity to infused bicuculline or 4-aminopyridine (4-AP) at the end of the kindling process, and attempted to correlate this measure with the rate, or extent, of kindling in each individual animal.

Groups of 14-20 male NMRI mice (22 g at the start of the experiment) were individually identified and kindled by repeated injection of PTZ (37 mg/kg, IP) Mondays, Wednesdays and Fridays for 17 days. Control groups received saline injections. 2 days after the last PTZ injection all mice were infused via a lateral tail-vein with bicuculline (0.05 mg/ml, 1 ml/min) or 4-AP (6 mg/ml, 0.7 ml/min) until first clonic convulsion was seen (around 25s in control animals). The threshold dose of infused convulsant was then calculated (Watson and Little, 1997). Results were expressed as mean ± SE, and compared with Student's t-test. Finally, the threshold value for each mouse was plotted against the number of clonic convulsions the individual mouse exhibited during the kindling processes (extent of kindling), and correlation performed by Spearman's rank correlation.

The results in table 1 show that kindled animals had significantly higher thresholds to both bicuculline and to 4-AP. A significant positive correlation between extent of kindling development and threshold was only seen with 4-AP ($p < 0.05$), not with bicuculline ($P > 0.1$). Body weights were not significantly different between the kindled and control groups ($P > 0.1$, data not shown).

Whilst it may seem surprising that there are elevated convulsion thresholds in kindled mice, this has been shown previously after bicuculline kindling by Nutt et al., (1982). Watson and Little (1997) also demonstrated increased 4-AP thresholds during the hyper-excitability of the ethanol withdrawal syndrome in mice. The present experiments demonstrated that the changes in sensitivity to infused 4-AP are likely to be related to the progressive development of PTZ kindling – or the behavioural expression of these seizures. The changes in sensitivity to bicuculline did not correlate with the number of observable behavioural seizures, but could be related to the number of PTZ injections, or alternatively the threshold changes may be a more rapid process, occurring after only a few injections of PTZ. Further investigations would be needed to examine these possibilities.

These experiments demonstrate the value of a correlative approach to experimental design, and indicate that there are measurable and temporally different changes occurring during PTZ kindling. Such knowledge will allow the further investigation of processes underlying the development of kindling, and selectivity of drug actions on these processes. Whether similar processes also occur in other kindling models, or other models of epileptogenesis, has yet to be discovered.

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Table 1: Threshold convulsive doses (mean ± s.e. as mg/kg) of bicuculline and 4-Aminopyridine measured 2 days after PTZ kindling in mice. (* $P < 0.05$ cf control group)

Infused Drug	Theshold in control group (mg/kg)	Theshold in kindled group (mg/kg)
Bicuculline	0.68 ± 0.02	0.77 ± 0.02 *
4-Aminopyridine	60.8 ± 1.9	66.2 ± 2.1 *

38P DIAZEPAM TOLERANCE DEVELOPMENT AND mRNA EXPRESSION OF COMPONENTS OF THE NMDA RECEPTOR COMPLEX

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The mechanisms underlying benzodiazepine (BZD) tolerance remain unclear. Evidence supports a role for glutamate receptors in tolerance to BZDs. Thus NMDA receptor antagonists attenuate the development of tolerance to the sedative effects of diazepam (DZP) (File & Fernandes, 1994).

The aim of these studies was to determine whether there were regionally selective alterations in the levels of mRNA for the NMDA receptor subunits NR1 and NR2A and for PSD-95, a protein associated with the postsynaptic density at glutamatergic synapses, during the development of tolerance to DZP. Since learning-related mechanisms may contribute to tolerance development, studies were conducted in groups of rats where experience of DZP was either repeatedly paired with the test apparatus (contingent group; CG) or not (non-contingent group; NCG).

Male hooded Long Evans rats (250-300g) were randomly assigned to one of three treatment groups (n=18/group) each receiving 3 daily injections: control (con; sesame oil vehicle), acute drug (ac; two days vehicle, then DZP 15mg/kg s.c.), and subacute drug (sub; three days DZP 15mg/kg s.c.). On the first two days of testing, half of the rats in each treatment group (n=9) were placed in a holeboard apparatus one hour post injection for ten minutes (CG), and the other half received injections after the holeboard experience (NCG). On the third day, all groups of rats were tested in a holeboard apparatus for a ten minute period one hour after the appropriate injection when measurements of locomotor activity and head dips were made. Rats were then killed and the brains removed.

Behavioural data were analysed using two-way ANOVA followed by the Tukey post hoc test. Quantitative *in situ* hybridisation was carried out using radiolabelled oligonucleotide probes to 20μM brain tissue sections. Following subsequent densitometric analysis, data were analysed using one-way ANOVA followed by the Newman Keuls post-hoc test where appropriate.

In both the CG and NCG, acute treatment with DZP reduced ($p<0.05$) locomotor activity which returned to control levels in the subacute treatment group (total distance travelled (cm; mean±SEM): CG: con=4574±548, ac=2307±411, sub=5317±638; NCG: con=5494±406, ac=1570±303, sub=5577±547). There was no significant difference in the behaviour of the CG and NCG on the third day of treatment and thus the groups were pooled for neurochemical analysis. NR2A subunit mRNA levels were significantly reduced ($p<0.05$) in the pyramidal cell layer of the CA1 hippocampal field (con=0.378±0.012, sub=0.337±0.010). PSD-95 mRNA levels were significantly inceased in the motor cortex (con=0.146±0.006, sub=0.170±0.007) and the pyramidal cell layer of the CA3 hippocampal field (con=0.233±0.009, sub=0.250±0.006) in the subacute treatment group compared with the control group. Subacute DZP treatment did not change NR1 mRNA levels in these regions. These results suggest that tolerance to the sedative effects of DZP is accompanied by regionally selective alterations in the NMDA receptor complex, and support a role for the involvement of glutamatergic processes in BZD dependence.

L.C. holds a Caledonian PhD Scholarship

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39P DIAZOXIDE IN THE TREATMENT OF SCHIZOPHRENIA: NOVEL APPLICATION OF POTASSIUM CHANNEL OPENERS IN THE TREATMENT OF SCHIZOPHRENIA

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Schizophrenia is a very common disorder, affecting 1% of the world population (Mohammadi & Akhondzadeh, 2001). For most of this century the causes of schizophrenia have been largely unknown. The relative ineffectiveness of dopamine antagonists to treat some symptoms of schizophrenia has promoted many investigators to postulate the involvement of the neuronal system in the pathophysiology of this disease. It has been suggested that the dopamine-coupled ATP-sensitive channels may function by hyperpolarizing cells during metabolic stress, a function that may be disrupted in people with schizophrenia (Pongs, 1992; Lin et al., 1993). Therefore application of potassium channel openers/activators may be beneficial in schizophrenia (Allen & Etcheberrigaray, 1998). Diazoxide is a potassium channel opener from benzothiadiazine derivative related to the thiazide diuretics (Lin, et al., 1993). The purpose of the present investigation was to access the efficacy of diazoxide, a potassium channel opener, as an adjuvant agent in the treatment of schizophrenia.

42 patients who met the DSM IV (Diagnostic and Statistical Manual of Mental Disorders (4th edn)) criteria for chronic schizophrenia completed the study. Patients were allocated in a random fashion, 21 to haloperidol 20 mg/day plus diazoxide 200 mg/day and 21 to haloperidol 20 mg/day plus placebo for a 8-week, double-blind, placebo-controlled study. The minimum score of 60 on positive and negative syndrome scale (PANSS) was required for entry into the study (Kay et al., 1987). Patients were assessed by a psychiatrist at baseline and after 2, 4, 6 and 8 weeks after the medication started. The mean decreases in PANSS score from baseline was used as the main outcome measure of response of schizophrenia to treatment. Repeated measures analysis of variance (ANOVA) with a two-tailed post hoc Tukey mean comparison test was performed in the change from baseline for positive and negative scores. To compare the outcome of two groups in the same week, an unpaired Student's t-test with a two-sided P value was used. Results are presented as mean ± S.E.M. Differences were considered significant with $P<0.05$.

Although both protocols significantly decreased the score of the positive, negative and general psychopathological symptoms over the trial period, the combination of haloperidol and diazoxide showed a significant superiority over haloperidol alone in the treatment of positive (the difference between the two protocols was significant at week 8, $t=1.993$, d.f. $=40$, $p=0.05$) and general psychopathology symptoms (the difference between the two protocols was significant at week 8, $t=2.396$, d.f. $=40$, $p=0.0214$) as well as PANSS total scores (the difference between the two protocols was significant at week 8, $t=3.501$, d.f.$=40$, $p=0.0012$). No significant differences were observed between the two protocols on the negative scores.

The results of this study present a novel application for potassium channel openers/activators in the neuropsychiatric disorders and diazoxide may be an effective adjuvant agent in the management of schizophrenia.

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40P SELEGILINE IN THE TREATMENT OF ATTENTION DEFICIT HYPERACTIVITY DISORDER IN CHILDREN: A DOUBLE BLIND RANDOMIZED AND CONTROLLED TRIAL

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Attention deficit hyperactivity disorder (ADHD) is a common disorder of childhood that affects 6% to 10% of school-aged children. Conventional stimulant medications are recognized by both specialists and parents as a useful symptomatic treatment. Nevertheless, approximately 30% of ADHD children treated with them do not respond adequately or cannot tolerate the associated adverse effects. Such difficulties highlight the needs for alternative safe and effective medications in the treatment of this disorder (Goldman et al., 1998). Selegiline is a type B Monoamine Oxidase (MAO-B) inhibitor that is metabolized to amphetamine and methamphetamine, stimulant compounds that may have utility in the treatment of ADHD (Jankovic, 1993). We undertook this study to further evaluate, under double-blind and controlled conditions, the efficacy of selegiline for ADHD in children.

A total of 24 children (18 boys and 6 girls) between the ages of 4-9 (mean ± SD was 7.36 ± 1.46) with ADHD as defined by DSM IV (Diagnostic and Statistical Manual of Mental Disorders (4th edn)) were randomized to selegiline or methylphenidate dosed on a age and weight-adjusted basis at selegiline 5 mg/day (under 5 years) and 10 mg/day (over 5 years) (group 1) and methylphenidate 1 mg/kg/day (group 2) for a 4 week double blind clinical trial. The principal measure of the outcome was the Teacher and Parent ADHD Rating Scale (Dupaul, 1991). Patients were assessed by a child psychiatrist at baseline, 14 and 28 days after the medication started. Repeated measured analysis of variance with a two-tailed post-hoc Tukey mean comparison test was performed on the change in Teacher and Parent ADHD Rating Scale scores from baseline. To compare the reduction of score of Teacher and Parent ADHD Rating Scale at week 4 compared to baseline, an unpaired two-sided Student's t-test was used. Results are presented as mean ± SEM differences were considered significant with $P\leq0.05$. To compare the dropout rate a two-sided Fisher's exact test was performed.

A repeated measures analysis of variance showed a significant effect of both protocols on the Parent and Teacher ADHD Rating Scale. No significant differences were observed on the reduction of scores of the Parent and Teacher ADHD Rating Scale at week 4 compared to baseline in the two groups ($t=0.26$, d.f. = 17, $P= 0.79$; $t=0.28$, d.f. = 17, $P= 0.78$ respectively). In the selegiline and methylphenidate groups the number of dropouts were 1, and 4, respectively. Although the number of dropout in the methylphenidate group was higher than the selegiline group, no significant difference was observed in the two groups ($P = 0.31$)

The results of this study must be considered preliminary, but they do suggest that selegiline may be beneficial in the treatment of ADHD.

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