1P ROLE OF ENDOTHELIN-1 IN MODULATION OF CARDIAC FUNCTION IN ENDOTOXAEMIA

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It is now known that the endothelium plays a pivotal role in the peripheral vascular dysfunction in sepsis and septic shock, with the release of vasoactive mediators, including endothelin-1 (ET-1) and nitric oxide (NO) (Warner & Kelm, 1996, Thiemermann *et al* 1997). The role of such mediators in the pathogenesis of myocardial dysfunction in septic shock remains controversial. The aims of the current study were to determine the effects of ET-1 on myocardial function using the isolated ejecting heart model.

Male Wistar rats (250-300g) were randomized to pre-treatment with vehicle (DMSO 4.5% i.p), the ET_A receptor antagonist ABT-627 (10mg^{-1}kg^{-1}i.p), or the ET_B receptor antagonist, ABT-192621 (20mg^{-1}kg^{-1}i.p). 60 minutes later, animals were randomised to control or endotoxaemic (LPS 20mg^{-1}kg^{-1}i.p) groups. 4 hours later, animals were anaesthetised with sodium pentobarbitone (100mg^{-1}kg^{-1}i.p) and killed by cervical dislocation. Hearts were rapidly removed and immersed in ice-cold gassed [95%O_2:5%CO_2] Krebs' solution. Aortae were cannulated, and hearts perfused with gassed Krebs' at 37°C in Langendorff mode for 30 mins. Perfusion was then switched to the ejecting mode via the left atrium (Neely *et al*, 1967), and measurements taken for aortic flow (Afl), coronary flow (Cfl), peak left ventricular (LV) pressure and LV end-diastolic pressure. After a period of equilibration, cardiac function was assessed in response to ET-1 (10^{-10}M).

All data are shown in Table 1 as mean ±sem. $p<0.05$ was taken as significant when comparing treatment vs. control, and denoted as *, one-way ANOVA. *n=4-7* in each group. Endotoxaemia resulted in reduction in both Afl and Cfl when compared with controls.

Pre-treatment with ABT-627 had no significant effect on flows in hearts from control animals, however, in hearts from endotoxaemic rats, ABT-627 entirely restored Cfl to that of controls. ABT-192621 reduced Cfl in control hearts, however this did not reach statistical significance. ABT-192621 had no significant effect in hearts from endotoxaemic animals. Pre-treatment with either ABT-627 or ABT-192621 did not affect Afl in either control or endotoxaemic groups.

Table 1. Afl and Cfl (μL/min) measured 24 mins post-ET-1

	control		LPS	
	Afl	Cfl	Afl	Cfl
Nil (vehicle)	55.4±1.6	14.9±0.9	36.5±3.0*	9.4±1.1*
+ET-1	35.8±3.5*	9.4±0.5*	31.2±2.0*	7.9±1.1*
ABT-627	52±2.2	16.1±0.8	33.8±0.6*	14.5±1.5
+ET-1	51.4±2.3	15.9±0.7	32.8±0.6*	14.0±0.8
ABT-192621	57.2±1.6	12.5±0.6	33.3±0.4*	8.6±0.7*
+ET-1	50.2±3.8	10.6±0.6	23.8±3.2*	6.2±0.4*

These observations show that the reduction in Cfl associated with sepsis (Warner & Kelm, 1996) which is masked by iNOS activity (Price *et al*, this meeting) is mediated by ET-1, acting on ET_A receptors. By contrast, the reduction in Afl which is also masked by iNOS activity (Price *et al*, this meeting) is independent of ET-1. These data suggest that ET_A receptor antagonists may have some beneficial effects in maintaining coronary flow in sepsis, but would not be expected to have a significant effect on cardiac output.

This work was supported by the British Heart Foundation and the Wellcome Trust

Neely *et al*, (1967), *Am J Physiol.* 212:804-814
Thiemermann *et al*, (1997), *Gen Pharmacol.* 29, 684-688
Warner & Kelm (1996), *Inflam Res.* 5, 51-53

2P EFFECT OF SELECTIVE iNOS INHIBITION ON CARDIAC FUNCTION IN ENDOTOXAEMIA

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It is widely accepted that excess amounts of nitric oxide (NO) produced via the induction of inducible nitric oxide synthase (iNOS) contribute to the peripheral vascular changes of septic shock (Thiemermann *et al*, 1997). Although NO has been implicated in the physiological control of myocardial function, controversy remains as to its role in the pathophysiology of cardiac dysfunction in septic shock. The aims of the current study were to investigate the role of iNOS in the modulation of cardiac function in endotoxaemia using the isolated ejecting heart model.

Control or endotoxaemic (LPS 20mg^{-1}kg^{-1} i.p 4h) male Wistar rats (250-300g) were anaesthetised with sodium pentobarbitone (100mg^{-1}kg i.p) and killed by cervical dislocation. Hearts were rapidly removed and immersed in ice-cold gassed [95%O_2:5%CO_2] Krebs' solution. Aortae were cannulated, and hearts perfused with gassed Krebs' at 37°C in Langendorff mode for 30 mins. Perfusion was then switched to the ejecting mode via the left atrium (Neely *et al*, 1967), and measurements taken for aortic flow (Afl), coronary flow (Cfl), peak left ventricular (LV) pressure and LV end-diastolic pressure. After a period of equilibration, cardiac function was assessed in response to L-NAME (10^{-3}M) or the selective iNOS inhibitor, 1400W (10^{-5}M). All experiments were performed in the presence/absence of the NOS substrate L-arginine (10^{-3}M).

Results for Afl and Cfl are shown in Table 1. All data are shown as mean ± sem. $p<0.05$ is taken as significant and denoted as * when comparing with control values, one-way ANOVA. *n=5-8* in each group. Endotoxaemia resulted in reduction in both Afl and Cfl.

In hearts from control animals, L-arginine and 1400W had no effect on flows. In contrast, in hearts from endotoxaemic animals, in the absence of L-arginine, 1400W had no effect, however, L-arginine increased Afl and Cfl an effect that was reversed with 1400W.

Table 1: Afl and Cfl (μL/min) measured 24 mins post-treatment

	control		LPS	
treatment	**Afl**	**Cfl**	**Afl**	**Cfl**
nil	60.7±3.8	13.89±1.3	32.8±0.4*	10.1±0.6*
L-NAME	23.4±2.9*	8.5±1.2*	9.7±0.7*	5.6±0.35*
1400W	57.4±2.8	13.56±0.9	32.7±2*	10.6±0.7*
L-arginine	56.8±1.3	15.88±1.7	45.8±1.6	13.6±1.2
L-NAME	37±2.0*	10.5±0.8*	24.7±2.7*	8.2±0.2*
1400W	54.6±1.6	15.15±0.4	34.8±1.9*	10.4±0.6*

These observations show that L-arginine is rate-limiting for the production of NO via iNOS in the heart in endotoxaemia. Moreover, when iNOS activity is compromised, in this setting the cardiotoxic effects of sepsis are revealed. These findings suggest that, in the heart, iNOS activity is protective in sepsis, and that the administration of inhibitors of NO production (including iNOS selective) would compromise function. The results obtained in this study may help to explain the detrimental effects of NOS inhibition in clinical trials of septic shock (Grover *et al*, 1999).

This work was supported by the British Heart Foundation and the Wellcome Trust

Grover *et al*, (1999), *Crit Care Med.* 27(1)A33
Neely *et al*, (1967), *Am J Physiol.* 212:804-814

3P LUTEOLIN INHIBITS LIPOPOLYSACCHARIDE (LPS)-INDUCED LETHAL TOXICITY AND PRO-INFLAMMATORY MOLECULE EXPRESSION

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Flavonoids are naturally occurring polyphenolic compounds that are known to process anti-oxidant, anti-tumor, anti-angiogenic, anti-inflammatory, anti-allergic and anti-viral properties (Formica and Regelson, 1995; Wang et al., 1998). The aim of the present study was to screen a panel of flavonoids *in vitro* for their ability to inhibit pro-inflammatory cytokine expression and to test the most potent of these agents in an animal model of sepsis. For our initial screen of flavonoids we stimulated RAW 264.7 cells with LPS and measured tumor necrosis factor-α (TNF-α) release in the supernatants. From the compounds tested (myricetin, catechin, hesperetin, luteolin, luteolin-7 glucoside, chrysin, eriodictyol and quercetin) luteolin (Lut) was the most potent in inhibiting TNF-α production (IC_{50} < 1μM). Moreover, Lut blocked the production of interleukin-6 (6±2% of the LPS value) and nitric oxide (8.2±0.5%) in response to LPS from RAW 264.7.

Administration of luteolin to C57Bl/6 mice (0.2mg/Kg, i.p.) 30 min prior to the LPS injection (S. *enteritis*, 800μg/25gr, i.p.) resulted in a marked reduction in plasma TNF-α levels (undetectable, 11.1±2.5 ng/ml and 1.1±0.2 ng/ml for control, LPS and Lut plus LPS, n=4-5). Lut also blocked the expression of intercellular adhesion molecule expression in the liver following LPS treatment, as assessed by western blotting. Moreover Lut prevented LPS-induced lethal toxicity in mice (4.1% surviving in the LPS group and 44.8% in the Lut plus LPS after 6 days, n=27-30).

To investigate the molecular mechanism(s) by which Lut inhibits LPS signaling, we determined its ability to interfere with total protein tyrosine phosphorylation, as well NF-κB activation. Exposure of RAW 264.7 cells to LPS led to a time-dependent increase in tyrosine phosphorylation that was attenuated by Lut pretreatment (10μM for 30 min). Since the expression of many pro-inflammatory molecules is dependent on NF-κB activation, we tested the effects of Lut on IκBα phosphorylation and degradation. Treatment of macrophages with LPS resulted in increased IκBα phosphorylation and reduced the levels of IκBα. Pretreatment of cells with Lut abolished the effects of LPS on IκBα. In addition, LPS-stimulated luciferase activity from macrophages transfected with a vector coding for the luciferase reporter gene under the control of κB cis-acting elements was abolished by luteolin pretreatment. We conclude that luteolin inhibits total tyrosine phosphorylation, NF-κB-mediated pro-inflammatory gene expression and LPS-induced lethal toxicity in mice.

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4P ROLE OF NITRIC OXIDE ON THE DELAYED CARDIOPROTECTION AFFORDED BY LIPOPOLYSACCHARIDE IN THE RAT HEART *IN VIVO*

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In most species myocardial protection associated with "classic" ischaemic preconditioning (IPC) lasts approximately 2 h (Murry *et al.*, 1991). It has recently been reported that a delayed phase of protection appears 24 h after IPC, referred to as delayed preconditioning (DP, Baxter *et al.*, 1996). Nitric oxide (NO) generation by inducible NO synthase (iNOS) has been proposed as both a trigger and a distal mediator of DP (Bolli *et al.*, 1998). The aim of this study was to investigate whether iNOS/NO is involved in DP afforded by lipopolysaccharide (LPS). Furthermore, we studied the effects of dexamethasone (Dex, 3 mg kg^{-1} i.p., inhibits induction of iNOS) or L-NIL (3 mg kg^{-1} bolus + 3 mg kg^{-1} h^{-1} i.v. infusion, selective inhibitor of iNOS activity) on the infarct size (IS) reduction afforded by LPS.

Male Wistar rats (200-280 g) were divided into two experimental studies: Study I: Rats (n=3-4 each group) were pretreated with saline (1 ml kg^{-1} i.p.) or LPS (*E. coli*, 1 mg kg^{-1} i.p.). Rats were anaesthetised (thiopentone sodium, 120 mg kg^{-1} i.p.) and hearts or plasma samples were taken after 2, 4, 8, 16 or 24 h, frozen and subjected to Northern blot (iNOS, Frank *et al.*, 1999) or nitrate/nitrite (Zacharowski *et al.*, 2000) analysis, respectively. Study II: Rats (n=5-9 each group) were treated with saline or LPS as above. After 16 h, rats were anaesthetised (as above), tracheotomised and ventilated (tidal volume, 10 ml kg^{-1}; 70 strokes min^{-1}; inspiratory oxygen-concentration, 30%; positive end-expiratory pressure, 1-2 mmHg). The carotid artery or jugular vein were cannulated to measure mean arterial blood pressure or for the administration of drugs, respectively. The chest was opened by a left-sided thoracotomy, the pericardium incised and a suture placed around the left anterior descending coronary artery (LAD). Rats were allowed to stabilise for 30 min before LAD-occlusion (25 min) and reperfusion (2 h). After the reperfusion period, the LAD was re-occluded and 1 ml of Evans blue dye (2% w v^{-1}) injected into the jugular vein to determine the perfused and the non-perfused (area at risk) myocardium. IS was assessed by incubation of heart slices with *p*-nitro-blue tetrazolium (0.5 mg ml^{-1}). Study groups: **(1)** saline; **(2)** LPS; **(3)** Dex 30 min prior to LPS; **(4)** LPS, L-NIL 30 min prior to ischaemia; **(5)** LPS 2 h prior to L-NIL. Statistics: *P* determined by one or two-way ANOVA followed by Bonferroni's test.

Study I: LPS caused a time-dependent increase in mRNA levels and activity of iNOS (Table 1). Study II: Mean AR and haemodynamic parameters of all groups were not significantly different (data not shown). LPS caused a significant reduction in IS (Table 2). Treatment with Dex or L-NIL reduced, but did not abolish, the cardioprotective effects of LPS-pretreatment.

Table 1: mRNA (iNOS, expressed as fold induction over control) and nitrate/nitrite (n/n, μMol l^{-1}) data. * $P<0.05$ vs. saline.

	saline	2h LPS	4h LPS	8h LPS	16h LPS	24h LPS
iNOS	1	12±4*	24±10*	12±6*	1	1
n/n	40±2	38±1	120±17*	377±10*	328±9*	121±40*

Table 2: Area at risk (AR, %) and infarct size (IS, %) data. * $P<0.05$ vs. saline; † $P<0.05$ vs. LPS.

	(1)	(2)	(3)	(4)	(5)
IS	61±4	13±4*	41±6*†	33±4*	46±4†
AR	49±4	54±2	53±2	47±3	52±4

We propose that NO acts as a trigger and to a lesser degree as a mediator of DP induced by LPS in the rat heart *in vivo*.

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5P DIFFERENT WALL FRAGMENTS OF GRAM-POSITIVE OR NEGATIVE BACTERIA INDUCE DELAYED PRECONDITIONING IN THE RAT HEART *IN VIVO*

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Pretreatment of rats with a low dose of lipopolysaccharide (LPS) or lipoteichoic acid (LTA) for 16h protects the heart against ischaemia (I) and reperfusion (R) injury (Brown *et al.*, 1989; Zacharowski *et al.*, 1999 & 2000). This phenomenon is referred to as delayed preconditioning (DP, Baxter *et al.*, 1996). Other agents such as monophosphoryl lipid A or RC-552 (both have similar lipid A moieties when compared to LPS) also induce DP. The cell wall of Gram-positive bacteria contains LTA and peptidoglycan (PepG), which when given together to rats, causes multiple organ failure (MO). However, LTA or PepG alone does not cause MO (De Kimpe *et al.*, 1995). The aim of this study was to investigate the delayed cardioprotective effects of different bacterial wall fragments in the rat *in vivo*. Furthermore, we compared these effects with the infarct size (IS) reduction afforded by classic ischaemic preconditioning (IPC).

Male Wistar rats (230-330 g) were pretreated i.p. (n=5-9 each group) for 16h as follows: **(1)** saline (1 ml kg^{-1}); **(2)** LPS (*E. coli*, 0.1 mg kg^{-1}); **(3)** LPS (*E. coli*, 1 mg kg^{-1}); **(4)** LTA (*S. aureus*, 0.1 mg kg^{-1}); **(5)** LTA (*S. aureus*, 1 mg kg^{-1}); **(6)** LTA (*B. subtilis*, 1 mg kg^{-1}); **(7)** PepG (*S. aureus*, 3 mg kg^{-1}); **(8)** PepG (*S. aureus*, 10 mg kg^{-1}) or **(9)** IPC (3x2min I + 2x5 + 1x10min R). Rats were then anaesthetised (thiopentone sodium, 120 mg kg^{-1} i.p.), tracheotomised and ventilated (tidal volume: 10 ml kg^{-1}, 70 strokes min^{-1}, inspiratory oxygen-concentration: 30%, positive end-exspiratory pressure: 1-2 mmHg). The carotid artery or jugular vein were cannulated to measure mean arterial blood pressure or for the administration of drugs, respectively. The chest was opened by a left-sided thoracotomy, the pericardium incised and a suture was placed around the left anterior descending coronary artery (LAD). Rats were allowed to stabilise for 30min and subsequently the LAD was occluded for 25min and then reperfused for 2h. After the reperfusion period, the LAD was re-occluded and 1 ml of Evans blue dye (2% w v^{-1}) was injected into the jugular vein to determine the perfused and the non-perfused (area at risk, AR) myocardium. IS was determined by incubation of heart slices with *p*-nitro-blue tetrazolium (0.5 mg ml^{-1}). Statistics: * $P<0.05$ vs. control, one or two-way ANOVA followed by Bonferroni's test.

The mean AR were similar in all groups studied. When compared to control, LPS, LTA and IPC caused a significant reduction in IS (Table 1). There were no difference in the haemodynamic parameters of any of the groups studied (data not shown).

Table 1: Area at risk (AR, %) and infarct size (IS, %) data.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
IS	58±3	38±8	23±4*	24±6*	22±5*	33±10	45±11	38±12	24±4*
AR	45±4	49±4	51±4	52±2	53±3	52±2	53±2	53±4	48±4

Thus, wall fragments of Gram-positive or Gram-negative bacteria induce DP. The cardioprotective effects of LTA (*S. aureus*, high and low dose) and LPS (high dose) are similar to those afforded by IPC. Further experiments are warranted to investigate whether a lower dose of LTA still induces DP.

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6P DESOGESTREL REDUCES VENTRICULAR FIBRILLATION INDUCED BY REPERFUSION BUT NOT BY ISCHAEMIA

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The risk of myocardial infarction may be lower in users of oral contraceptives containing third generation progestogens such as desogestrel, compared to second generation products, such as norethisterone (Lewis *et al.*, 1996). Cardiac arrhythmias are a serious consequence of acute myocardial ischaemia and infarction. We have therefore compared the effects of desogestrel and norethisterone on ischaemia- and reperfusion-induced arrhythmias in ovariectomised, anaesthetized rats.

Female Wistar rats (200-290g) were ovariectomized under Hypnorm/diazepam anaesthesia. After 12 to 16 days, rats (280-390g) were treated with norethisterone or desogestrel at doses of 0.01, 0.03 or 0.1 mg 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 cannulated 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 arrhythmias occurring in the first 25 min of ischaemia were quantified. Separate groups of rats receiving 0.01, 0.03 or 0.1 mg kg^{-1} norethisterone, 0.03mg kg^{-1} desogestrel or vehicle were used to study reperfusion-induced arrhythmias. Five min after occlusion of the coronary artery the ligature was released to allow blood flow back into the myocardium and the reperfusion-induced arrhythmias were monitored for 10 min.

Desogestrel, but not norethisterone, reduced reperfusion-induced ventricular fibrillation but neither progestogen significantly altered the incidence of ischaemia-induced arrhythmias (Table 1). Neither compound had significant effects on heart rate or blood pressure.

Table 1. The effect of norethisterone or desogestrel (0.03mg kg^{-1}) on the incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) during ischaemia or reperfusion

	Ischaemia		Reperfusion	
	VT	VF	VT	VF
Control	100%	20%		
Norethisterone	91%	18%	85%	46%
Control	83%	33%	76%	67%
Desogestrel	100%	33%	58%	25%*

n=12-13, *P<0.05 compared to control, Fisher's exact test.

These results indicate that desogestrel reduced reperfusion-induced arrhythmias when given once daily for 7 days. Previously, we found that the same dose of desogestrel, but not norethisterone, significantly decreased platelet aggregation (Fraser & Coker, 1999). Other drugs that interfere with platelet aggregation can reduce reperfusion- but not ischaemia-induced arrhythmias (Shaw & Coker, 1996; 1997). It can therefore be concluded that the ability of desogestrel to reduce reperfusion- but not ischaemia-induced arrhythmias could be related to reduced platelet aggregation.

Supported by the British Heart Foundation (PG/97084).

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7P AMP579, AN ADENOSINE A_1 AND A_{2A} RECEPTOR AGONIST, ATTENUATES LETHAL REPERFUSION INJURY IN RAT HEART VIA THE P42/P44 MAPK PATHWAY

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Various peptide growth factors, including transforming growth factor-β1, insulin-like growth factor and cardiotrophin-1, have been shown to limit reperfusion injury by activating "survival" signalling pathways (Yellon and Baxter, 2000). Such kinase pathways include p42/p44 mitogen-activated protein kinase (MAPK). AMP579 ([1S-[1a, 2b, 3b, 4a (S*)]]-4-[7-[[2-(3-chloro-2-thienyl)-1-methylpropyl] amino]-3H-imidazo [4,5-b]-pyridinyl-3yl] cyclopentane carboxamide) is a combined adenosine A_1 and A_{2a} receptor agonist, reported to limit myocardial infarct size when administered during reperfusion (Smits et al., 1998). The mechanism of protection is not clearly defined but inhibition of recruitment and adhesion of neutrophils has been proposed. We hypothesised that AMP579 protects during reperfusion by a primary cytoprotective action in the heart, independent of effects on circulating neutrophils and requiring activation of p42/p44 MAPK. To test this hypothesis, we studied AMP579 in a neutrophil-free model of ischaemia-reperfusion, using PD98059 a specific inhibitor of p42/p44 MAPK activation.

Male Sprague-Dawley rats (300-400 g) were anaesthetised with pentobarbitone sodium (60 mg/kg). Hearts were excised and rapidly perfused in the Langendorff mode with modified Krebs-Henseleit buffer. Temperature was maintained close to 37 °C throughout. Following stabilisation, regional ischaemia was induced by snaring the left main coronary artery for 35 min. Reperfusion was instigated for 120 min after which the risk zone was delineated with Evans blue and the infarct delineated by triphenyltetrazolium staining. Computerised planimetry was used to assess infarct size as a percentage of the ischaemic risk zone (I/R %). Control hearts received no intervention. To assess the protective effects of AMP579 at reperfusion, hearts were randomised to receive AMP579 1 μM which was perfused for 65 min, beginning 5 min before reperfusion. To inhibit activation of p42/p44 MAPK, PD98059 10 μM was co-perfused in some hearts during the same period.

AMP579 caused a marked increase in coronary flow rate during early reperfusion, consistent with coronary vasodilatation. This vasodilator effect was not abolished by PD98059. AMP579 also caused a marked but transient bradycardia during the first 30 min of reperfusion. Thereafter, rate pressure product was similar in all experimental groups. Infarct size is shown in table 1.

Table 1: Infarct size

	control	AMP579	PD98059+ AMP579	PD98059
I/R (%)	57.0 ± 3.1	31.5 ± 5.0*	55.2 ±1.0	54.1 ± 6.5
n	9	6	6	6

Data are mean ± s.e.mean of n experiments. * $P < 0.05$ versus control group (1 way ANOVA)

AMP579 during early reperfusion significantly limited infarct size, an action that was clearly neutrophil-independent. The infarct-limiting effect of AMP579 was abolished by PD98059, suggesting that activation of p42/p44 MAPK is essential for the protective effect of AMP579 at reperfusion.

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8P AMP579, AN ADENOSINE A_1 AND A_{2A} RECEPTOR AGONIST, LIMITS INFARCT SIZE IN RABBIT HEART *IN VIVO* WHEN GIVEN AT REPERFUSION: ROLE OF A_{2A} RECEPTOR ACTIVATION

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AMP579 ([1S-[1a, 2b, 3b, 4a (S*)]]-4-[7-[[2-(3-chloro-2-thienyl)-1-methylpropyl] amino]-3H-imidazo [4,5-b]-pyridinyl-3yl] cyclopentane carboxamide) is a novel adenosine analogue with agonist activity at both A_1 and A_{2a} receptors. The compound has been reported to limit myocardial infarct size when administered during reperfusion (Smits et al., 1998). Previous work in our own and other laboratories suggests that selective adenosine A_1 receptor agonists do not limit infarct size when given at reperfusion (Thornton et al., 1992; Baxter et al., 2000). Therefore, we hypothesised that the protective action of AMP579 during reperfusion is related to A_{2a} agonist activity, rather than A_1 agonist activity. We tested this hypothesis in a rabbit model of acute myocardial infarction.

Male New Zealand White rabbits were anaesthetised with pentobarbitone sodium (40 mg/kg), and artificially ventilated. A sternotomy was performed and the heart exposed. A silk suture was placed around an anterior branch of the left coronary artery. Regional myocardial ischaemia was induced by occluding the coronary artery for 30 min. Rabbits were assigned to treatment with AMP579 (30 μg/kg iv bolus 10 min prior to reperfusion followed by 3 μg/kg/min for 70 min) or vehicle control. Two groups of animals were randomised to receive the selective A_{2a} receptor antagonist ZM241385 1 mg/kg iv bolus 5 min prior to AMP579 or vehicle treatment. A further group of rabbits was treated with the selective A_{2a} agonist CGS216803 30 μg/kg iv bolus 10 min prior to reperfusion followed by 3 μg/kg/min for 70 min. After 180 min reperfusion, the ischaemic risk zone was delineated ex vivo by Evans blue exclusion and the infarcted tissue was delineated by triphenyltetrazolium staining. Computerised planimetry was used to assess infarct size, expressed as a percentage of the risk zone (I/R %).

Treatment with either AMP579 or CGS21680 resulted in a reduction in mean arterial pressure approximating 20 mm Hg that was sustained during the period of infusion. Pretreatment with ZM241385 completely abolished this depressor effect of AMP579. Infarct size data are presented in table 1.

Table 1: Infarct size

treatment	vehicle	AMP	ZM +vehicle	ZM +AMP	CGS
I/R (%)	45.1 ±3.8	29.6 ±3.8*	47.4 ±6.3	38.2 ±4.9	44.7 ±5.8
n	11	15	6	8	6

Data are mean ± s.e.mean of n experiments. * $P < 0.05$ versus vehicle treated group (1 way ANOVA)

The infarct-limiting effect of AMP579 was attenuated by the A_{2a} receptor antagonist. However, CGS21680, a selective A_{2a} agonist with equipotent haemodynamic activity, did not limit infarct size. We conclude that selective A_{2a} receptor activation during reperfusion is not sufficient to limit myocardial infarct size but may contribute, in part, to the protective action of AMP579.

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9P NITRIC OXIDE SYNTHASE AND HEME OXYGENASE MODULATION OF CARDIAC ANAPHYLAXIS *IN VITRO*

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Nitric oxide (NO) and carbon monoxide (CO) are endogenously formed from L-arginine and heme catabolism by nitric oxide synthase (NOS) and heme oxygenase (HO), respectively. Relaxin (RLX) is a peptide hormone secreted mainly by the corpus luteum, whose relaxing effects on myometrium and the vascular system have been accounted for the induction of inducible NOS (iNOS), thus leading to an increased generation of NO. Hemin, an endogenous protoporphyrin, stimulates the inducible isoform of heme oxygenase (HO-1), leading to an increase in CO production. Both NO and CO modulate the release of histamine induced by immunological and non-immunological stimulation from serosal mast cells (Masini *et al.*, 1994; Ndisang *et al.*, 1999). Cardiac anaphylaxis *in vitro* is a reliable model of immune response and the changes of myocardial functions are the results of the release of pro-inflammatory and vasoactive mediators from cardiac mast cells. Here we report on the effects of RLX and hemin treatment on cardiac anaphylaxis *in vitro*.

Guinea pig hearts from actively sensitised animals were perfused in a Langendorff apparatus and challenged with egg albumin. Records were taken of the strength of contraction, rate and coronary outflow. Histamine content in the perfusates and in the heart was detected fluorimetrically; nitrite were detected by means the Griess reaction. In cardiac homogenates iNOS and HO-1 activities were detected measuring ^{3}H-citrulline and bilirubin formation respectively. Cyclic GMP levels were detected by RIA using ^{125}I-labelled cyclic GMP, tissue calcium levels by means of atomic absorption. RLX (30 ng/ml) was added to the perfusion fluid 60 min before antigen challenge. The animals were treated with hemin (4 mg/Kg i.p.) 16 hours before anaphylaxis.

RLX fully abates the increase of heart rate and strength of contraction induced by egg albumin, and causes a less sustained coronary constriction and a more pronounced coronary dilatation 20 min after anaphylaxis. Consistently, the release of histamine induced by antigen is significantly diminished in the presence of RLX (176±29 vs. 225±31 ng/ml in the first 5 min; n=8; $p<0.05$). RLX significantly increases cGMP levels (390±42 vs. 125±36 fmol/mg protein; n=6; $p<0.001$) and reduces the antigen-evoked increase of cardiac calcium. The treatment of the animals with hemin increases significantly the amount of bilirubin produced (560±32, in treated vs 116±4 ng/mg protein in control animals; n=4; $p<0.001$). Moreover, the effects of egg albumin on contraction, rate and coronary flow are completely inhibited by hemin-treatment. The effect of hemin were reverted by Zn-protoporphyrine IX (50 ug/Kg i.p.), an inhibitor of HO-1. These data support the modulation of anaphylactic reactions by iNOS and HO-1 activation.

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10P OXIDATIVE STRESS REDUCES THE PHARMACOLOGICAL ACTIVITY OF THERAPEUTICS WITH ANTIOXIDANT PROPERTIES

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Omapatrilat (OP) is a novel combined inhibitor of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) developed for the treatment of hypertension and heart failure (Weber., 1999). Through simultaneous inhibition of ACE and NEP, OP prevents enzymatic degradation of bradykinin. Since bradykinin is an important trigger of classical preconditioning (PC) (Goto et *al.*, 1995) the aim of this study was to examine if OP, through its ability to augment bradykinin levels, lowers the threshold for triggering PC.

Male Sprague Dawley rats (350-450g) were deeply anaesthetised with pentobarbitone sodium (50mg.kg^{-1}, i.p) and killed. Hearts were excised and Langendorff perfused with crystalloid buffer and subjected to 35 minutes of regional ischaemia followed by 120 minutes of reperfusion. Control hearts received no further intervention. PC was induced with 5 min global ischaemia/10 min reperfusion (1 x 5 PC) prior to regional ischaemia. Subthreshold PC consisted of 2 min global ischaemia/10 min reperfusion (1 x 2 PC). OP (10μM) was perfused for 5 min before and after 1 x 2 PC and washed out for 5 min prior to regional ischaemia. Bradykinin B_2 receptor involvement was assessed using Hoe 140 (1μM) which was co-perfused with OP or given alone. Infarct size was determined using tetrazolium staining and expressed as a percentage of the risk zone. Data are highlighted in table 1.

Table 1 Infarct Size

GROUP (n)	*INFARCT SIZE (%)*
Control (17)	53.4±2.0
1x 2 PC (9)	48.4±3.8
1x 5 PC (11)	21.5±3.5*
OP (6)	34.6±1.5*
OP + 1x2 PC (7)	19.7±2.5*
Hoe 140 + OP + 1x2 PC (7)	46.5±6.0
Hoe 140 + OP (6)	53.8±5.0
Hoe 140 (8)	51.5±4.2

Infarct size is expressed as a percentage of the risk zone (%). Results are stated as mean±s.e.of mean of (n) experiments (one way ANOVA, followed by Fisher's protected least significant difference test) * = $P<0.01$ vs control

Subthreshold PC did not limit infarct size. OP given alone conferred modest protection. However, when subthreshold PC was combined with OP (1x2 PC + OP), infarct limitation was comparable to that seen with 1x 5 PC. This protective effect was abolished by Hoe 140, consistent with a role for bradykinin B_2 receptor activation in conferring protection.

These data suggest that OP may have useful cardioprotective properties in patients with coronary syndromes at risk of myocardial infarction.

Z.E holds a British Heart Foundation Studentship (FS/98075)

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11P OXIDATIVE STRESS REDUCES THE PHARMACOLOGICAL ACTIVITY OF THERAPEUTICS WITH ANTIOXIDANT PROPERTIES

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Cardiovascular diseases (Dhalla N.S. *et al.*, 2000), including hypertension, heart failure and post ischaemic reperfusion injury are associated with the generation of increased amounts of reactive oxygen species (ROS). Various antioxidant defences counteract ROS actions, minimising oxidative tissue damage. Some therapeutics have proven to possess additional antioxidant properties and it was suggested that the radical-scavenging properties might contribute to their therapeutical benefit.

The present study was performed to explore the pharmacological activity of agents with antioxidant properties after exposure to electrolysis-induced oxidative stress.

We investigated two agents with known radical scavenging properties, the α,β adrenoceptor antagonist carvedilol and the ACE-inhibitor captopril, and compared their pharmacological effects with prazosin, timolol and lisinopril, respectively, compounds without scavenging qualities, in the presence of ROS. For this purpose rat left atria and rat thoracic aortae were mounted in organ baths and connected to a force transducer. After a priming procedure the aortic strips were incubated with either captopril (3μM), lisinopril (300nM), carvedilol (100nM) or prazosin (5nM), and isolated left atria with carvedilol (14nM) or timolol (50nM) for 15 min. Subsequently, electrolysis was performed for 15 s with a constant current of 15 mA in aortic preparations and for 30s 30mA in atrial preparations.15 min later concentration-response curves with angiotensin I and the α_1-adrenoceptor agonist phenylephrine were constructed.

In isolated left atria the pD_2 values of isoproterenol in the presence of saline or carvedilol were 8.6 ± 0.1 and 7.4 ± 0.3, respectively, and after exposure to electrolysis-induced oxidative stress the values amounted to 8.3 ± 0.2 and 7.0 ± 0.3, oxidative stress the values amounted to 8.3 ± 0.2 and 7.0 ± 0.2, respectively, and after exposure to electrolysis-induced oxidative stress the values amounted to 8.3 ± 0.2 and 7.0 ± 0.2, respectively. A reduction of the β-adrenoceptor blocking activity of carvedilol after electrolysis was obtained (dose ratio 28.4 ± 3.6 vs. 15.3 ± 1.1, n = 6 - 8, $p < 0.05$), whereas the activity to timolol proved to be insensitive for exposure to oxidative stress (dose ratio 50.8 ± 5.9 vs. 48.1 ± 11.7, n = 6 - 8, ns.).

In aortic strips the pD_2-values of phenylephrine of 7.6 ± 0.1, 5.7 ± 0.1 and 6.1 ± 0.1 were established under control conditions, in the presence of carvedilol and prazosin, respectively. Exposure to ROS altered the pD_2 values to 7.3 ± 0.2, 5.6 ± 0.2 and 5.9 ± 0.1, respectively.

Oxidative stress impaired the α_1-adrenoceptor blocking activity of carvedilol significantly (dose ratio 87.4 ± 12.3 vs. 44.7 ± 13.2, n = 5 - 7, $p < 0.05$), whereas that of prazosin remained unaffected(dose ratio 36.1 ± 7.8 vs 30.2 ± 8.8, n = 6 - 7, n.s.).

Under control conditions and in aortic strips incubated with captopril and lisinopril the pD_2-values of angiotensin I amounted to 7.9 ± 0.1, 6.5 ± 0.1, 6.2 ± 0.1, respectively. Subsequent to electrolysis these values were 7.7 ± 0.1,7.7 ± 0.1 and 6.4 ± 0.1, respectively. The exposure to ROS completely diminished the ACE-inhibiting effects of captopril(dose ratio 34.4 ± 14.9 vs. 1.0 ± 0.2, n = 5, $p < 0.05$). Oxidative stress did not impair the effects of lisinopril (dose ratio 60.5 ± 23.5 vs. 18.1 ± 21.0, n = 5, n.s.).

We have demonstrated that electrolysis-induced oxidative stress is an appropriate model to investigate the effects of ROS on cardiac and vascular tissue and to study the possible effect of therapeutics with antioxidant properties. From our data we conclude that agents with radical scavenging properties are more susceptible to ROS-induced degradation and accordingly to loss of their primary pharmacological activity.

Dhalla N.S. et al. (2000) *J. Hypertension*, 18: 655-673

12P THE STABLE NITROXYL RADICAL TEMPONE REDUCES OXIDATIVE STRESS-MEDIATED RENAL DYSFUNCTION IN THE RAT *IN VIVO* AND *IN VITRO*

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Generation of reactive oxygen species (ROS) has been implicated in the pathogenesis of renal ischaemia-reperfusion (I/R) injury (Weight *et al.*, 1996). TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl) is a stable, water soluble nitroxyl radical which has been shown to be metabolised to its 4-hydroxy derivative, TEMPOL, in the rat *in vivo* (Kroll and Borchert, 1999). We have previously demonstrated that TEMPOL, which is a ROS scavenger (Laight *et al.*, 1997), can exert beneficial actions against I/R and oxidative stress-mediated renal dysfunction and injury *in vivo* and *in vitro* (Chatterjee *et al.*, 2000). The aims of this study were to investigate the effects of TEMPONE on (i) the renal dysfunction mediated by I/R of rat kidneys *in vivo* and (ii) the cellular injury of rat renal proximal tubular (PT) cells exposed to oxidative stress in the form of hydrogen peroxide (H_2O_2) *in vitro*.

For *in vivo* studies, 36 male Wistar rats (220-310 g) were anaesthetised with sodium thiopentone (120 mg kg^{-1} i.p.). After performing a midline laparotomy, rats were divided into 3 groups; (i) 'Shams', in which rats were maintained under anaesthesia for the duration of the experiment, (ii) 'I/R only', in which rats underwent bilateral clamping of the renal pedicles for 45 min followed by reperfusion for 6 h and (iii) 'I/R + TEMPONE', in which rats underwent I/R, but were administered an i.v. bolus of TEMPONE (100 mg kg^{-1} in saline) at the start of I/R followed by an infusion of TEMPONE (30 mg kg^{-1} h^{-1} in saline) throughout I/R. At the end of each experiment, plasma samples were collected from each rat and levels of urea and creatinine were measured (Vetlab Services, Sussex).

For *in vitro* studies, PT cells were isolated from the kidney cortex of 6 male Wistar rats (250-300 g) using collagenase digestion, differential sieving and Percoll density centrifugation. PT cells were cultured on 24 well plates in Minimum Essential Medium (MEM) containing 10% (v v^{-1}) fetal calf serum. Once confluent, cultures were divided into three groups; (i) PT cell incubated with MEM only ('Untreated'), (ii) PT cells treated with 1 mM H_2O_2 for 3 hours ('H_2O_2 only') or (iii) PT cells treated with 1 mM H_2O_2 and 1 or 10 mM TEMPONE ('TEMPONE + H_2O_2'). Cellular injury was assessed spectrophotometrically by measurement of the mitochondrial-dependent conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan.

Renal I/R produced significant increases in plasma urea and creatinine concentrations which, were significantly reduced by infusion of TEMPONE during I/R (Table 1). Incubation of rat PT cell cultures with 1 mM H_2O_2 for 3 hours significantly inhibited mitochondrial respiration (Table 2). Incubation with 1 and 10 mM TEMPONE significantly reduced the H_2O_2-mediated inhibition of mitochondrial respiration (Table 2).

Thus, I/R of rat kidneys produces significant renal dysfunction, which can be significantly reduced by administration of TEMPONE. Furthermore, TEMPONE reduces H_2O_2 (and therefore ROS)-mediated cellular injury in primary cultures of rat PT cells. We therefore propose that TEMPONE may be beneficial in renal disorders mediated by oxidative stress. We also suggest that the beneficial action provided by TEMPONE may involve, to some extent, its conversion to TEMPOL followed by scavenging of ROS.

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Table 1 (*in vivo*)	*N*	Plasma Urea (mmol L^{-1})	Plasma Creatinine (μmol L^{-1})
Sham	11	5 ± 1	44 ± 4
I/R only	11	26 ± 1*	191 ± 6*
I/R + TEMPONE	12	19 ± 1* +	154 ± 11* +

Table 2 (*in vitro*)	*N*	Mitochondrial respiration (% control)	
		1 mM TEMPONE	10 mM TEMPONE
Untreated	6	100	100
H_2O_2 only	6	17 ± 1*	17 ± 1*
TEMPONE + H_2O_2	6	28 ± 3*	37 ± 6* +

Table 1: Effect of I/R and TEMPONE on biochemical indicators of renal dysfunction (*P<0.05 vs. Sham, + P <0.05 vs. I/R only). Table 2: Effect of TEMPONE on H_2O_2-mediated inhibition of mitochondrial respiration (*P <0.05 vs. Untreated, + P <0.05 vs. Control). Data are expressed as mean ± s.e. mean of *N* rats, analysed using one-way ANOVA followed by the Bonferroni's post significance test. A P value of less than 0.05 was considered to indicate significance.

13P PRESYNAPTIC REGULATION OF NORADRENERGIC AND CHOLINERGIC NEUROTRANSMISSION IN THE ILEAL MYENTERIC PLEXUS OF$\alpha_{2A/D}$-ADRENOCEPTOR KNOCKOUT MICE

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Studies on α_2-adrenoceptor knockout mice have shown that both α_{2A} and α_{2C} subtypes are required for the presynaptic control of noradrenergic transmission in the central nervous system and heart, and that the sympathetic nervous system is abnormally regulated in $\alpha_{2A/D}$-adrenoceptor knockout mice (Altman *et al.*, 1999; Hein *et al.*, 1999). Since α_2-adrenoceptors play a prominent role in the pathophysiology of digestive functions, the present study examined their role in the presynaptic regulation of noradrenergic and cholinergic neurotransmission in the ileum of both control (NMRI) and $\alpha_{2A/D}$-knockout ($\alpha_{2A/D}$-KO) mice.

Myenteric plexus longitudinal muscle (MPLM) strips of ileum were prepared from adult male NMRI and $\alpha_{2A/D}$-KO mice (age: 8 weeks). Tissues were preincubated either with ^{3}H-noradrenaline (^{3}H-NA) for 30 min or ^{3}H-choline (^{3}H-Ch) for 45 min, and then superfused in the presence of desipramine 1 μM (^{3}H-NA) or hemicholinium-3 3 μM (^{3}H-Ch). Stimulation periods (S_1 to S_6; from t=64 min every 18 min) consisted of either 30 pulses at 50 Hz (^{3}H-NA) or 60 pulses at 1 Hz (^{3}H-acetylcholine; ^{3}H-ACh). The α_2-adrenoceptor agonist medetomidine (0.1–1000 nM) was added at increasing concentrations 12 min before $S_{2\text{-}6}$. Antagonists, when used, were present throughout superfusion at 300 nM. Results are given as mean ±s.e. mean.

In NMRI mice, control ^{3}H outflow at S_1 was 0.42±0.04% (^{3}H-NA; n=23) and 0.46±0.04% (^{3}H-ACh; n=25). Medetomidine decreased the evoked transmitter release with maximal inhibition (sigmoid curve fitting) of 57% (^{3}H-NA; n=10) or 40% (^{3}H-ACh; n=14). In the presence of the α_2-adrenoceptor antagonists phentolamine or rauwolscine, the concentration-response curves to medetomidine were shifted to the right. The estimated pK_d values were 8.7 (^{3}H-NA) and 9.2 (^{3}H-ACh) for phentolamine and 8.3 (^{3}H-NA) and 7.7 (^{3}H-ACh) for rauwolscine. Phentolamine and rauwolscine slightly enhanced ^{3}H-NA release ($S_{1(PHE)}$= 0.56±0.10%; n=15; $S_{1(RAU)}$= 0.55±0.06%; n=16), but did not modify ^{3}H-ACh output ($S_{1(PHE)}$= 0.51±0.05%; n=11; $S_{1(RAU)}$= 0.47±0.05%; n=12). In $\alpha_{2A/D}$-KO mice, control ^{3}H outflow at S_1 accounted for 0.28±0.04% (^{3}H-NA; n=17) and 0.52±0.03% (^{3}H-ACh; n=21). Medetomidine did not modify the evoked ^{3}H-NA release, and significantly inhibited the evoked ^{3}H-ACh output only when assayed at 10 nM (-14±3.87 %; n=8).

The present results suggest that in the digestive tract both noradrenergic and cholinergic axon terminals are equipped with α_2-adrenoceptors belonging almost exclusively to the $\alpha_{2A/D}$ subtype, whereas non-$\alpha_{2A/D}$-adrenoceptor subtypes are absent or represent a very minor component. In this respect, both auto- and hetero-α_2-adrenoceptors of mouse ileum differ from those of central nervous and peripheral tissues where an admixture of $\alpha_{2A/D}$ with non-$\alpha_{2A/D}$ subtypes was always clearly detected (Trendelenburg *et al.*, 1999).

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14P RESPONSES TO *E*-CAPSAICIN IN DETRUSOR TISSUE FROM BOTH WILDTYPE AND VANILLOID RECEPTOR-1 NULL MICE

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Given that vanilloid receptor (VR) heterogeneity exists (Liu & Simon, 1996), it is necessary to understand the relative contributions of VR subtypes to capsaicin-sensitive responses in native tissues. We have designed a method for detecting and measuring the effects of capsaicin in mouse isolated bladders and have compared our findings with those obtained using the VR1 null ($VR1^{-/-}$) mouse (Davis *et al.*, 2000).

2-4 bladder strips were cut in a longitudinal direction (dome to base) from adult C57 Black 6 mice (20-40 g). Each was suspended in 5 ml tissue baths under a 5 mN load for isometric recording and bathed in Krebs' solution maintained at 37°C and bubbled with 5% CO_2 in O_2. Data acquisition and analysis were performed using Biopac Systems, Inc. MP100 hardware and AcqKnowledge software.

In the presence of guanethedine (3 μM) and atropine (1 μM), E-capsaicin (0.1-30 μM, 15 min) evoked concentration-dependent contractions of bladder strips from wildtype ($VR1^{+/+}$) mice (EC_{50} 0.1 μM) which were maximal at 1-10 μM (23 ±2 % of carbachol response, 100 μM, n=14). Only 56% of bladder strips responded to capsaicin (54/96 bladder strips, N=25). Capsaicin (1 μM) evoked contractions were not abolished by tetrodotoxin (TTX, 3 μM) or the VR, NK1 and NK2 receptor antagonists, capsazepine (10 μM), L-732, 138 (1 μM) and MDL 29,913 (3 μM), respectively (n=4 each). Strips from $VR1^{-/-}$ were unresponsive to capsaicin (1-10 μM, n=14).

Electric field stimulation (EFS, 0.5 ms pulse width, 30 s duration supramaximal voltage, 90 V), induced frequency-dependent (1-50 Hz), TTX-sensitive (3 μM), nonadrenergic noncholinergic (NANC) contractions in both $VR1^{+/+}$ and $VR1^{-/-}$ mice. At 1 Hz, these were prolonged and approximately monophasic; at 5-50 Hz they were triphasic, characterised by a transient phasic, tonic and low amplitude post-stimulus contraction. We measured the initial contraction height (peak A) and the remaining contraction heights after 30 s EFS (peak B) and 10 s (peak C) post EFS. In $VR1^{+/+}$ mice, pretreatment with capsaicin (10 μM, 15 min) had no effect on the height of peak A but reduced the heights of peak B and C at frequencies ≥ 5Hz (n=8, P<0.01 each). In $VR1^{-/-}$ mice, similar treatment with capsaicin did not significantly affect the height of peak A and C over the frequency range, but revealed a capsaicin-sensitive component in peak B, at 20 Hz (n=14, P=0.07) and 50 Hz (n=14, P<0.01, Mixed Analysis of Variance). Vehicle controls (DMSO) were superimposable with frequency response curves constructed prior to capsaicin treatment (n=8, P>0.05) in both $VR1^{+/+}$ and $VR1^{-/-}$ bladders.

Our results support the observations previously reported for rats (Meini & Maggi, 1994). Further, we have identified a frequency-dependent, capsaicin-sensitive component in the NANC contraction of the $VR1^{-/-}$ mouse urinary bladder, which suggests an effect not mediated via the native VR1 subunit. Further studies are required to determine the mechanism of this effect.

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15P ANTAGONIST POTENCIES AT CHIMERIC HUMAN AND RAT $P2X_7$ RECEPTORS

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Species differences in antagonist potencies exist between $P2X_7$ receptor orthologues. KN62 exhibits 1000 fold (Humphreys *et al.*, 1998), and PPADS 10 fold (Chessell *et al.*, 1998), selectivity for human over rat $P2X_7$ receptors. In this study we have engineered chimeric and mutated receptors to investigate which regions of the receptor contribute to the antagonist binding sites.

Rat (R) and human (H) $P2X_7$ receptor cDNAs were bisected at amino acid 255 to produce HR $P2X_7$ and RH $P2X_7$ chimeric receptors (Thompson *et al.*, 2001). Site-directed mutagenesis was performed using a QuikChange mutagenesis kit. Receptor function was studied at 22°C by measuring 2'-and 3'-O-(benzoylbenzoyl)-ATP (BzATP) stimulated ethidium accumulation into HEK293 cells transfected with the various constructs (Michel *et al.*, 2000). Cells were grown in 96 well plates and studied in sucrose buffer containing (mM): 10 Hepes, 10 glucose, 5 KCl, 0.5 $CaCl_2$, 280 sucrose (pH 7.4). Antagonists were preincubated with cells for 30min. In some studies, cells were pretreated with 1unit.ml^{-1} apyrase for 1h. Data are the mean±s.e.mean of 4-7 experiments. Antagonist pIC_{50} values were determined against an EC_{50} concentration of BzATP.

In electrophysiological studies, the chimeric receptors formed functional channels (Thompson *et al.*, this meeting). BzATP also stimulated ethidium accumulation in cells expressing these channels with pEC_{50} values of 6.3±0.1 (R), 5.9±0.1 (H), 5.6±0.1 (RH) and 5.6±0.1 (HR). KN62 inhibited responses at the human (pIC_{50} 6.3±0.1) and HR $P2X_7$ (pIC_{50} 6.0±0.3) receptors. In contrast, KN62 (10μM) had no effect at rat and RH $P2X_7$ receptors.

This indicates that KN62 sensitivity resides in the region preceding amino acid (a.a) 255. KN62 is also an antagonist of mouse $P2X_7$ receptors. Since there are only 10 a.a. in the extracellular region of the rat sequence (up to a.a 255) that differ from the human and mouse receptors, several of these residues in the rat receptor were mutated to the corresponding a.a. in the human and mouse receptor. These were G86S, H130S and I136K. EC_{50} values for BzATP at these mutant receptors were not significantly different from the wildtype rat $P2X_7$ receptor. However, they all remained insensitive to KN62.

PPADS was also examined at the chimeric receptors. In these studies it was necessary to treat cells with apyrase due to tonic activation of $P2X_7$ receptors by endogenously released ATP. In the apyrase treated cells the effects of KN62 were similar to those obtained in non-apyrase treated cells, with pIC_{50} values of 6.7±0.2 (H), 6.8±0.2 (HR) and <5 (RH). PPADS inhibited BzATP stimulated ethidium accumulation at all receptors, with pIC_{50} values of 8.0±0.1 (H), 7.0±0.2 (rat), 7.4±0.1 (RH) and 7.5±0.1 (HR).

In conclusion, it is likely that KN62 interacts within a region of the human $P2X_7$ receptor, preceding a.a. 255, which differs from that present in the rat orthologue. PPADS appears to interact with residues either side of a.a 255. This is not unexpected since PPADS appears to interact with the ATP binding site (Michel *et al.*, 2000), which is now thought to be formed from regions either side of a.a 255 and close to the two transmembrane domains of the receptor (Ennion *et al.*, 2000).

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16P EVIDENCE FOR DIFFERENTIAL EXPRESSION OF $P2X_1$ AND $P2X_3$ PURINE RECEPTORS IN THE DETRUSOR MUSCLE AND UROTHELIUM, RESPECTIVELY, IN THE RAT AND HUMAN URINARY BLADDER

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The aetiology of urinary incontinence in 30% of women affected is unknown, but it has been shown to be associated with ageing. Its pathophysiology is undetermined, but it may be as a result of altered sensory neuromodulation. Adenosine 5'-triphosphate (ATP) is well recognised as a neurotransmitter in smooth muscle preparations. There is evidence to show that ATP causes both bladder contractions and to have a sensory role in processing physiological information in the urinary bladder (Ferguson *et al.*, 1997). These effects are likely to be mediated by P2X receptors, namely $P2X_1$ and $P2X_3$. We have carried out studies using immunohistochemistry and Western analysis to investigate the distribution of these ligand-gated ion channels in rat and human urinary bladders.

Urinary bladders were obtained from young (150-220g) and old (880-1200g) female Sprague-Dawley rats. Human bladder tissue was obtained from female and male donor subjects (n=4, age range 31-56 years). Sections of bladder from both species were incubated with subtype-specific antibodies to $P2X_1$ (Vulchanova *et al.*, 1996) and $P2X_3$ (Kidd *et al.*, 1998) receptors. Western analysis was carried out on urothelial and detrusor muscle tissue lysates as previously described (Sellers, 1999). Control experiments were performed involving either the omission or pre-incubation of the primary antibody with the specific protein blocking peptide. Specificity of the antibodies was determined by cross-reactivity experiments using lysates from HEK-293 cells expressing the human recombinant $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$ or $P2X_7$ receptor types.

Bands with apparent molecular masses of 50 and 70 kDa for $P2X_1$ and of 50 and 55-64 kDa for $P2X_3$ recombinant receptors were obtained on Western analysis using the appropriate subtype-specific antibodies. No cross-reactivity could be detected using the antibodies against lysates from wild type HEK cells or those expressing the other receptor types.

$P2X_1$ receptor immunostaining was found on detrusor muscle fibres in both rat and human urinary bladder. In contrast, $P2X_3$ receptor immunoreactivity was found predominantly in the urothelium of both species. Western analysis of rat tissue lysates showed staining patterns that were similar to those observed for the respective recombinant receptor type. Strong immunoreactivity was observed for the $P2X_3$ receptor in the urothelium, which could not be detected in detrusor muscle (Figure 1), whereas the converse localisation was observed for the $P2X_1$ receptor staining. In addition, a comparison of urothelium from young and old rats showed a marked change in the immunoreactive pattern obtained with the $P2X_3$ receptor antibody. This suggests that a change in the covalent modification and/or expression of the receptor had occurred on ageing (Figure 1).

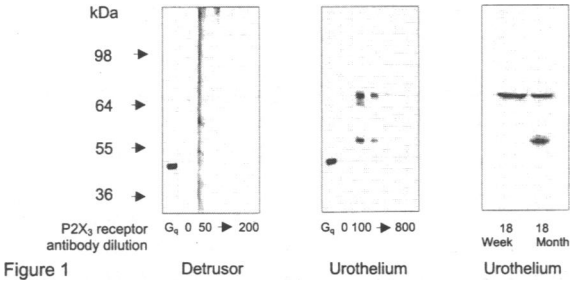

Figure 1

In conclusion, our data indicates the presence of the $P2X_3$ receptor within the urothelial cell, but not in the detrusor muscle of the urinary bladder in both age groups and species studied. However, there appears to be a difference in the $P2X_3$ receptor, as determined by Western analysis, in the elderly, when compared to the young, rats.

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17P PRENATAL EXPOSURE TO CARBON MONOXIDE IMPAIRS MYELINATION AND SPHINGOMYELIN HOMEOSTASIS IN THE SCIATIC NERVE OF RAT OFFSPRING

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Our previous findings have shown an impairment of ion channel maturation in peripheral nerves of rats born to dams exposed to carbon monoxide (CO) during pregnancy (Carratù *et al.*, 1993). Therefore, further experiments have been carried out in order to investigate possible consequences of prenatal CO exposure on myelination and sphingomyelin homeostasis in the rat sciatic nerve. The influence of prenatal CO on motor activity has been also explored.

Pregnant Wistar rats were exposed to 0 or 150 ppm of CO mixed with air from day 0 to day 20 of pregnancy as previously described (Cagiano *et al.*, 1998). Subgroups (5/group) of pregnant CO (0, and 150 ppm)-exposed rats were implanted under anaesthesia (Equithesin, 3 ml kg^{-1} i.p.) with catheters in the abdominal aorta. Maternal blood HbCO levels were measured on gestational day (GD) 20 by the spectrophotometric method described by Rodkey *et al.* (1979). Male rats (1 pup per litter from eight different litters per age and per treatment group) exposed in utero to CO (0, 150 ppm) were sacrificed on postnatal days 3, 8, 18, 40 and 90. Sciatic nerves were removed and processed for computer-assisted morphometric analysis (Schafer & Friede, 1988). Concentrations of sphinganine (intermediate of complex sphingolipid biosynthesis) and sphingosine (intermediate of complex sphingolipid turnover) were determined by the HPLC-OPA reagent fluorescence method (Riley *et al.*, 1994). Motor activity was recorded in Macrolon cages by infrared monitoring, according to the technique previously described by Tamborini *et al.* (1989).

The results show that pregnant rats exposed to CO (150 ppm) exhibit a significant increase in blood HbCO levels (mean % ± S.E.) on GD 20 with respect to controls (0 ppm: 1.8 ± 0.04; CO 150 ppm: 16.02 ± 0.68*; * P < 0.001, two-tail Student's t-test). Prenatal CO exposure produced a significant reduction in myelin sheath thickness (mm ± S.E.) of sciatic nerve fibers in 40- and 90-day-old rats with respect to controls (40-day-old rats: 0 ppm: 0.83±0.04; CO 150 ppm: 0.6±0.03*; 90-day-old rats: 0 ppm: 1.13±0.04; CO 150 ppm: 0.75±0.04*; *P<0.01, two-tail Dunnett's t-test). Developmental exposure to CO significantly increased sphingosine (SO) levels (ng/mg ± S.E.) in the sciatic nerve of 90-day-old rats with respect to controls (0 ppm: 1.34±0.26; CO 150 ppm: 2.33±0.28*; *P<0.05, overall one-way ANOVA; n=6), without altering sphinganine (SA) concentrations (F = 0.69, df = 1/10, n.s.; n=6). Consequently, the SA/SO ratio was significantly reduced in CO-exposed offspring (0 ppm: 0.62±0.03; CO 150 ppm: 0.41±0.03*; *P<0.01; n=6). Finally, prenatal CO exposure did not significantly impair motor activity. Overall one-way ANOVA for activity counts showed the following differences: F = 0.25, df = 1/10, n.s.; n=6.

In conclusion, the present findings show that a CO exposure level resulting in maternal blood HbCO concentrations equivalent to those experienced by human cigarette smokers produces subtle and long-lasting alterations in sphingomyelin homeostasis, which, in turn, could affect myelin maturation and/or turnover in the sciatic nerve of male rat offspring.

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18P ACETYLCHOLINESTERASE BLOCKADE DOES NOT ACCOUNT FOR THE CARDIAC INHIBITORY EFFECTS OF THE ANTI CANCER DRUG IRINOTECAN

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Irinotecan, a camptothecin derivative, acts as an inhibitor of DNA topoisomerase I and is currently used for the treatment of advanced colorectal cancer (Rothenberg & Blanke, 1999). The cytotoxic activity of this drug depends mostly on its active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38). In clinical settings, irinotecan can evoke toxic cholinergic effects consisting mainly of bradycardia, visual impairment, diaphoresis and abdominal cramps (Gandia *et al.*, 1993). It has been proposed that this syndrome might be ascribed to a direct blockade of acetylcholinesterase (AChE; Dodds & Rivory, 1999). The present study investigates: 1) the effects of irinotecan on heart rate and blood pressure; 2) the putative role played by AChE in the cardiac actions of irinotecan.

The experiments were carried out on male Wistar rats (200±20 g) anaesthetized with urethane (13.5 mmol.kg^{-1} i.p.). Catheters were surgically placed in the trachea and left carotid artery to ensure a patent airway and for recording of cardiovascular parameters. Diastolic blood pressure was monitored by a pressure transducer. Heart rate was triggered by the blood pressure signal and expressed as beats min^{-1} (bpm). Some animals were subjected to bilateral cervical vagotomy at the time of surgical preparation. A set of experiments was performed in rats with systemic ablation of capsaicin-sensitive- sensory nerve fibers: animals were given a total dose of 400 μmol kg^{-1} capsaicin s.c. in 2 days and used 10 days later. Test drugs were administered by i.v. route. Results are given as mean of 6 experiments ± s.e.mean (ANOVA followed by Student-Newman-Keuls test).

In controls, basal heart rate and blood pressure were 403.6±16.3 bpm and 66.4±4.5 mm Hg. Atropine (9 μmol kg^{-1}) or vagotomy did not affect blood pressure, but they increased heart rate (456.5±11.7 bpm, p<0.05; 462.3±12.8 bpm, p<0.05), indicating the presence of an inhibitory vagal tone. Irinotecan (1-20 urnol kg^{-1}) reduced heart rate and blood pressure, with maximal effects at 10 μmol kg^{-1} (317.7±11.6 bpm, p<0.05; 38.3±4.1 mm Hg, p<0.05). SN-38 (20 μmol kg^{-1}) did not modify both parameters (394.6±18.4 bpm; 71.5±6.1 mm Hg). The inhibitory actions of irinotecan (10 μmol kg^{-1}) were prevented by atropine (390.1±12.999 bpm; 63.6±5.1 mm Hg) or vagotomy (410.7±13.1 bpm; 68.3±4.8 mm Hg). Pretreatment with capsaicin did not alter heart rate or blood pressure but prevented the effects of irinotecan (416.5±18.3 bpm; 73.2±5.3 mm Hg). In both capsaicin-untreated and treated rats, the AChE blocker, physostigmine (0.3 μmol kg^{-1}), decreased heart rate (306.3±14.8 and 323.6±16.5 bpm, p<0.05) and increased blood pressure (92.7±6.2 and 98.4±5.7 mm Hg, p<0.05).

The present results indicate that irinotecan, but not its active metabolite SN-38, enhances the activity of vagal cholinergic fibers involved in the inhibitory control of heart rate and blood pressure. However, a blockade of AChE does not appear to account for the inhibitory actions of irinotecan on cardiovascular functions. It is rather suggested that irinotecan triggers a reflex parasympathetic discharge to the heart mediated by activation ofcapsaicin-sensitive afferent fibers.

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19P EFFECT OF SENNA ON GROWTH OF ABERRANT CRYPT FOCI AND TUMOURS IN THE RAT COLON

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Anthranoid-containing laxatives are suspected of causing large intestinal tumours in rats after long-term, high dose administration (Westendorf et al., 1990; Mascolo et al., 1999). The aim of the present study was to investigate the capacity of senna pod extract (SE) to induce the development of aberrant crypt foci (ACF), which are generally considered as putative preneoplastic lesions (Bird, 1995), and of tumours in the rat colon.

Experiments were performed on male Wistar rats weighing 120±10 g at the start of the study. The animals were randomly divided into 6 groups (see table 1). Azoxymethane (AOM, 7.5 mg kg^{-1}), used as initiating agent, to evaluate SE acting as tumour promoter, was administered (i.p.) on day 1 and 5 of treatment. SE (from Cassia angustifolia, containing about 50 % of sennoside B) was given by intragastric gavage every day for two years. At the end of the treatment, animals were killed and the colon removed and analysed for the determination of ACF and tumours as previously described (Bird, 1995).

Results are summarised in Table 1. AOM given alone induced the expected appearance of ACF and tumours. In contrast, SE (30 and 60 mg kg^{-1}), given alone (groups 3 and 4), was unable to induce ACF and tumours growth. When the treatment with SE was coupled with the initiating treatment with AOM (groups 5 and 6), a significant reduction of number of ACF and tumours was observed.

Table 1. Induction of aberrant crypt foci (ACF) and tumours in rats exposed for two years to senna pod extract (SE) with or without azoxymethane (AOM).

Group and Treatment	ACF $colon^{-1}$	Tumour $colon^{-1}$
1 Control	0	0
2 AOM 7.5 mg kg^{-1}	90.3±12.6	2.20±0.29
3 SE 30 mg kg^{-1} day^{-1}	0	0
4 SE 60 mg kg^{-1} day^{-1}	0	0
5 AOM 7.5 mg kg^{-1}+ SE 30 mg kg^{-1} day^{-1}	25.0±4.22[a]	1.25±0.18[a]
6 AOM 7.5 mg kg^{-1}+ SE 60 mg kg^{-1} day^{-1}	34.2±7.04[a]	1.35±0.21[b]

Means ± s.e. mean; n=10 for each experimental group. [a]$p<0.01$and [b]$p<0.05$ vs group 2 (ANOVA followed by Dunnett's test)

It is concluded that senna was devoid of tumour-initiating activity. On the contrary, senna might exert an anti-tumoural activity on rat colon carcinogenesis.

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20P FUNCTIONAL CHARACTERISATION OF α1-ADRENOCEPTOR SUBTYPES IN HUMAN SKELETAL MUSCLE RESISTANCE ARTERIES

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Recently we reported that contractile responses to noradrenaline (NA) in human skeletal muscle resistance arteries (SkMRAs) are predominantly mediated by α_1-adrenoceptors (Jarajapu *et al.*, 2001). In this study the α_1-adrenoceptor subtypes were functionally characterised using agonists NA (non-selective) and A61603 (α_{1A}-selective), the antagonists prazosin (α_1-selective), 5-methyl-urapidil (α_{1A}-selective, 5MU) and BMY7378 (α_{1D}-selective) and the alkylating agent, chloroethylclonidine (CEC) (Hancock 1996).

SkMRAs (normalised diameter $L_{0.9}$ = 307±10 μm, n=56/21 no. of segments/no. of patients) were isolated from the non-ischaemic areas of limbs amputated for critical limb ischaemia. Arterial segments were mounted on a small vessel wire myograph in physiological saline solution at 37°C continuously bubbled with carbogen and normalised according to Mulvany & Halpern (1977). Propranolol (1 μM), corticosterone (3 μM), cocaine (3 μM) and RS79948 (0.1 μM) (α_2- selective antagonist) were present throughout the experimental protocol. Arterial segments were activated by 123 mM KCl twice and 10 μM NA. Cumulative concentration response curves (CRCs) were obtained for NA and A61603 in the absence and presence of the antagonists. Arterial segments were washed for 60 min after 30 min incubation with CEC.

Prazosin and 5MU competitively antagonised the responses to NA giving pA_2 values of 9.18 (n=10) and 8.48 (n=10) with Schild slopes of 1.3 (95% CL: 1.14-1.51) and 0.99 (95% CL: 0.74-1.25) respectively. NA exhibited biphasic CRCs in the presence of 300 nM 5MU indicating the presence of a 5MU-resistant receptor subtype. CEC (1 μM) had no effect on the CRCs to NA (n=5) showing the absence of α_{1B}- subtype but 10 μM CEC reduced the maximum responses to NA ($P<0.05$, n=8) and A61603 ($P<0.05$, n=5) to 69% of the control indicating an effect on the α_{1A}- subtype at higher conc. The potency of NA was not shifted by 10 and 100 nM BMY7378 but a small shift was observed at 1 μM BMY7378, giving a pK_B value of 6.5±0.3 (n=5). Baseline tension was not affected by 5MU and CEC during the incubation period of 30 min..

These results show that contractile responses to NA in human SkMRAs are predominantly mediated by the α_{1A}-adrenoceptor subtype. A small population of a 5MU-insensitive α_1-adrenoceptor subtype is also involved in these responses.

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21P NORADRENALINE-INDUCED HYPERTROPHIC RESPONSE IN CARDIOMYOCYTES FROM MONOCROTALINE-TREATED RATS

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Adult rat cardiomyocytes (CM) contain β- and α_1-adrenoceptors (AR). Noradrenaline (NA) is an agonist at α_1- and β_1-AR. Thus, we studied in CM a) whether simultaneous β_1-AR stimulation affects α_1-AR-mediated increase in rate of protein synthesis by NA, and b) whether this is changed in monocrotaline (MCT)-treated rats with right ventricular hypertrophy (RVH).

CM were (isolated from right and left ventricles of 12 weeks old male Wistar-rats), and rate of protein synthesis (PS; incorporation of [^{3}H]phenylalanine into CM) was determined as recently described (Pönicke et al., 2000).

In right (RV) and left (LV) ventricular CM NA (1 nM – 10 μM) concentration-dependently increased PS; increases at 1 μM NA and pEC_{50}-values [M] were (n=10) in RV: 38±7% and 6.6±0.1, in LV: 51±9% and 6.5±0.2. This effect was inhibited by 0.1μM prazosin, and significantly (paired t-test, $p<0.01$) enhanced by 0.3μM of β_1-AR selective CGP 20712A (CGP; RV: 61±8%, LV: 69±8%). Moreover, pEC_{50}-values [M] for NA increased significantly (RV: 7.3±0.2, LV: 7.2±0.1, $p<0.05$ vs. control). Furthermore, 1μM dobutamine and 10μM forskolin significantly suppressed NA-induced PS-increase.

Six weeks after a single injection of 60 mg/kg i.p. MCT rats developed marked RVH (LV:RV-weight 1.69±0.2 vs. 3.44±0.1 in control, n=29); some of these rats (ca. 35%) developed cardiac failure. In RV-CM of MCT-treated rats α_1-AR density (assessed by [^{3}H]-prazosin binding) was significantly reduced (14.1±2.0 vs. 27.0±2.6 fmol/mg protein, n=5-6, $p<0.01$) while in LV-CM of MCT-treated rats α_1-AR density was not considerably altered (22.8±1.6 vs. 26.3±2.8 fmol/mg protein, n=4-6). In addition, in membranes obtained from RV and LV of MCT-treated rats the immunodetectable amount of $G_{q/11}$-protein (assessed by Western-blots with QL-anti-serum) was decreased (RV: 1.2±0.1 vs. 2.1±0.1; LV: 1.5±0.1 vs. 2.0±0.1 arbitrary units, n=9, $p<0.01$). In RV- and LV-CM of MCT-treated rats concentration-response-curves for NA-induced PS-increase were shifted to the right (pEC_{50}-values [M] RV: 5.9±0.2, LV: 6.2±0.1) and PS-increases were markedly reduced (increases at 1 μM NA were in RV: 16±5%, in LV: 32±4%). In the presence of 0.3 μM CGP, however, concentration-response curves for NA-induced PS-increases were almost identical with those obtained in control-CM with CGP (increases at 1 μM NA and pEC_{50}-values [M] in RV (n=10): 58±6% and 7.1±0.1; in LV (n=16): 72±6% and 7.1±0.1).

We conclude that a) in adult rat RV- and LV-CM NA-induced PS-increase is inhibited by β_1-AR stimulation via a cyclic AMP-dependent pathway b) in MCT-treated rats NA-induced PS is decreased possibly due to enhanced β_1-AR inhibition and/or reduction in $G_{q/11}$-protein.

Pönicke, K. et al. Br. J. Pharmacol. 129: 1723-1731, 2000.

Supported by a grant of the Deutsche Forschungsgemeinschaft (Br 526/6-1).

22P LEUKOTRIENE B_4 IS AN INDIRECTELY ACTING VASOCONSTRICTOR IN THE GUINEA PIG PULMONARY ARTERY

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Leukotriene B_4 (LTB_4) is a chemotactic agent with contractile properties in the guinea pig lung parenchyma (Hansson et al., 1981; Piper and Samhoun, 1982). Since lung parenchymal preparations consist of both airway and vascular components and the contractile effects of LTB_4 on guinea pig airways are negligible (Dahlén et al., 1983), the aim of this study was to examine if LTB_4 had vascular effects. As two different subtypes of receptors for LTB_4 (BLT_1 and BLT_2) recently have been proposed (Yokomizo et al., 2000), we also assessed the effects of purported subtype selective receptor antagonists.

Ring preparations of left and right branches of main pulmonary arteries from male Dunkin Hartley guinea pig (300-450g) were set up in 5ml organ baths containing Tyrode's solution kept at 37 °C and gassed with 6.5% CO_2 in O_2. Mechanical responses were recorded isometrically using an EMKA data acquisition system and a resting tension of 10mN. Because of tachyphylaxis (Dahlén et al., 1983), LTB_4 was added as a single concentration in each experiment. Drugs were administered 30 min prior to application of LTB_4. Responses (mean±s.e.m.) are expressed as a percentage of a reference contraction to KCl (40mM), and the area under the contraction-time curves (AUC) used for global assessment of the contractions. Statistical analysis was performed by using Dunnett's post-hoc test. A p value of less than 0.05 was considered significant.

LTB_4 induced concentration-dependent contraction of the guinea pig pulmonary artery (30nM: 7.0±3.8% [n=4]; 300 nM: 22.5±4.2% [n=13]; 1000nM:42±2.8% [n=10]). The contractions to LTB_4 (1μM [n=7]) were rapid in onset with peak amplitude reached within 1 min ($AUC_{0\text{-}5min}$: 57.7±10.9 [n=7]). The combination of mepyramine (H_1 receptor antagonist, 1μM) and metiamide (H_2 receptor antagonist, 1μM) significantly inhibited the contractions to LTB_4 ($AUC_{0\text{-}5min}$: 19.7±6.1 [n=7]). The TP receptor antagonist BAYu3405 (3μM) significantly inhibited LTB_4 ($AUC_{0\text{-}5min}$: 27.0±9.4 [n=7]). The combination of BAYu3405 with antihistaminics abolished the contractions ($AUC_{0\text{-}5min}$: 1.2±0.5 [n=7]). Both the unselective BLT receptor antagonist, ONO-4057 (10μM [n=4]) and the proposed BLT_1 selective receptor antagonist, U-75302 (1μM [n=5]) significantly and markedly inhibited the contractions to LTB_4 ($AUC_{0\text{-}5min}$: 0.3±0.2 and 4.7±2.0, respectively).

In conclusion, LTB_4 contracted the guinea pig pulmonary artery and the mechanism of contraction involved release of both histamine and thromboxane. The results with BLT receptor antagonists suggest that the vascular effects of LTB_4 were mediated via a BLT_1 receptor.

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23P INVOLVEMENT OF HEAT SHOCK PROTEIN (Hsp-90) IN 17-BETA-OESTRADIOL-INDUCED VASORELAXATION *IN VITRO*

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17-β-oestradiol (E2) is known to stimulate nitric oxide synthesis in vascular endothelium but the molecular mechanism responsible for this effect remains to be elucidated. Recent studies have shown that in HUVEC E2 induces an Hsp-90-eNOS activation that is abrogated by geldanamycin, a potent Hsp-90 inhibitor, suggesting that Hsp-90 plays an important role in the rapid estrogen receptor-mediated modulation of eNOS (Shah *et al.*, 1999; Russell *et al.*, 2000). In this study we examined the possible participation of this pathway in the rat aorta rings *in vitro*.

Thoracic aorta was rapidly excised from male Wistar rats (Charles River, 200-250 g) stunned with CO_2 and sacrificed by exanguination. Vascular tissue was cleaned from fat and rings of 2-3 mm width were cut and placed in organ baths filled with oxygenated (95% O_2-5% CO_2) Krebs solution at 37°C and then connected to an isometric transducer under resting tension of 0.5 g. After standardisation of the tissue with PE (10^{-6} M), a stable contraction of the tissue was obtained with PE (10^{-6} M) and a cumulative concentration-response curve to Ach (10^{-8} -10^{-5} M) was performed to check the presence of an intact endothelium. After washing the tissue was contracted with PE (10^{-6}M) and a cumulative concentration-response curve (0.01-10 μM) to E2 was performed. Concentration-response curves to E2 were also obtained in presence of L-NAME (100 μM), tamoxifen (10 μM) and geldanamycin (10 μM). All results are expressed as mean ± s.e.m. and analysed by using two way analysis of variance (ANOVA) followed by Bonferroni post-test.

E2 (0.01-10 μM) induced a concentration-dependent and endothelium-dependent vasodilatation (max relaxation 63 % ± 5.8, n=6) that was inhibited by 15 minutes pre-incubation of the rings with L-NAME (100 μM) (max relaxation 19% ± 7.5, n=6, $p<0.001$); E2 had no effect on rings without endothelium. When rings were incubated with geldanamycin (20 μM) for 15 minutes there was a significant reduction in E2-mediated vasodilatation (max relaxation 33 % ± 3.5, n=6, $p<0.001$). In addition, 10 minutes pre-incubation of the rings with E2 receptor antagonist tamoxifen (10 μM), was able to inhibit significantly E2-induced vasorelaxation (max relaxation 41 % ± 5.9, n=6, $p<0.05$).

These findings suggest that Hsp-90 plays a key role in rapid oestrogen receptor-mediated regulation of eNOS.

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24P FUNCTIONAL RESPONSE OF APELIN-13, THE NOVEL ENDOGENOUS LIGAND FOR THE ORPHAN RECEPTOR APJ, IN HUMAN INTERNAL MAMMARY ARTERY: COMPARISON WITH NATRIURETIC PEPTIDES.

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Endothelin-1 (ET-1) is a potent, long-acting vasoconstrictor peptide continuously released by the endothelium. Plasma levels of ET-1 are increased in cardiovascular disease. Apelin-13 has recently been discovered as the endogenous ligand for the orphan receptor APJ (Tatemoto *et al.*, 1998), which is localised to the human cardiovascular system (Katugampola & Davenport, 2000). Our objective was to determine the functional role of apelin-13 in human arteries and to compare the effects with the known vasodilators the natriuretic peptides (atrial; ANP, brain; BNP, and C-type; CNP).

Sections of internal mammary artery from eight patients undergoing coronary artery bypass operations were cut into 3mm rings, denuded of their endothelium, and mounted in 5ml organ baths for the measurement of isometric tension (37°C; bathed in oxygenated Krebs' solution). Initial responses were obtained with 100mM KCl. Constrictions were induced with a submaximal concentration of ET-1, and once the responses reached a plateau three-fold cumulative concentration-response curves to the natriuretic peptides (0.1nM-300nM), and apelin-13 (0.1-1000nM) were constructed. Results were expressed as % relaxation of constrictor response ± s.e.mean. EC_{50} values were determined using the iterative curve-fitting software Fig P (Biosoft, Cambs, UK) and expressed as geometric means with 95% confidence interval (CI).

ET-1 potently constricted all segments tested. Only 4 of the 5 vessels tested reponded to BNP and only responders are included in the graph and analysis. The four peptides reversed the ET-1-mediated constriction with differing potencies (CNP> ANP> apelin-13> BNP; Figure 1), with EC_{50} values of 3.09 (95% CI: 0.55-17.5), 22.3 (95% CI: 8.96-55.4), 22.1 (95% CI 3.95-123) and 49.5 (95% CI: 3.98-615) nM respectively. The magnitude of response to the highest concentration of NP also differed with ANP> CNP> BNP> apelin-13 (E_{MAX} 106.6±2.3, 53.2±6.3, 42.0±14.7 and 28.4±5.6% respectively).

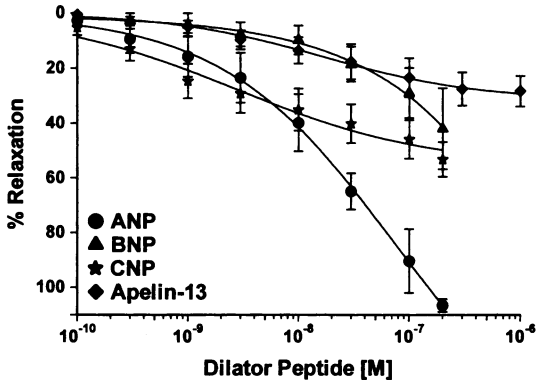

Figure 1 Cumulative concentration-response curves to NPs and apelin-13, reversing constrictions induced by ET-1 (n = 4-6 individuals).

Of the compounds tested, only the circulating hormone ANP fully reversed established constrictions induced by ET-1 in human internal mammary artery *in vitro*. At the concentration tested, BNP, CNP, and the novel orphan receptor ligand apelin-13 partially reversed responses to ET-1, with apelin-13 having the lowest maximum response of the peptides tested. The vasodilator action of apelin-13 *in vivo* remains to be determined.

Tatemoto *et al.*, 1998 *Biochem Biophys Res Comm*, **251**, 471-476.
Katugampola & Davenport 2000 *Br J Pharmacol*, **131**, 85P.

25P THE EFFECT OF NEUROPEPTIDE Y (NPY) ON THE GTPase ACTIVITY OF RAT LEFT VENTRICLE Gi PROTEINS.

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Neuropeptide Y (NPY) is a highly conserved 36 residues peptide belonging to a family of structurally related peptides. Neuropeptide Y and its receptors are distributed among the central nervous system and in the periphery. It represents the most abundant neuropeptide in the heart; by activating at least two different Gi proteins-coupled receptors (Millar *et al*, 1994), NPY modulates heart activity (McDermott *et al.* 1993). Moreover, recent findings have demonstrated the ability of amphiphilic peptides, including NPY, to directly activate G_i proteins thus suggesting the involvement of alternative or additive mechanisms to the classical receptor-mediated pathways (Grundemar *et al*, 1994).

The aim of this work was to investigate the effect of NPYon the GTPase activity of Gi proteins of rat left ventricle. Either receptor-mediated or direct activation of Gi proteins would justify the NPY-dependent reduction of adenylate cyclase activity measured in rat heart (Millar *et al*, 1994).

To investigate the possible activation of GTPase activity, we prepared membranes from the left ventricle of adult male Wistar rats (150-250). The GTPase activity of membrane proteins (3-10 μg) was measured according to Ogadaki *et al.* (1999) by the release of ^{32}Pi from [γ-^{32}P]GTP. High affinity hydrolysis of labelled nucleotide (from 0.1 to 0.8 μM) was measured after 15-min incubation in medium with or without Mg^{2+} ions. Basal and NPY-induced GTPase activities were calculated as the difference between the high (low GTP) and low affinity hydrolysis (100 μM cold GTP). Results are the mean ± s.e. of 3-5 different experiments performed in triplicate. NPY, from 0.1 to 50 nM of concentration, stimulated basal GTPase activity of rat left ventricle, reaching a maximum at 10 nM. The stimulation was evident only when GTPase activity was measured in the absence of Mg^{2+} ions: In this case basal GTP activity increased from 50 ± 15 (basal) to 288 ± 38 pmoles / mg of proteins/15 min in NPY (10 nM) pretreated membranes. A similar effect was obtained using NPY (18-36, 1 μM). Under these experimental conditions, NPY shifted Km of the enzyme from 0.522 ± 0.100 μM to 0.051* ± 0.020 μM and Vmax: from 534 ± 25 to 415 ± 49 pmoles / mg of proteins/15 min (means ± s. e. of 4 experiments performed in duplicate). In the presence of Mg^{2+} ions a significant increase of basal GTPase activity was also observed (from 50 ± 15 to 399 ± 45 pmoles/mg of proteins/15min), but NPY did not produce any change of the enzymatic activity.

The present results suggest that an alternative or additive mechanism to receptor activation could be hypothesised for NPY also in the rat heart

*Significantly different from control (Student's t test $p<0.05$)

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Millar *et al.* Am J. Physiol. 266: C1271-1277, 1994

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26P INVOLVEMENT OF PROTEIN KINASE C (PKC) IN THE EFFECT OF ANTIARRHYTHMIC PEPTIDE (AAP) ON CARDIAC GAP JUNCTIONS

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Antiarrhythmic peptides are a group of synthetic oligopeptides derived from the natural AAP (GP-4Hyp-GAG) (Dhein & Tudyka, 1995), which prevent ischemia-induced ventricular fibrillation (Dhein et al., 1994) by enhancing gap junction current (Müller et al., 1997). Since this current can be regulated by PKC in the heart, the purpose of this study was to evaluate the role of PKC in the effect of the synthetic AAP10 (GAG-4Hyp-PY-$CONH_2$). Isolated pairs of adult male guinea pig cardiomyocytes were superfused with saline and submitted to double cell patch clamp. Each cell was connected to a switch clamp amplifier (SEC05, npi, Tamm, Germany), whole cell configuration was established and holding potential V_H adjusted to -40 mV. While cell 2 was kept at V_H, the potential of cell 1 was clamped to potentials from -50 to +50 mV relative to V_H (200 ms pulses). Under these conditions, the gap junction current (I_{GJ}) can be determined in cell 2 (Dhein, 1998). I_{GJ} was measured under control conditions every 5 min. for 90 min (n=7) in absence or presence of 50 nM AAP10 (added to the bath). In order to inhibit PKC, experiments were repeated with either bisindolylmaleimide I (BIM, 0.2 μM), CGP54345 (10 μM, specific PKCα inhibitor) or HBDDE (50 μM, inhibitor of PKCγ and α) or monensin (2 μM, inhibits transfer of connexins from Golgi to membrane) in the pipette. In addition, the ability of AAP10 to activate PKC was investigated in lysates of cardiomyocytes. Moreover, we investigated the phosphorylation of Cx43 in HeLa-cells transfected with Cx43 under 50 nM AAP10. Initial gap junction conductance G_{GJ} was 38±5 nS. We found that under control conditions, there was a continuous decline in G_{GJ} by -0.5 to -2.5 nS/min, which was reversed to an increase by +0.5 to +1.0 nS/min in the presence of AAP10. This AAP10-induced increase in G_{GJ} could be completely inhibited by BIM and by HBDDE, but only partially by CGP54345. Monensin also significantly suppressed the AAP10 effect. In addition, we found that AAP10 significantly activated PKC in lysates of cardiomyocytes, which could also be suppressed by HBDDE. In Cx43 transfected HeLa cells exposure to 50 nM AAP10 led to increased ^{32}P-incorporation in Cx43. This could be completely prevented by BIM. Thus, we conclude that AAP10 enhances G_{GJ} via PKC, predominantly PKCγ and partially PKCα, leading to enhanced phosphorylation of Cx43. This phosphorylation may cause either enhanced gap junction conductivity with increased I_{GJ} or -as suggested by the sensitivity to monensin- increased incorporation of channels into the membrane.

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supported by the DFG (Dh 3/9-1)

27P REGIONAL HAEMODYNAMIC RESPONSES TO FLUOXETINE OR NISOXETINE IN CONSCIOUS SPRAGUE-DAWLEY RATS

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The novel anti-obesity drug, sibutramine (Connoley *et al.*, 1999), inhibits noradrenaline (NA) and 5-HT reuptake from nerve terminals (Luscombe *et al.*, 1989), and modulates splanchnic haemodynamics (Gardiner *et al.*, 1998). The purpose of this study was to compare the haemodynamic effects of selective inhibition of 5-HT reuptake (with fluoxetine) and NA reuptake (with nisoxetine).

Male Sprague-Dawley rats (350-450 g) were anaesthetised (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.) and had pulsed Doppler flow probes implanted to monitor coeliac, mesenteric and hindquarters blood flows. At least 10-14 days later, animals were re-anaethesised and had catheters implanted in the distal abdominal aorta, via the ventral caudal artery (for measurement of heart rate and blood pressure) and i.p. cavity (for drug administration). Experiments were initiated on the following day and were continued over the subsequent 3 days. On the first day, each animal received either a high dose (30 mg kg^{-1}) or low dose (10 mg kg^{-1}) of the test drug, on the second day animals received vehicle (0.5 ml sterile water + 0.5 ml 5 % dextrose), and on the final day, the other dose of test drug was administered.

Table 1 summarises some of the results. Baseline values for the fluoxetine and nisoxetine groups, respectively, were: heart rate (HR; beats min^{-1}) 356±13, 327±9; blood pressure (BP; mm Hg) 99± 3, 100± 2; vascular conductance ([kHz mm Hg^{-1})]10 ^{3}), coeliac (CVC) 129±9, 87±7 (P<0.05; Mann-Whitney Test), mesenteric (MVC) 91±7, 84±6, and hindquarters (HVC) 51±7, 50±3. Fluoxetine and nisoxetine caused pressor and tachycardic effects, and vasoconstriction in the coeliac and mesenteric vascular beds. The pressor effects of both drugs were most apparent at a dose of 10 mg kg^{-1}. Nisoxetine had little effect on the hindquarters vascular bed, whereas fluoxetine produced a substantial vasodilatation.

Table 1: Cardiovascular changes (Δ) 1 and 5 min after fluoxetine or nisoxetine. HR; BP; CVC, MVC, HVC (%). Values are mean ± s. e. error of mean; $^{*}P$<0.05 vs. baseline (Friedman's Test); $^{\#}$ P <0.05 vs. 1 min value (Friedman's Test); $^{\circ}$ P <0.05 vs. fluoxetine (Mann-Whitney Test, with Holm-Bonferroni correction) (Ludbrook, 1998).

Fluoxetine (n=8,7)	**10 mg kg^{-1}**		**30 mg kg^{-1}**	
	1 min	5 min	1 min	5 min
ΔHR (beats min^{-1})	23 ±10	67 ±10$^{*\#}$	21±12*	46±13*
ΔBP (mm Hg)	10 ±4*	13 ±6*	10±5*	-5±5$^{\#}$
ΔCVC (%)	-12±7	-3 ±10$^{\#}$	-13±7*	14±10$^{\#}$
ΔMVC (%)	- 36 ±7*	-21±9$^{*\#}$	-33±4*	0±9$^{\#}$
ΔHVC (%)	26 ±5*	30 ±7*	23±9*	21±9*
Nisoxetine (n=7)	**10 mg kg^{-1}**		**30 mg kg^{-1}**	
ΔHR(beats min^{-1})	15 ±6	-4 ±5°	16 ±7	24±14*
ΔBP (mm Hg)	18 ±5*	6 ±2$^{\#}$	24±4$^{*\circ}$	1±2$^{\#}$
ΔCVC (%)	-21±4*	-17±7*	-33±5$^{*\circ}$	-20±2$^{*\circ}$
ΔMVC (%)	-30±5*	-12±3$^{*\#}$	-46±3*	14±6$^{*\#}$
ΔHVC (%)	-5 ±4°	5 ±7$^{\#\circ}$	-3±7°	13±6*

It has yet to be determined whether the co-administration of these drugs produce haemodynamic changes similar to sibutramine.

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28P MODULATION OF ANANDAMIDE EVOKED RELAXATION IN THE RAT ISOLATED MESENTERIC BED BY THE PGI_2 ANALOGUE CICAPROST

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Anandamide is the endogenous ligand for the cannabinoid CB_1 receptor. In addition to its CNS effects, activation of CB_1 receptors leads to a profound changes in the peripheral vasculature, where it causes vasodilatation and hypotension (Randall and Kendall, 1998, Hillard, 2000). Previously, it has been shown in the rat isolated mesenteric bed that ATP induced vasodilatation is in part mediated by CB1 receptors (Stanford and Mitchell, 1999). Under these conditions, prostacyclin and NO are also released. The aim of this study was to investigate any of the possible effects that NO or PGI_2 may have on the actions of anandamide in the rat isolated mesenteric bed.

Male Wistar rats (250-300g) were anaesthetised with sodium pentobarbitone (100 mgkg^{-1}; *i.p.*) and killed by cervical dislocation. The mesenteric artery was cannulated and the mesentery excised. The bed was perfused at a constant rate with Kreb's buffer heated to 37°C and gassed with 95% O_2, 5% CO_2. Perfusion pressure, measured by an arterial cannula, was raised to approx. 120 mmHg by methoxamine ($1x10^{-5}$M) added to the perfusate. The effects of 1-4μl volume injections of anandamide ($3x10^{-11}$ to $1x10^{-7}$ M) were recorded. Some experiments were performed in the presence of threshold concentrations of sodium nitroprusside (SNP) ($3x10^{-9}$M) or the prostacyclin analogue cicaprost ($3x10^{-10}$M). All data is shown as mean±S.E.Mean. Statistical significance was determined by two-way ANOVA.

Anandamide induced concentration dependent relaxation of the perfused mesenteric bed (EC_{50} $1.6x10^{-8}$M, n=8). In the presence of cicaprost ($3x10^{-10}$M) which induced a 13.15±1.0% reduction in perfusion pressure, the concentration response to anandamide was shifted to the right (EC50 $6.125x10^{-8}$M, P=0.0043, n=5) (Fig. 1A). In the presence of SNP ($3x10^{-9}$M) which induced a 17.385±3.8% reduction in perfusion pressure, the EC50 value for anandamide was not altered (Fig B).

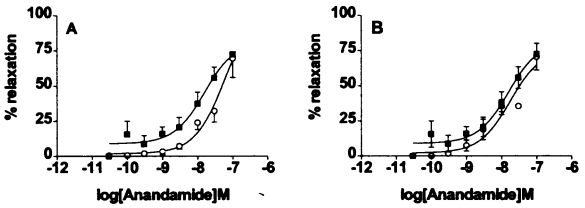

Figure 1.Demonstrates the effect of anandamide on the rat isolated mesenteric bed in the presence and absence (filled squares) of A) cicaprost $3x10^{-10}$M (open diamonds), B) SNP $3x10^{-9}$M (open circles).

Here we have demonstrated that cicaprost but not SNP has an inhibitory effect on anandamide induced relaxation. The mechanisms by which it has this effect are yet to be elucidated.

The authors would like to acknowledge Dr S.Price for invaluable technical assistance.

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29P PEROXISOME PROLIFERATOR-ACTIVATED (PPAR) γ LIGANDS INDUCE VASCULAR SMOOTH MUSCLE CELL APOPTOSIS

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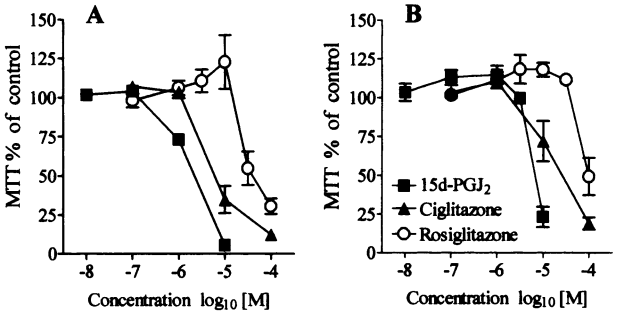
The nuclear receptor, peroxisome proliferator-activated receptor (PPAR)γ is expressed in endothelial cells, where it mediates ligand induced apoptosis (Bishop-Bailey & Hla, 1999). Here we describe the expression and effects of PPARγ ligands on the proliferation of vascular smooth muscle cells (SMC) isolated from adult rat aorta (WKY3M-22), which resemble medial SMC, or from neonatal rat aorta (WKY12-22), which resemble a developmental/intimal phenotype (Lemire et al., 1994).

WKY3M-22 and WKY12-22, gifts from Dr. David Han (University of Washington, Seattle), were maintained in DMEM supplemented with 2 mM glutamine, penicillin (1000 IU.ml^{-1}), streptomycin (1 mg.ml^{-1}), and 10% FCS (37°C; 5% CO_2; 95% air). PPARγ expression was measured by Northern blot analysis, and by immunoflorescence as previously described (Bishop-Bailey & Hla, 1999). Cells in 96-well plates were serum starved for 48h, before treatment for 48h with the PPARγ ligands, 15-deoxy-$\Delta^{12,14}$ (15d)-PGJ_2 (1-10μM), ciglitazone (1-100μM), or rosiglitizone (a gift from Dr. Steven Smith, SmithKline-Beecham; 1-100μM). Cell death was assessed by the MTT assay, and nuclear morphology was assessed by staining with Hoechst 33258, as previously described (Bishop-Bailey & Hla, 1999). Drug vehicle (0.1% DMSO) had no effect on any parameter measured.

PPAR-γ mRNA and protein were expressed in WKY12-22 and WKY 3M-22 SMC. All the PPARγ ligands induced cell death with a rank order of potency of 15d-PGJ_2 > ciglitazone> rosiglitizone in both WKY12-22 (Figure 1A) and WKY3M-22 (Figure 1B) SMC. The PPARγ ligands were approx. 3-10 fold more potent on WKY 12-22 than WKY 3M-22 SMC. Cell death was characterised as apoptosis, by the presence of condensed nuclei after treatments.

Figure 1. PPARγ ligand induced cell death (% of control MTT value) in WKY12-22 (A) and WKY3M-22 (B) vascular smooth muscle cells. Data is mean ± s.e.m. of n=9-20 determinations from 7 experiments.



PPARγ is more greatly expressed in intimal SMC *in vivo* after balloon angioplasty (Law et al., 2000). Here we show that PPARγ ligands induce cell death in 'intimal' and 'medial' SMC. The use of PPARγ agonists may therefore be a novel therapy for the excessive intimal cell proliferation seen in vascular pathologies such as restenosis following angioplasty.

Funded by the BHF (FS/99047).

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30P BLOCKADE OF PRE- AND POST-SYNAPTICALLY LOCATED AT1 -RECEPTORS: A COMPARISON BETWEEN LOSARTAN, IRBESARTAN, TELMISARTAN, VALSARTAN, CANDESARTAN, EPROSARTAN AND EMBUSARTAN

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Numerous studies have shown that angiotensin II (Ang II) enhances sympathetic nervous transmission. The pithed rat is a high-renin model, in which endogenously generated angiotensin II facilitates neurally mediated increments in vascular resistance. The objective of the present study was to compare the sympatico-inhibitory properties of several AT_1-receptor blockers (**los**artan, **irb**esartan, **telmi**sartan, **val**sartan, **cande**sartan, **epro**sartan and **embu**sartan) with the inhibition of the direct vasoconstrictor effect of exogenous Ang II in the pithed rat model.

To investigate blockade of pre-synaptic AT_1-receptors we studied the effect of AT_1-blockade on the sequelae of electric stimulation of the thoraco-lumbar sympathetic outflow (0.25-8 Hz). To investigate blockade of postsynaptic AT_1-receptors we studied the effect of the AT_1-antagonists on dose-response curves (DRC) elicited by exogenous Ang II. In addition, the effect of AT_1-blockade on post-synaptic α-adrenoceptor mediated responses was studied using exogenously administered noradrenaline (NA).

Male Wistar rats weighing 260–310 g were used. The animals were anaesthetized using hexobarbital 100 mg/kg i.p., pithed and subsequently artificially respirated. After 15 minutes, either saline or the AT_1-antagonists were administered i.v. Another 15 minutes later, spinal cord stimulation was applied or DRC to Ang II (i.v.) and NA (i.v.) were constructed. Blood pressure was monitored via a carotid arterial catheter. n=6-8 per group Doses of AT_1 blockers used were 0.003-60 mg/kg. The stimulation-induced increase in diastolic blood pressure (DBP) as well as the Ang II elicited DBP response could be dose-dependently reduced by all AT_1-receptor blockers.

The order of potency with respect to sympatico-inhibition was epro > cande = embu = telmi = val > los > irbe. (comparison of doses which at 2 Hz reduced ΔDBP by 20 mmHg (50% of maximum inhibition, '>' signifies $p<0.05$, Students t-test, see table 1). The order of potency regarding inhibition of Ang II-induced DBP-increase was cande > embu = val = epro = telmi > irbe > los (comparison of pA_2-values, '>' signifies $p<0.05$, Students t-test, see table 1). None of the AT_1-blockers had any effect on pressor responses to exogenous NA (see table 1). We conclude that the facilitating effect of Ang II on sympathetic neurotransmission is mediated by pre-synaptically located AT_1-receptors. The order of potency regarding this sympatico-inhibition differs from the order of potency regarding inhibition of the direct pressor effects of Ang II.

These findings suggest differences in affinity of the AT_1-blockers for the pre- and postsynaptic AT_1-receptor.

AT_1-antagonist	Sympatholytic Dose ED_{20} (-log mol/kg)	Ang II blockade pA_2 (-log mol/kg)	NA response EC_{50} (-log mol/kg)
Control			8.52 ± 0.09
Eprosartan	6.32 ± 0.12	7.20 ± 0.03	8.61 ± 0.05
Candesartan	5.77 ± 0.10	8.01 ± 0.01	8.29 ± 0.09
Embusartan	5.62 ± 0.13	7.25 ± 0.16	8.38 ± 0.07
Telmisartan	5.53 ± 0.12	7.01 ± 0.10	8.41 ± 0.09
Valsartan	5.50 ± 0.12	7.20 ± 0.17	8.32 ± 0.09
Losartan	5.12 ± 0.11	5.81 ± 0.20	8.42 ± 0.10
Irbesartan	4.76 ± 0.11	6.40 ± 0.05	8.47 ± 0.06

Table 1. Potency of the AT_1-antagonists concerning sympatico-inhibition (left column) and inhibition of Ang II induced DBP-increase (middle column). Effect of AT_1-blockade on NA-responses (right column). Values shown as mean ± SEM.

31P TOLCAPONE, ENTACAPONE AND NITECAPONE STIMULATE RENAL D1-LIKE RECEPTORS AND DECREASE RENAL TUBULAR SODIUM ABSORPTION

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In recent years, several nitrocatechol derivatives (tolcapone, entacapone and nitecapone) have been developed and found to be highly selective and potent inhibitors of catechol-*O*-methyltransferase (COMT). More recently, natriuretic properties were described for two of these compounds (entacapone and nitecapone), though this was not accompanied by major increases in the urinary excretion of dopamine (Eklöf et al., 1997; Hansell et al., 1998). The aim of the present work was to evaluate whether natriuresis produced by nitrocatechol derivatives is dependent on the enhanced availability of renal dopamine or direct stimulation of dopamine receptors. Adult male Wistar rats were given the COMT inhibitor (tolcapone, entacapone and nitecapone, 30 mg kg^{-1}, p.o.) or vehicle (10 % Tween 80, in saline). Fifteen minutes later, all animals were submitted to an oral hydration (tap water, 20 ml kg^{-1}, p.o.) in order to promote diuresis. In some experimental groups, rats were given the D_1–like receptor antagonist Sch 23,390 (2x30 μg kg^{-1}, i.p. in saline) 15 min before the administration of COMT inhibitors and repeated 3 hours later. The interaction of nitrocatechol derivatives with D_1–like receptors was evaluated by their ability to displace [^{3}H]-Sch23390 binding from membranes of rat renal cortex (Galbusera et al., 1988) and cAMP production (Cheng et al., 1990) in Opossum kidney (OK) cells (ATCC 1840-CRL). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. Urinary excretion of sodium (μmoles h^{-1}) was markedly increased by all three nitrocatechol derivatives: vehicle, 55.0±5.6; tolcapone, 97.5±9.3; entacapone, 98.4±9.3; nitecapone, 120.5±12.6. Pretreatment with the selective D_1–like receptor antagonist Sch 23390 completely prevented their natriuretic effects. Tolcapone and entacapone did not change the urinary excretion of dopamine, whereas nitecapone produced a 2-fold increase in urinary dopamine (from 2.2±0.7 to 4.5±0.3 μmoles h^{-1}). Tolcapone, entacapone, nitecapone and dopamine were found to displace [^{3}H]-Sch 23390 from rat renal cortical membranes with IC_{25} values of 38 (19, 79), 60 (24, 149), 65 (21, 196) and 147 (72, 303) μM, respectively. In OK cells, all three nitrocatechol derivatives at (100 μM) significantly increased cyclic AMP accumulation (results are percent of control values: tolcapone, 352±102%; entacapone, 229±69%; nitecapone, 220±7%) and reduced Na^+/H^+ exchange (results are percent of control values: tolcapone, 64±14%; entacapone, 66±14%; nitecapone, 9±8%) and Na^+-K^+-ATPase (results are percent of control values: tolcapone, 65±4%; entacapone, 48±5%; nitecapone, 38±13%) activities, this being prevented by blockade of D_1–like receptors. In conclusion, stimulation of D_1–like dopamine receptors and inhibition of Na^+/H^+ exchange and Na^+-K^+ ATPase activities by nitrocatechol COMT inhibitors may contribute to natriuresis produced by these compounds.

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Supported in part by grant PRAXIS/2/2.1/SAU/1836/95

32P EFFECT OF MELANOCORTIN AGONISTS ON JOINT INFLAMMATION

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In this study we challenged the hypothesis that melanocortin type 3 receptor (MC3-R) agonists possessed a therapeutic potential in gouty arthritis by testing their activity in a novel model of crystal induced inflammationin the rat knee joint.

Male Sprague Dawley rats (220-250g) were anaesthetized and injected intra-articular (i.a.) with a 50 μl suspension of 20 mg/ml monosodium urate (MSU) crystals in the right knee, whilst the left knee received 50 μl PBS as a control. The animals were then killed by CO_2 exposure at different time points (2-72 h post injection). The knee joint was exposed, the size of the joint measured with a caliper, and the arthritic score (0= normal and 4= severe damage) calculated. The knee joint was then lavaged with 1 ml PBS supplemented with 3 mM EDTA. After centrifugation, cell-free supernatants were stored at –20C prior to analysis for interleukin (IL)-1β and IL-6 content by specific ELISA (R&D Systems, Abingdon, UK). The resulting pellet was resuspended in 100 μl of wash buffer and 100 μl Turk's solution and differential cell count performed using a Neubauer haematocytometer. Rats were pre-treated (30 min) with the synthetic MC3/4-R agonist MTII (100 μg s.c.) [1], the natural MC3-R agonist γ_2-melanotropic stimulating hormone, (γ_2-MSH; 1 $mgkg^{-1}$ i.a.) [2] or adrenocorticotropin (ACTH;1-5 μg i.a. or 20-100 μg s.c). Clinically relevant drugs were also used such as colchicine (3 $mgkg^{-1}$ s.c.), dexamethasone (1 $mgkg^{-1}$ p.o.) or indomethacin (3 $mgkg^{-1}$ p.o.). In all these experiments rats were killed at the 16 h time-point and samples analyzed as previously stated. Values were analyzed by ANOVA and Bonferroni test with *P<0.05 taken as significant.

PMN migration into the rat knee joint occurred in a time dependent manner with a peak at 16 h (3.6 ± 0.82 x 10^5 PMN ml^{-1}), well above the values of PBS-treated rats (0.33 ± 0.08 x10^5 PMN ml^{-1}) (n=6, *P<0.05). Similar differences were measured for joint size and arthritic score. The 16 h-PMN influx was preceded by cytokine release: IL-1β and IL-6 peaked at 6 h with 107 ± 37 and 124 ± 37 pg/ml^{-1}, respectively. Undetectable levels were found in PBS treated knee joints. The synthetic MC3/4-R agonist MTII and colchicine caused a 69% and 48% reduction in PMN migration respectively compared to the control values (3.0 ± 0.62 x 10^5 $PMNml^{-1}$, n=5; *P<0.05). This was associated with a respective 96% and 83% reduction in IL-1β release (and a 91% and 55% reduction in IL-6 levels) (*P<0.05). The natural agonist γ_2-MSH inhibited the inflammatory response with a 71% reduction in PMN migration (n=4, *P<0.05). Dexamethasone and indomethacin also inhibited PMN migration by 86% and 49%, respectively (control values: 4.24 ± 0.72 x10^5 PMN ml^{-1}), without affecting cytokine levels. Systemic ACTH was effective reducing cell migration from 6.04±0.45 to 1.88±0.17 x10^5 PMN ml^{-1} at 100 μg dose (n=5; *P<0.05). At 5 μg, local ACTH caused ~40% reduction in all parameters.

In conclusion, we report a novel model of rat joint inflammation sensitive not only to drugs used for the management of human gouty arthritis, but also to agonists of the MC3-R.

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[2] Russelli-Reifruss *et al.*, *Proc. Natl. Acad. Sci.* **90**: 8856-8860

This work was supported by the Arthritis Research Campaign (grant P0537). MP is an ARC Post-doctoral fellow whereas RJF is a Wellcome Principal Research Fellow.

33P ANNEXIN-1-DERIVED PEPTIDES PROTECT AGAINST MYOCARDIAL ISCHAEMIA REPERFUSION INJURY

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Annexin 1 (ANX-A1; also termed lipocortin 1) belongs to a family of calcium and phospholipid binding proteins structurally characterized by a conserved 70 amino acid sequence repeated four or eight times. This core is preceded by a unique N-terminal domain that varies among different lipocortin peptides. Expression or secretion of ANX-A1 can be stimulated by glucocorticoid and exogenously applied ANX-A1 has been shown to reduce neutrophil trafficking in several animal models of acute inflammation, including reperfusion injury of the rat mesentery (Cuzzocreaa *et al.* 1995) and myocardium (D'Amico *et al.*, 2000). A recent study has suggested that ANX-A1 peptides acts through the formyl peptide receptor (FPR) on neutrophils to down-regulate their activation (Walther *et al.* 2000). In the current study, we assessed i) the potential inhibitory actions of three peptides derived from the N-terminal region (peptides Ac2-26, Ac2-12 and Ac2-6) in a rat model of myocardial reperfusion injury, and ii) whether FPR blockade could alter the protective effect of peptide Ac-2-26.

Male Sprague-Dawley rats (200-220 g) were anaesthetised with Inactin™ (120 mg kg^{-1}, i.p.; RBI) and subjected to the following procedure: cannulation of the left jugular vein for drug administration; tracheotomy; cannulation of the right carotid artery for blood pressure measurement; thoracotomy and exposure of the heart; placement of a silk ligature around the left anterior descending coronary artery (LADCA) close to its origin. Rats were artificially ventilated with room air at a rate of 54 strokes min^{-1} and a stroke volume of 1.0 to 1.5 ml $100\ g^{-1}$. After a 30 min stabilisation period, LADCA was occluded for 25 min, and then a 2 h reperfusion period allowed. The LADCA was then re-occluded, and Evans blue dye (1 ml of 2% wv^{-1}) injected i.v. to stain the area at risk (AR). The AR was calculated and expressed as a percentage of the total left ventricular weight. The extent of tissue necrosis was then assessed by staining with *p*-nitro-blue tetrazolium (0.5 mg ml^{-1}, 20 min at 37°C) as described (D'Amico *et al.*, 2000). The infarct size (IS), necrotic tissue, was expressed as a function of the AR mass (IS/AR) and as a function of the total left ventricular weight (IS/LV). In selected experiments, the AR was collected for assessment of endogenous ANX-A1 levels by Western blot analysis. All peptides were given i.v. at the end of the ischaemic period. Statistical differences were assessed by unpaired Student's t-test.

In vehicle-treated animals, occlusion and reopening of LADCA produced an IS/AR ratio of 53 ± 2% (n=10). This figure was reduced by 1 $mgkg^{-1}$ Ac2-26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK; 3050 Da) (Cuzzocrea *et al.*, 1997) and Ac2-12 (1354 Da) treatments to 30 ± 2% (n=5, P<0.05) and 37 ± 2% (n=5, P<0.05), respectively. However, the IS/AR ratio was not affected by peptide Ac2-6 (577 Da) with a value of 56 ± 5% (n=4). None of the treatments affected systemic blood pressure and heart rate (not shown). To investigate the possible involvement of FPR in the action of ANX-A1, a selective inhibitor of FPR was used. N-t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc2; Sigma) was administered at the dose of 0.4 mg kg^{-1} together with the lower dose of peptide Ac2-26 (0.5 mg kg^{-1}). The IS/AR ratio for Ac2-26 alone was 42 ± 0.7% (n=5, P<0.05 compared to control). Boc2 significantly reversed the protective action of peptide Ac2-26 against myocardial infarct with an IS/AR ratio of 59 ± 3% (n=5). Treatment of rats with Boc2 alone had no effect on myocardial infarct size, the ISR ratio was 55 ± 3% (n=5, not significantly different from control). Western blotting analysis demonstrated presence of immunoreactive ANX-A1 in the myocardial tissue of vehicle-treated animals as seen 2 h after reperfusion. Rats treated with a protective dose of peptide Ac2-26 (1 mg kg^{-1}) showed apparent reduction in band intensities (n=3; not shown).

In conclusion, we propose that ANX-A1-derived peptides are beneficial in the treatment of myocardial pathologies associated with ischemia/reperfusion. The bioactive region resides within amino acid 2 to 12 of ANX-A1. Finally, an involvement of FPR in the protective effect displayed by these peptides can be envisaged.

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This work was supported by the Joint Research Board of Bart's Hospital (grant XMKZ). RJF is a Principal Research Fellow of the Wellcome Trust, whereas MP is supported by the ARC

34P DEXAMETHASONE INDUCES SERINE PHOSPHORYLATION OF ANNEXIN 1 IN PITUITARY CELLS AND SUPPRESSES ACTH RELEASE BY A PKC-DEPENDENT MECHANISM.

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Annexin 1 (ANXA1, lipocortin 1), which is produced by non-secretory folliculostellate (FS) cells in the anterior pituitary gland, acts as a paracrine mediator of glucocorticoid action in the rat neuroendocrine system (Taylor *et al.*, 1995) where its biological activity requires amino acid sequences in the N-terminal region (John *et al.*, 1998). As this region includes serine (ser) and tyrosine (tyr) phosphorylation sites we are investigating the effects of changes in phosphorylation (P) status on the biological activity of ANXA1 in the pituitary gland. The present study focused on PKC-dependent ser-P and examined the effects of (a) a PKC inhibitor (PKC residues 19-36, 5μM) on the inhibitory actions of dexamethasone (dex, 0.1μM) and $ANXA1_{Ac2\text{-}26}$ (6.7μM) on the release of ACTH from rat anterior pituitary tissue *in vitro* and (b) dex on the cellular disposition and P status of ANXA1 in this tissue and in a human pituitary FS cell line (PDFS, Danilla *et al.*, 2000).

The pituitary tissue and PDFS cells were incubated according to established protocols. ACTH released (expressed as pg/mg tissue) was determined by radioimmunoassay; ANXA1 was detected by western blot analysis and fluorescence activated cell (FAC) analysis, using specific antisera to distinguish between ser-P and non-P species of the protein. Band density within blots (analysed by computerised densitometry) was normalised to the steroid-free control (100). Forskolin (0.1μM - 1mM, 60min) caused concentration-related increases in ACTH release from the pituitary tissue. The responses to a submaximal concentration (100μM, basal 81.8 ± 27.7; forskolin 238.7 ± 23; P<0.01 n=6, Duncan's test) were reduced by pre-incubation (2h) with dex (103.5 ± 16.4 P<0.01) or $ANXA1_{Ac2\text{-}26}$ (163.3 ± 22.3, <0.05). Concomitant addition of the PKC inhibitor reduced the inhibition of ACTH release induced by the steroid (169.2 ± 22.2, P<0.05) and $ANXA1_{Ac2\text{-}26}$ (275.7 ± 49.1). Blockade of PKC also prevented the dex-induced translocation of ANXA1 from the cytoplasm to the outer cell membrane, a process which is critical to ANXA1 action in the anterior pituitary gland. Parallel studies showed abundant ANXA1 expression by PDFS cells; as in normal pituitary tissue, the bulk of the protein was contained within the cells but a small proportion was found on the cell surface. Dex (1μM) caused a transient increase in ser-P-ANXA1 within the cells which was maximal at 30 min (band density 146.3 ± 0.8 *vs.* control 100, P<0.01, n=3) and which was followed by a marked increase in total ANXA1 expression (maximal at 3h); the steroid also promoted the translocation of ser-P-ANXA to the cell surface (298 ± 2.5 *vs.* control 100, n=3). Preliminary studies using FAC analysis to measure cell surface ser-P-ANXA1 yielded a similar profile of data.

Together, these data suggest that PKC-dependent ser-P is important in mediating the effects of dex and ANXA1 on anterior pituitary function.

We are grateful to the Wellcome Trust for financial support.

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35P TACHYKININ ACTIVATION OF HUMAN MONOCYTES FROM NORMAL AND RHEUMATOID PATIENTS: EFFECTS OF CYCLOSPORIN A

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We previously demonstrated that NK_1 and NK_2 receptors are present on human monocytes (MO), substance P (SP) and neurokinin A (NKA) inducing superoxide anion production and tumor necrosis factor-alpha (TNF-α) mRNA expression (Brunelleschi *et al.*, 1998). Moreover, in MO isolated from patients with rheumatoid arthritis (RA), NKA and the selective NK_2 receptor agonist [β-Ala^8]-NKA(4-10) triggered an enhanced respiratory burst (Brunelleschi *et al.*, 1998). Cyclosporin A (CsA) is an immunosuppressant drug used for the treatment of RA, which has been shown to reduce TNF-α production in human MO and to block T-lymphocyte activity, type1 T-helper cells being preferentially suppressed (Schmidt *et al.*, 1994).

Therefore, we decided to evaluate the effects of CsA on MO challenged with tachykinins or other stimuli, e.g., phorbol 12-myristate 13-acetate (PMA) or lypopolisaccharide (LPS). Adherent MO, isolated from normal donors, were challenged, in the presence or absence of CsA , with the stimuli for 24 hr; TNF-α release, which was selected as a parameter of MO activation, was measured by ELISA. TNF-α release was also determined in MO from CsA-treated (Sandimmun^R Neoral^R, 2.5 mg/kg/die *per os*) RA patients at basal time (T_0, before the first CsA administration), two weeks (T_1) and four weeks (T_2) on CsA treatment. Informed consent was obtained from the participants and protocol was approved by a local ethic committee.

CsA inhibited, in a dose-dependent (0.01 –500 ng/ml) manner, TNF-α release from MO: at 100 ng/ml, a concentration in the pharmacological range employed for RA treatment, CsA reduced to 45 reduced to 45 ± 2 % and 56 ± 3% PMA- and LPS-evoked TNF-α release, respectively, and almost completely abolished (about 90% inhibition) the ones induced by SP, NKA or [β-Ala^8]-NKA(4-10).

In MO from RA patients treated with CsA, PMA-evoked TNF-α release was 1264 ± 250 pg/ml at T_0 and dropped to 513 ± 265 pg/ml (about 60% inhibition; n = 6; $p < 0.05$ vs. baseline) at T_1. It was further reduced to 312 ± 49 pg/ml (more than 70% inhibition; n =6; $p < 0.01$ vs. baseline) at T_2. Similar results were obtained by evaluating LPS-evoked TNF-α release. Tachykinin-evoked TNF-α release in MO collected from RA patients was similarly reduced after CsA treatment. Baseline values for tachykinin-evoked TNF-α release (e.g., 94± 8 pg/ml for SP 10^{-7}M; n = 6) were higher ($p< 0.05$) than those obtained from MO isolated from healthy donors (e.g., 42 ± 9 pg/ml for SP 10^{-7}M; n = 6). CsA treatment significantly reduced ($p < 0.05$), in a time-dependent manner (about 50- 60% inhibition at T_1 and 80-90% inhibition at T_2), tachykinin-evoked TNF-α release from MO collected from RA patients, with minimal variations among the three tachykinins used.

These data demonstrate that MO isolated from RA patients, when challenged *in vitro* with tachykinins, secrete greater amounts of TNF-α as compared to control cells and that CsA, either *in vitro* than *ex-vivo*, potently inhibits TNF-α release from human MO, possibly contributing to its clinical efficacy in RA.

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36P RESPECTIVE EFFECTS OF cAMP ELEVATION VERSUS cGMP ELEVATION ON GMCSF RELEASE BY HUMAN SYNOVIOCYTES

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Granulocyte-macrophage colony-stimulating factor (GMCSF) is a pro-inflammatory cytokine. Synovial fluid concentrations of GMCSF are significantly higher in patients with rheumatoid arthritis (al Janadi *et al.*, 1996). Synoviocytes are a likely source of GMCSF in the inflamed arthritic joint and its local production is the first indication of disease activity. GMCSF contributes to the pathogenesis of rheumatoid arthritis by activating leukocytes and neutrophils, increasing their maturation and survival. Prostanglandin E_2 (PGE_2), a product of cyclooxygenase activity, inhibits the production of GMCSF in synovial fibroblasts (Agro *et al.*, 1996; Breese *et al.*, 1999). In vascular cells cAMP mediates the effect of COX-2 activity in the regulation of GMCSF release. However, the relative role of cGMP elevating drugs on GMCSF in these cells is not known. Thus, the aim here was to determine the relative roles of cAMP and cGMP in GMCSF production by human synoviocytes.

Synovium was obtained from patients undergoing routine surgery for mechanical injuries. Explants of synoviocytes were cultured in DMEM fortified with 2 mM glutamine and 20 % foetal calf serum, and incubated at 37 °C and 5 % CO_2. Explanted synoviocytes were identified by morphology and cultured to confluency. Cells were serum starved for 24 h and then treated with or without serum plus a cytokine mixture of tumour necrosis factor-α, interleukin-1β and interleukin-6 (all 10 ng ml^{-1}). In addition the following drugs were administered: **study 1)** IBMX (10^{-5} M, non specific phosphodiesterase (PDE) inhibitor), Ro-201724 (10^{-4} M, cAMP specific PDE IV inhibitor) or zaprinast (10^{-4} M, cGMP specific PDE V inhibitor) and sodium nitroprusside (SNP, 10^{-4} M), in the presence or absence of indomethacin (10^{-5} M); **study 2)** IBMX (10^{-5} M), IBMX plus PGE_2 (10^{-8} M), Ro-201724 (10^{-4} M), Ro-20-1724 plus PGE_2 (10^{-8} M), and PGE_2 were added to cells in the presence of indomethacin only. Production by the synovoiocytes of PGE_2 and GMCSF were measured by radioimmunoassay and specific sandwich ELISA, respectively. Cell viability was assessed by the ability of cells to reduce MTT to formazan.

Study 1: PGE_2 and GMCSF release from unstimulated cells was undetectable. In the presence of cytokines 6±1 ng ml^{-1} PGE_2 and 119±41 pg ml^{-1} GMCSF was released (n=6). None of the drugs had any significant effect on PGE_2 production. However, IBMX or Ro-201724 significantly reduced GMCSF release (control, 119±41; IBMX, 21±8; Ro-20-1724, 34±12 pg ml^{-1}, one way ANOVA, $P<0.05$), respectively. Neither zaprinast nor SNP had any effect on the release of GMCSF. When indomethacin was also added PGE_2 production was undetectable (<0.075 ng ml^{-1}), where as GMCSF levels significantly increased to 571±104 pg ml^{-1} (paired t-test, <0.05). None of the drugs had any additional effects in the presence of indomethacin.

Study 2: Addition of exogenous PGE_2, Ro-20-1724 or IBMX alone, had no effect on the release of GMCSF. However, Ro-20-1724 (control, 1543±302; Ro-20-1724, 640±172 pg ml^{-1}) or IBMX (control, 1229±241; IBMX, 335±94; pg ml^{-1}) in combination with PGE_2 significantly reduced the production of GMCSF (unpaired t-test, $P<0.05$, n=4).

Therefore, these results suggest that cAMP but not cGMP regulates GMCSF release in cytokine-stimulated human synoviocytes.

This work was funded by NicOx, S.A France

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37P A COMPARISON OF THE RESPONSES OF RAW 264.7 MACROPHAGES TO LIPOPOLYSACCHARIDE AND INFECTION WITH *SALMONELLA TYPHIMURIUM*

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An important aspect of the pathogenesis of *Salmonella typhimurium* is the ability of the bacteria to survive and grow in macrophages. These cells respond to lipopolysaccharide (LPS). Lipid A, the toxic part of the LPS molecule, is important in mediating lethality in a murine model of typhoid fever (Khan *et al.*, 1998). LPS induces the release of the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF-α), via activation of several signalling pathways. These include activation of cJun N-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK), extracellular-signal-regulated kinase (ERK; Widman *et al.*, 1999) and inhibitor of kappa B kinase (IKK). Infection with *S. typhimurium* activates ERK (Procyk *et al.*, 1999), but its effects on JNK, p38 and IKK in macrophages are unclear. The aim of this study was to compare the effects of LPS and *S. typhimurium* on the responses of RAW 264.7 cells.

Murine macrophage-like cells (RAW 264.7) were either treated with LPS (*Escherichia coli*, Sigma; 1 μg/ml) or live *S. typhimurium* at multiplicities of infection (moi) of 0.05-50. TNF-α release (at 3h) was determined by ELISA (R&D). JNK, p38 MAPK and IKK activation (data expressed as fold increase in activity over basal) was measured by *in vitro* kinase assays (Alldridge et al., 1999). Each value represents the mean ±sem of at least 3 independent experiments.

LPS induced TNF-α release (2538.5±526 pg/ml). Infection with *S. typhimurium* induced moi-dependent release of TNF-α (expressed as a % of LPS stimulated TNF-α release: moi 0.05, 6.8±2.1%; moi 0.5, 95.9±8.2%; moi 5, 102.1±13.9%; moi 50, 59.4±7.5%).

LPS and *S. typhimurium* activated p38 MAPK, JNK and IKK. LPS activation of p38 MAPK peaks at 15 min (21±3.5) and is reduced by 60 min (1.1±0.2). *S. typhimurium* activates p38 MAPK by 15 min (8.9±3.3) but, in contrast to LPS treatment, this activation is sustained (60 min, 15.9±6.4). JNK was activated by both LPS and *S. typhimurium* by 15 mins (23.9±14.7; 2±0.1 respectively) and activation was still present at 60 min (15.2±6.8; 3.1±0.6, respectively). LPS and *S. typhimurium* activated IKK at 15 mins (13.5±3.7; 5.1±2.7, respectively) and is sustained at 90 min (9.1±1.9; 9.6±4.2, respectively).

Our data shows that both LPS and live *S. typhimurium* induce TNF-α release from RAW 264.7 cells. Both LPS and bacterial infection activates IKK and JNK with a similar time course. However live *S. typhimurium* causes sustained activation of p38 MAPK in contrast to the transient LPS-induced activation of this kinase.

This work was supported by the BBSRC and Wellcome Trust.

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38P MACROPHAGE STIMULATING PROTEIN (MSP) ACTIVATES HUMAN MACROPHAGES

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Macrophage Stimulating Protein (MSP), originally described to stimulate chemotaxis of murine resident peritoneal macrophages, is the ligand for the Ron receptor, a member of the Met subfamily of tyrosine kinase receptors (Gaudino *et al.*, 1994). MSP was later demonstrated to inhibit NO release and iNOS induction in LPS- and/or IFN-γ-stimulated murine peritoneal macrophages (Chen *et al.*, 1998), but its effects on human macrophages have never been evaluated.

Therefore, we decided to investigate Ron expression (evaluated by RT-PCR and immunoprecipitation followed by immunoblotting) and MSP effects on human mononuclear phagocytes of different origin: circulating monocytes (MO; isolated from heparinized venous blood by Ficoll-Paque gradient centrifugation and purified by adhesion), monocyte-derived macrophages (MDM; prepared from MO cultured for 7-8 days), alveolar macrophages (AM; isolated from broncho-alveolar lavage) and peritoneal macrophages (PM; collected from ascitic fluid of cirrhotic patients). Superoxide anion (O_2^-) production was selected as a marker of cell activation and was measured spectrophotometrically by evaluating SOD-inhibitable cytochrome C reduction. Human recombinant MSP was expressed in Sf9 insect cells by the baculovirus expression system and its biological activity was tested by Ron tyrosine phosphorylation and by scatter assay on Madin-Darby Canine Kidney (MDCK) cells expressing Ron. hrMSP activity was expressed as scatter units, one scatter unit corresponding to the 1:250 dilution.

In the range 0.5 – 100 scatter units/ml, MSP had no effect on MO, while it dose-dependently evoked O_2^- production from MDM: maximal production (42 ± 3 nmol cytochrome C reduced/10^6 cells; n = 5) was obtained at 100 scatter units/ml, ED_{50} value being 30 ± 2 scatter units. Similar dose-dependent effects were observed also in PM and AM, maximal O_2^- production by MSP 100 scatter units/ml approaching 40 nmol cytochrome C reduced/10^6 cells (n = 4-6). In AM, MSP-induced respiratory burst was higher than the ones evoked by PMA 10^{-6} M (24 ± 7 nmoles cytochrome C reduced/10^6 cells; n = 6) or FMLP 10^{-6}M (6.3 ± 1 nmoles cytochrome C reduced/10^6 cells; n =6). Consistent with these observations, the mature Ron protein was expressed by the MSP responsive cells (MDM, AM and PM), but not by the unresponsive MO. To evaluate the pathways involved in NADPH oxidase activation, we used different signal transduction inhibitors. MSP-evoked O_2^- production is mediated by tyrosine kinase activity, as the tyrosine kinase inhibitor genistein reduced by 30% MSP-evoked respiratory burst while the tyrosine phosphatase inhibitor sodium orthovanadate potentiated it. MSP-evoked respiratory burst also requires the activation of Src but not phosphatidylinositol 3-kinase (PI-3 kinase), since the selective Src inhibitor PP1 inhibited to about 50% this effect, while the PI-3 kinase inhibitor wortmannin was inactive.

These data demonstrate that MSP induces the respiratory burst in human macrophages of different origin, but not in human monocytes, suggesting MSP/Ron complex as a possible marker for human mature macrophages.

Chen, YQ. *et al.* (1998) *J. Immunol.* **161**, 4950- 4959
Gaudino, G. *et al.* (1994) *EMBO J.* **13**, 3524- 3532.

39P IL-4, BUT NOT IFNγ, DIFFERENTIALLY REGULATES GM-CSF AND G-CSF RELEASE FROM STIMULATED ARTERIAL AND VENOUS SMOOTH MUSCLE CELLS

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Granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF) can promote leukocyte survival. We have recently shown that human vascular smooth muscle cells (SMCs) release GM-CSF and G-CSF when stimulated with inflammatory cytokines (Stanford *et al.*, 2000). Here we have investigated the effect of IL-4 and IFNγ on the release of GM-CSF and G-CSF.

Samples of human saphenous vein and internal mammary artery were cultured as described previously (Stanford *et al.*, 2000). Cells were treated with increasing concentrations of IL-4 (0.01-10ng/ml) or IFNγ (0.01-100ng/ml) in the presence and absence of IL-1β (1ng/ml). After 24 hours medium was removed from cells. GM-CSF and G-CSF levels were measured by ELISA.

Basal release of GM-CSF and G-CSF from arterial and venous SMCs was undetectable in the presence, and in the absence, of IL-4 or IFNγ. As observed previously IL-1β stimulated GM-CSF and G-CSF release. IL-4 inhibited GM-CSF release but potentiated G-CSF release by stimulated arterial (GM-CSF, 1296±38 vs. 641±21pg/ml; G-CSF, 8851±152 vs. 13997±173pg/ml: mean±sem, n=3) and venous (Figure 1a) SMCs. IFNγ inhibited the release of both GM-CSF and G-CSF by stimulated arterial (GM-CSF, 829±35 vs. 241±18pg/ml; G-CSF, 1481±114 vs. 443±22pg/ml) and venous (figure 1b) SMCs.

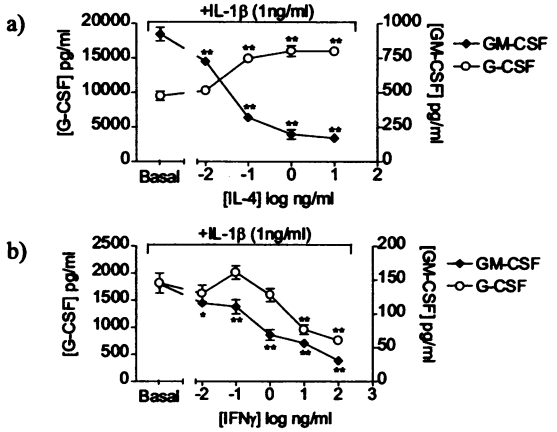

Figure 1 Effect of a) IL-4 and b) IFNγ on GM-CSF and G-CSF release from stimulated venous SMCs. Data represents n=3 from 1 patient. Similar results were obtained using cells cultured from 2 other patients. One-way ANOVA vs. basal(post-test Dunnett): *P<0.05, **P<0.01.

GM-CSF and G-CSF preferentially activate different populations of leukocytes. Thus, pathways that increase the release of one and inhibit the release of another will have profound effects on the populations of leukocytes present at the site of inflammation. Here we have identified IL-4, but not IFNγ, as one such pathway.

Stanford *et al.* (2000). *Br J Pharmacol.* 129:835-38.

This work was funded by grants from the BHF and the Wellcome Trust.

40P REGULATION OF THE RELEASE OF COLONY-STIMULATING FACTORS FROM HUMAN AIRWAY SMOOTH MUSCLE CELLS BY PROSTAGLANDIN E_2

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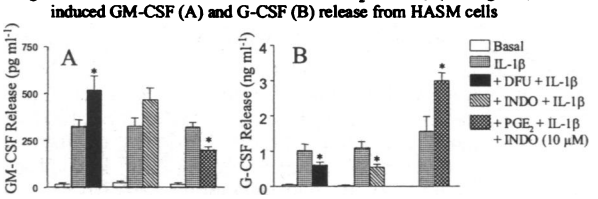
Human airway smooth muscle (HASM) cells are believed to contribute to inflammation by releasing prostanoids and cytokines such as granulocyte/macrophage-colony stimulating factor (GM-CSF) in response to the cytokine, IL-1β (Belvisi *et al.*, 1997; Saunders *et al.*, 1997). In the present study we have determined whether HASM cells release other colony-stimulating factors (CSFs) and if cyclo-oxygenase (COX) products regulate these processes.

HASM cells from donor tracheae were cultured in 96 well plates. At sub-confluence cells were growth-arrested for 24h before treatment with the drugs under investigation (diluted in DMEM containing 3% FCS). At 24h the supernatants were assayed for CSFs by ELISAs (Stanford *et al.*, 2000). None of the drugs affected cell viability as determined by mitochondria-dependent reduction of MTT to formazan. The vehicle for DFU and indomethacin (0.01% DMSO), and PGE_2 (0.01% ethanol) had no effect on CSF release.

IL-1β (0.001-10 ng ml^{-1}) evoked a concentration-dependent release of GM-CSF (E_{max} = 335.8 ± 96.9 pg ml^{-1}; EC_{50} = 0.011 ± 0.007 ng ml^{-1}) and G-CSF (E_{max} = 2866.6 ± 240.8 pg ml^{-1}; EC_{50} = 0.062 ± 0.05 ng ml^{-1}) from HASM cells whereas the elaboration of macrophage colony-stimulating factor (M-CSF) was not increased above the basal level (~8 pg ml^{-1}). The selective COX-2 inhibitor, DFU, and the non-selective COX inhibitor, indomethacin (each 10μM; maximally effective concentration) augmented IL-1β-stimulated release of GM-CSF by 59.6% and 42.4% respectively (Fig. 1A). In contrast, DFU and indomethacin inhibited IL-1β-induced G-CSF production by 43.3% and 49.7% respectively (Fig. 1B).

The effects of indomethacin were antagonised by PGE_2, the major COX product released from HASM cells (Belvisi *et al.*, 1997). Thus, PGE_2 (10 μM) inhibited GM-CSF release by 37.9% whereas it augmented the release of G-CSF by 87.7% in IL-1β-treated HASM cells (Fig. 1).

Figure 1: The effects of COX inhibitors and PGE_2 on IL-1β (0.01 ng ml^{-1}) induced GM-CSF (A) and G-CSF (B) release from HASM cells



Data were analysed by a one way ANOVA followed by Dunnett's multiple comparisons test. *P<0.05 significant difference from IL-1β-treated cells; n = 6 - 15 determinations from 2 - 5 donors.

The results of the present study establish that IL-1β promotes the release of GM-CSF and G-CSF from HASM cells, which is differentially regulated by prostanoids, following the induction of COX-2. These data are, therefore, consistent with studies performed with human vascular smooth muscle cells (Stanford *et al.*, 2000). We speculate that the elaboration of the eosinophil survival factor, GM-CSF, from stimulated HASM treated with NSAIDs could explain 'aspirin-sensitive'asthma.

Belvisi M.G., Saunders M.A., Haddad, E.B. *et al* (1997) *Br. J. Pharmacol.*, 120, 910-916.
Saunders, M.A., Mitchell, J.A., Seldon, P.M. *et al.* (1997) *Br. J. Pharmacol.*, 120, 545-546.
Stanford S.J., Pepper J.R. & Mitchell J.A. (2000) *Br. J. Pharmacol.*, 129, 835-838.

41P EFFECTS OF 5-AMINOISOQUINOLINONE, A WATER-SOLUBLE, POTENT INHIBITOR OF THE ACTIVITY OF POLY (ADP-RIBOSE) POLYMERASE ON P-SELECTIN AND INTERCELLULAR ADHESION MOLECULE-1 IN LUNG INJURY

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Poly (ADP-ribose) synthetase (PARP), a nuclear enzyme activated by strand breaks in DNA, has been show to play an important role in the pathogenesis of inflammation. The aim of the present study was to evaluate whether PARS activity may modulate neutrophil infiltration. In this study we demonstrate a role of PARS in neutrophils recruitment in acute inflammation induced by zymosan-activated plasma. Intrathoracic stimulation with zymosan-activated plasma leads to an increase in neutrophils infiltration at 24 h. Treatment with the new potent PARP inhibitor, 5-amino-isoquinolinone (5-AIQ) provides protection against lung injury by inhibiting the expression of P-selectin and intracellular adhesion molecules-1 (ICAM-1) and, consequently, by inhibiting the recruitment of neutrophils into the injured lung.

Furthermore, using *in vitro* studies, we demonstrated that in human endothelial cells, oxidative or cytokine-dependent expression of P-selectin and ICAM-1 is reduced by pharmacological inhibition of PARS by 5-AIQ.

These findings provide the first evidence that PARS activation participates in neutrophil-mediated lung injury regulating the expression of P-selectin and ICAM-1 in acute inflammation.

Table 1. Effect of 5-AIQ treatment on neutrophil accumulation triggered by ZAP

Group	Neutrophils (x 10^6/cavity)	Myeloperoxidase (U/g wet tissue)
Sham + Vehicle	0.003±0.004	12±4
Sham + 5-AIQ	0.004±0.002	13±3.2
ZAP + Vehicle	1.99±0.2*	82.9±4*
ZAP + 5-AIQ	0.25±0.1°	21±4.2°

*P<0.01 *versus* Sham. °P<0.01 *versus* ZAP. All values in the figure and experiment carried out are expressed as mean ± standard error (s.e.m.) of the mean of n=10 animals for group Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Student's unpaired t-test. Non-parametric data were analyzed with the Fisher's exact test. A p-value less than 0.05 was considered significant.

42P EVIDENCE FOR AN ANTI-PROLIFERATIVE ROLE OF PEROXISOME-PROLIFERATOR-ACTIVATED RECEPTORS IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Airways diseases such as asthma and COPD are mediated in part by inappropriate pro-proliferative responses of airway smooth muscle cells. The nuclear receptors, peroxisome proliferator-activated receptors (PPAR) inhibit vascular smooth muscle cell growth. However, the effects of PPAR ligands on human airway smooth muscle (HASM) cells have not been addressed. Thus, we have investigated the effects of the PPARα ligand, WY-14643, the PPARγ ligand, ciglitazone and the endogenous PPARγ ligand 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2; Bishop-Bailey, 2000) on proliferation of HASM cells in culture.

HASM cells were cultured in 96 well plates (Belvisi *et al.*, 1997). At sub-confluence the cells were serum-deprived for 24 h and then treated with test drugs diluted in DMEM in the absence and presence of 3% fetal calf serum (FCS). Proliferation of HASM cells was determined by measuring [^{3}H]-methyl-thymidine incorporation (1μCi/well; specific activity 25Ci/mmol; pulsed for 6hrs at 24hr).

In the absence of FCS basal proliferation of HASM cells was low (1502±168cpm, n=30) and WY-14643, ciglitazone or 15d-PGJ_2 (100μM) did not induce proliferation (848±173 cpm, 404±63.9cpm, 643±62.62 cpm respectively, n=30). FCS (3%) induced proliferation (14080±2130 cpm; n=30) and under these conditions, WY-14643 or 15d-PGJ_2 induced modest increases of this response (Figure 1). The effect of 15d-PGJ_2 was bi-phasic with inhibitory responses observed at higher concentrations (Figure 1). By contrast, ciglitazone induced only inhibitory effects on proliferation (Figure 1). The vehicle (0.1% or 1 % DMSO) had no effect on proliferation

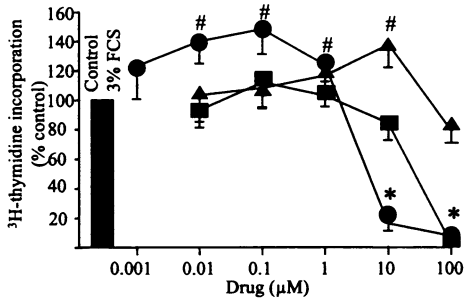

Figure 1: The effect of ciglitazone (■), WY-14643 (▼); 15d-PGJ_2 (●) on HASM cell proliferation induced by 3% FCS. Data are normalised to control (3% FCS) and analysed by a one-sample t-test. P<0.05; # increased;* decreased compared to control. n=6-9 using cells from 2-3 donors.

These observations suggest that HASM cells express PPARα and γ receptors. Moreover, activation of PPARγ receptors inhibits proliferation whereas activation of PPARα receptors induces proliferation. Thus, activation of PPARγ receptors in vivo may act to limit the hyperplasia associated with airway remodelling observed in airway disease.

Belvisi, M.G., Saunders M.A., Haddad E-B. *et al.* (1997). *Br. J. Pharmacol.*, 120, 910-916.

Bishop-Bailey D. (2000) *Br. J. Pharmacol.*, 129, 823-834.

This work was funded by the Wellcome Trust and the BHF.

43P CASPASE-MEDIATED CLEAVAGE OF MST1 DURING APOPTOSIS OF HUMAN EOSINOPHILS

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In preliminary studies we have found that constitutive apoptosis of human eosinophils is temporally associated with the activation of a 36 kDa protein kinase that phosphorylates myelin basic protein (MBP) in "in-gel" renaturation assays. The aim of the present study was to identify and characterise this enzyme and to assess its role in apoptosis.

Eosinophils were purified from the peripheral blood of steroid-naïve asthmatic donors and cultured without cytokines, in the absence and presence of an apoptosis-promoting Fas–activating monoclonal antibody, CH-11 (100 ng ml^{-1}), or a survival-enhancing cytokine, granulocyte/macrophage colony-stimulating factor (GM-CSF; 10 pM) for varying times up to 24h. In other experiments, eosinophils were treated with catalase (0.3 & 3 mg ml^{-1}) or the caspase inhibitor, z-Asp-CH_2-DCB (20, 100 & 200 μM), in the absence and presence of CH-11. At appropriate times apoptotic cells were enumerated and "in-gel" assays, and immunoprecipitation were performed as described previously (Kankaanranta *et al.*, 2000). The vehicle (0.1% DMSO) for z-Asp-CH_2-DCB had no effect on apoptosis, the expression of MBP kinases or on cell viability.

In the absence of haematopoeitic cytokines, eosinophils underwent spontaneous apoptosis (5.2 ± 1.5% & 39.5 ± 4.5%, n = 6 at 4 and 24h respectively) that was associated with the appearance of a p36 MBP kinase (p36 MBPK). Temporally, p36 MBPK was activated at 4h and increased in a time-dependent manner up to 24h. CH-11 enhanced p36 MBPK activity (by 338 ± 103% at 20h, n = 3) over that seen in untreated cells whereas p36 MBPK activity was not detected in cells treated with GM-CSF. Catalase inhibited spontaneous (from 20.9 ± 5.0% to 10.2 ± 1.0%, n = 2) and CH-11 (from 29.2 ± 6.3% to 12.9 ± 3%, n = 3)-induced apoptosis and activation of p36 MBPK (to 17 ± 17% and 53 ± 22% of untreated cells respectively). In addition, z-Asp-CH_2-DCB (200 μM) protected against spontaneous (from 47.2 ± 2.5% to 2.5 ± 0.4%, n = 3, $P < 0.05$) and CH-11 (from 54.7 ± 4.0% to 2.2 ± 0.6%, n = 3, $P < 0.05$)-induced apoptosis and the activation of p36 MBPK (to 12 ± 8% and 24 ± 14% of vehicle-treated eosinophils respectively, n = 3).

A constitutively-active 63 kDa MBP kinase (p63 MBPK) was detected in freshly-purified eosinophils. During spontaneous- and CH-11-induced apoptosis, and GM-CSF-enhanced survival, p63 MBPK was transiently increased at 4h over baseline. However, at 20h, CH-11 decreased p63 MBPK activity to 47 ± 15% (n = 3) of control, whilst at 24 h, GM-CSF increased this activity by 191 ± 23% (n = 3) when compared to untreated cells. In apoptotic eosinophils the reduction in p63 MBPK activity coincided with the appearance of p36 MBPK. To identify these MBPKs, antibodies to kinases bearing similar characteristics were screened in immunopreciptation studies. Polyclonal antibodies to the N-terminal catalytic domain of Mst1, immunprecipitated both p63 and p36 MBPK, while detectable p63 MBPK kinase activity was immunoprecipitated with antibodies raised against the N-terminal region of Mst2.

Collectively, these results suggest that Mst1 accounts, in part, for p36 and p63 MBPK activities and that p36 is a caspase cleavage product of p63 MBPK. However, the possibility that Mst2 or another kinase also contribute to these activities cannot be excluded. In addition, the data implicate p36 MBPK in propagating pro-apoptotic signals in response to H_2O_2 and via caspase-mediated pathways in human eosinophils.

Kankaanranta, H., de Souza, P.M., Giembycz, M.A., *et al.*, (2000). *Methods.Mol.Med.*, **44**, 99-110.

44P EFFECT OF CANNABINOID AGONISTS ON INTESTINAL MOTILITY IN A CHRONIC MODEL OF INTESTINAL INFLAMMATION

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Cannabinoid CB_1 receptors are located on enteric nerves (Pertwee, 1999) and their activation can mediate the inhibition of excitatory transmission and the reduction of intestinal motility (Izzo et al. 2000). Our goals were to evaluate the effect of the cannabinoid receptor agonists (CP 55,940 and cannabinol) on intestinal motility and to measure cannabinoid CB_1 receptor expression in a chronic model of intestinal inflammation.

Male ICR mice (24-26 g) received orally two doses of croton oil (10 μl $mouse^{-1}$ in two consecutive days). Four days after the first administration of croton oil, mice received (orally) 0.1 ml of a black marker and after 20 min they were killed to assess upper gastrointestinal transit (Puig & Pol, 1998). Cannabinoid drugs (4 μl in dimethyl sulfoxide) were administered intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.) 20 min before marker administration. For intracerebroventricular injections (Puig & Pol, 1998), mice were briefly anaesthetised with enflurane. Cannabinoid CB_1 receptor expression in the jejunum was studied by Western blot analysis.

Both CP 55,940 (0.03-10 nmol $mouse^{-1}$) and cannabinol (10-3000 nmol $mouse^{-1}$) produced a significant dose-related inhibition of transit in croton oil-treated mice (15±5-80±4% inhibition for CP 55,940 and 5±4-70±4 inhibition for cannabinol, n= 10-12, $P<0.05$-0.01) and both agonists has a lowed ED_{50} value compared to the corresponding i.p. treatment in control mice [ED_{50} (nmol $mouse^{-1}$): CP 55,940 4.75±0.38 in control mice, 2.15±0.32 in croton oil-treated mice, $P<0.01$; cannabinol 2280±187 in control mice, 1295±112 in croton oil-treated mice, $P<0.01$). Administered i.c.v. CP 55,940 and cannabinol in croton oil-treated mice also decreased intestinal motility, but the ED_{50} values (2.35±0.31 nmol $mouse^{-1}$ for CP 55,940 and 1360±154 nmol $mouse^{-1}$ for cannabinol) were not statistically different from the corresponding ED_{50} values after i.p. administration. The inhibitory effect of i.p.-injected CP55,940 (0.3 nmol $mouse^{-1}$) or cannabinol (300 nmol $mouse^{-1}$) was counteracted by the cannabinoid CB_1 receptor antagonist SR141716A (16 nmol $mouse^{-1}$, i.p.), but not by the cannabinoid CB_2 receptor antagonist SR144528 (52 nmol $mouse^{-1}$, i.p.) or by the ganglion blocker hexamethonium (69 nmol $mouse^{-1}$, i.p.). Densiometric analysis of immunoreactive bands showed a significant increase (266±16, $P<0.05$, n=3) of cannabinoid CB_1 expression in the inflamed jejunum compared to control tissue.

It is concluded that chronic inflammation enhances the potency of cannabinoid receptor agonists probably by up-regulating cannabinoid CB_1 receptor expression in the small intestine.

Izzo A.A. et al. (2000) *Trends Pharmacol Sci.* 21,372.
Pertwee R.G. (1999) *Life Sci.* 65,597-605.
Puig M.M. & Pol G. (1998) *J. Pharmacol. Exp. Ther.* 287,1068-1075

45P PHARMACOLOGY OF BV8: A NEW PEPTIDE FROM AMPHIBIAN SKIN

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Amphibian skin contains numerous biologically active peptides, many of which are related to mammalian hormones or neurotransmitters. From skin secretions of *Bombina bombina*, we isolated a small protein termed BV8 (Mollay *et al.*,1999) related to a non-toxic constituent of the venom of black mamba. As deduced from cloned cDNAs, the murine and human BV8 homologues have identical amino-terminal sequences. BV8 mRNA is abundant both in human and in mouse testis. It is present also in other mouse tissues: brain, cardiac muscle, skeletal muscle, gastrointestinal tract, kidney, liver, lung and peripheral leukocytes.

Peptide injection (5 μg/rat) into the left lateral ventricle of rat brain (n = 8) shortened the tail withdrawal latency to radiant heat nociceptive stimulus by 50 ± 4.1 %. S.c. injection of BV8 (0.5-5 μg/rat) (n = 8) dose-dependently reduced the pain threshold to heat nociceptive stimulus, measured with tail-flick test (from -30 ± 2 % to -50 ± 6 %) and plantar test (from -32 ± 2 % to -45 ± 2 %) and to mechanical nociceptive stimulus measured with the paw pressure test (from -15 ± 2 % to -61 ± 5 %).

Mechanical hyperalgesia induced by 2 μg/rat, s.c., of BV8 was completely reverted by pre-treatment of rats with non-analgesic doses of the following compounds: 5 mg/kg s.c. indomethacin (83 ± 7%), 7.5 μg/kg i.p. clonidine (82 ± 5%) and 0.1 mg/kg, i.p. MK 801 (70 ± 5%) and 3 mg/kg, i.p. L-NAME (93 ± 8%). BV8 also induced characteristic hyperalgesic behaviour: vocalisation on touch, writhing and licking of thighs and abdomen. Northern blot of RNA from human leukocytes demonstrated an intense band of mRNA that hybridizes with the BV8 probe. This band was 2.5-fold increased after 24 hours exposure of peripheral neutrophils to 50 nM GM-CSF. Moreover, BV8 may be secreted by leukocytes, because the sequence of its precursor contains the typical signal peptide sequence for secretion (Wechselberger *et al.* 1999).Thus, BV8 could be a new mediator of leukocyte-dependent hyperalgesia.

The IASP guidelines on ethical standards for investigations of experimental pain in animals were followed.

Mollay *et al.*, Eur. J. Pharm. 374: 189196,1999

Wechselberger *et al.*, FEBS Lett. 462: 177-181, 1999

46P THE MAMMALIAN HOMOLOGUE OF THE NOVEL PEPTIDE BV8 IS EXPRESSED IN THE CENTRAL NERVOUS SYSTEM AND SUPPORTS NEURONAL SURVIVAL BY ACTIVATING THE MAP KINASE/ PI-3-KINASE PATHWAYS

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Previous studies have identified the mammalian homologue of Bv8 (mBv8), a small protein which was originally isolated from skin secretions of the frog, *Bombina Variegata*. We now show, by *in situ* hybridisation, that mBv8 RNA is widely expressed in the rodent CNS, with high levels being detected in layer II of the cerebral cortex, limbic regions, cerebellar Purkinje cells, and dorsal and ventral horns of the spinal cord. A similar pattern of distribution was found by examining the presence of mBv8 protein by immunocytochemistry. Moreover,we report that Bv8 exerts a protective activity in different models of neuronal degeneration. Addition of Bv8 (0.1 or 1 μM) to cultured cerebellar granule cells (from 8-day old Sprague Dawley male rats) (Nicoletti et al., 1986) reduced the extent of apoptotic death induced by switching the growing medium from 25 into 5 mM K^+(a model of developmental apoptosis, see D'Mello et al., 1993) (percentage of apoptotic cells as assessed by Hoechst 33258 staining: 50.22 ± 5.68 s.e.m. vs 30.35 ± 3.057 s.e.m., n=6, $p<0.01$ One-way ANOVA + Fisher's PLSD.). Bv8-induced neuroprotection was abolished by incubating the cultures with PD98059 (10 μM) and LY294002 (10 μM), which inhibit the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI-3-K) pathways, respectively. Exposure of mouse mixed cortical cultures (Rose et al., 1992) to Bv8 (0.1 μM) protected against neurodegeneration induced by applying to the culture either NMDA (100 μM) for 10 min (percentage of neuronal death as assessed by trypan blue exclusion: 100 ± 3 s.e.m. vs 53 ± 8 s.e.m. n=6, $p<0.01$) or a cocktail of drugs containing MK801 (10 μM) DNQX (30 μM) and nifedipine (100 μM) for 48 h (percentage of neuronal death as assessed by trypan blue exclusion: 100 ± 3 s.e.m. vs 46 ± 2 s.e.m., n=6, $p<0.01$). Again Bv8-induced neuroprotection was negated by treatment of the cultures with PD98059 (10 μM) and LY294002 (10 μM). Bv8 activated the MAPK pathway in cultured cerebellar granule cells, as revealed by Western blot analysis of phosphorylated p44/p42 MAPKs (Iacovelli et al., 1999) We conclude that mBv8 acts as an endogenous neurotrophic factor and supports neuronal survival through the activation of the MAPK/PI-3-K pathways.

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47P ADENOSINE A_1 RECEPTOR-MEDIATED INHIBITION OF CALCITONIN GENE-RELATED PEPTIDE RELEASE FROM RAT TRIGEMINAL NEURONES

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The mechanisms involved in the pathogenesis of migraine implicate major roles for calcitonin gene-related peptide (CGRP) and the trigeminovascular system. In the present study, adult rat trigeminal ganglion neurones in culture were used to compare a number of pharmacological agents on CGRP release without the complicating influences of other neuronal and non-neuronal factors. Trigeminal neurone cultures were prepared as described in detail previously (Eckert *et al.*, 1997). Neurones were plated onto poly-D-lysine and laminin-coated 6-well culture dishes or glass coverslips and cultured in the presence of mNGF 2.5S (50 ng ml^{-1}) and Ara-C (20 μM) for 4-6 days. CGRP was measured using a commercial enzyme immunometric assay kit (SPIbio, Massy, France).

Incubation of trigeminal neurones with forskolin (3 μM), for 30 min raised CGRP levels from 52 ± 7 to 260 ± 29 pg ml^{-1} (n=17 independent culture preparations). Significantly, the A_1 adenosine agonist, GR79236X, potently inhibited forskolin-stimulated CGRP release (pIC_{50}: 7.7 ± 0.1, maximal inhibition 65 ± 2.5 % at 300 nM, n=4) whereas the A_{2A} (CGS21680, 1 μM) and A_3 (2-Cl-IB-MECA, 1 μM) receptor-selective agonists were ineffective (Table 1). DPCPX (100 nM) did not alter forskolin-stimulated CGRP release but abolished the GR79236X-mediated (100 nM) inhibition of release. Forskolin-stimulated CGRP release could be closely correlated with the phosphorylation of the protein kinase A (PKA) substrate, cyclic AMP response element binding protein (CREB) as assessed by Western analysis using a phospho-specific antibody (Ser133) to CREB. Forskolin (3 μM) evoked a marked increase in the phosphorylation of CREB which was attenuated by GR79236X (100 nM), and consistent with parallel observations made for CGRP release, DPCPX (100 nM) reversed the inhibition of CREB phosphorylation by GR79236X.

Immunocytochemical studies and Western analysis confirmed the presence of A_1 adenosine receptors on trigeminal neurones. However, despite the additional detection of 5-HT_{1B} receptors on these cells, sumatriptan (1 μM) did not inhibit forskolin-stimulated CGRP release (Table 1) nor showed any effect on the concomitant CREB phosphorylation. In contrast, the μ-opioid receptor agonist fentanyl (0.1 μM) elicited a 74 ± 4 % reduction in CGRP levels (Table 1) and attenuated forskolin-stimulated CREB phosphorylation.

Table 1. Modulation of CGRP release (pg ml^{-1} 30 min^{-1}) from rat trigeminal neurones

Treatment	Basal	Forskolin	Forskolin + treatment	n
GR79236X (0.1 μM)	65 ± 7	*288 ± 12	#139 ± 23	4
CGS21680 (1 μM)	53 ± 12	*232 ± 18	238 ± 14	3
2-Cl-IB-MECA (1 μM)	37 ± 10	*188 ± 35	195 ± 37	4
Sumatriptan (1 μM)	37 ± 10	*193 ± 33	185 ± 10	4
Fentanyl (0.1 μM)	45 ± 10	*219 ± 36	#91 ± 18	4

* P<0.001 *versus* basal; # P<0.01 *versus* forskolin (Student's t test)

Forskolin-stimulated CGRP release and CREB phosphorylation was mimicked by the cell-permeable cAMP analogue, cpt-cAMP (1 mM), (basal CGRP release 64 ± 7 pg ml^{-1} 30 min^{-1}, +cpt-cAMP 163 ± 39 pg ml^{-1} 30min^{-1}, n=3, P<0.05). Pre-treatment of cultures (1 h) with the cell-permeable PKA inhibitor, *myr*PKI_{14-22} (100 μM), blocked forskolin-stimulated CGRP release and CREB phosphorylation (basal release 64 ± 7 pg ml^{-1} 30 min^{-1}, +forskolin 251 ± 35 pg ml^{-1} 30 min^{-1}, +forskolin +myrPKI_{14-22} 70 ± 5 pg ml^{-1} 30 min^{-1}, n=3).

Taken together, these data confirm the PKA-dependency of forskolin-stimulated CGRP release from rat trigeminal neurones and suggest that A_1 adenosine agonists warrant further investigation in animal models relevant to migraine.

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48P THE EFFECT OF GR190178, A SELECTIVE LOW-EFFICACY ADENOSINE A1 RECEPTOR AGONIST, ON THE TREATMENT OF NEUROPATHIC HYPERALGESIA IN THE RAT.

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Clinical studies in neuropathic patients have shown that i.v. adenosine alleviates spontaneous pain, tactile and thermal allodynia and mechanical hyperalgesia (Segerdahl *et al.*, 1995; Sollevi *et al.*, 1995). In addition, we have previously shown that the highly selective adenosine A1 agonist, GR79236, reverses the hypersensitivity to pain associated with nerve injury through a central site of action (Collins *et al.*, 1999). We have now examined the selective low-efficacy A1 agonist GR190178, N-(3-Fluoro-4-hydroxyphenyl)-5-methoxy adenosine, (Sheehan *et al.*, 2000), in the Chronic Constriction Injury (CCI) model of neuropathic hypersensitivity to pain in the rat (Bennett & Xie, 1988).

Briefly, under isoflurane anaesthesia, the common left sciatic nerve of male Random Hooded rats (180-200g) was exposed at mid-thigh level. Four ligatures of chromic gut (4.0) were tied loosely around the nerve with a 1mm spacing between each. The wound was then closed and secured with suture clips. The surgical procedure was identical for the sham operated animals except the sciatic nerve was not ligated. The effect of GR190178 on CCI-induced decrease in mechanical paw withdrawal threshold was measured using an algesymeter (Randall & Selitto, 1957). In each study, GR190178 or vehicle (0.25% methylcellulose) was dosed when the mechanical hypersensitivity had reached a stable maximum. Statistical analysis was carried out to compare the difference between the drug treated group and vehicle treated group using unpaired Student's t test (P<0.05).

GR190178 (1$mgkg^{-1}$ p.o., day 14 post-surgery) produced a complete (104%; P<0.05) and rapid reversal (1hr) of the CCI-induced decrease in paw withdrawal threshold. The paw withdrawal threshold for the sham operated animals compared to the GR190178 CCI animals was 103 ± 10g vs 96 ± 8g respectively, both n=10; P>0.05. This reversal was not maintained, declining 5hrs post a single dose and was similar to the vehicle treated CCI group 8hrs post dose (81 ± 8g vs 71 ± 5g respectively, both n=10; P>0.05). This effect was similar to that achieved with the high-efficacy agonist, GR79236, which also produced a rapid and significant reversal (Collins et al., 2000).A further study, showed GR190178 produced a dose-related reversal (ED_{50} = 0.6$mgkg^{-1}$ p.o.; n=7 per group) of the CCI-induced decrease in paw withdrawal threshold 1hr post dose.

In conclusion, the low-efficacy A1 agonist, GR190178 rapidly reversed the hypersensitivity to pain associated with nerve injury, similar to that observed with the high-efficacy adenosine A1 agonist, GR79236. These studies further support the hypothesis that adenosine A1 agonists may show utility in the treatment of neuropathic pain in man.

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Sollevi, A. *et al.*, (1995). Pain, *61* 155-158.

49P THE EFFECT OF THE SELECTIVE LOW-EFFICACY ADENOSINE A1 AGONIST GR190178, IN MODELS OF NOCICEPTIVE, ACUTE AND ESTABLISHED PAIN HYPERSENSITIVITY IN THE RAT

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We have previously described the efficacy of a highly selective adenosine A1 receptor agonist, GR79236 in animal models of acute and established inflammatory hypersensitivity (Clayton et al., 2000). We have now identified a low-efficacy adenosine A1 agonist, GR190178, N-(3-Fluoro-4-hydroxyphenyl)-5-methoxy adenosine, (Sheehan et al., 2000; Table 1), which we have evaluated in models of nociceptive, acute (carrageenan and Freund's Complete Adjuvant - FCA) and established inflammatory hypersensitivity (FCA).

Table 1. Relative intrinsic efficacy for GR79236 and GR190178.

Relative intrinsic efficacy (% relative to NECA; mean ± SEM, n≥3)		
	GR79236	GR190178
Ca2+ (FLIPR)	81 ± 4	45 ± 17

Male Random Hooded rats (180-220g) were fasted overnight. GR190178 was administered intraplantar 30mins prior to 100µl 2% carrageenan or 1h prior to 100µl of 1mg/ml FCA into the left hind paw. In a model of established inflammatory hypersensitivity, GR190178 was administered 2 days after FCA. The anti-hypersensitivity activity of GR190178 on carrageenan and FCA induced decrease in weight bearing on the inflamed left paw (dual channel weight averager. Clayton et al., 1997) was determined 3h and 6h respectively, after the inflammatory insult, or in the model of established inflammatory hypersensitivity 6h after GR190178. The effect of GR190178 on carrageenan and FCA induced paw oedema was assessed using a plethysmometer. The activity of GR190178 on carrageenan induced decrease in thermal paw withdrawal latency (Hargreaves et al., 1988) was also determined 3h post carrageenan. Anti-nociceptive activity was determined by investigating the effect of GR190178 on normal mechanical and thermal paw withdrawal thresholds. The effect of GR190178 on normal slow rearing activity was measured using the Benwick Activity Monitor, which detects animal movement (Locomotor activity – LMA). This was compared to its analgesic activity to allow calculation of a therapeutic index (TI). Statistical analysis was carried out to determine whether there was a significant difference between the vehicle treated and drug treated groups using unpaired Student's t test ($P<0.05$).

GR190178 inhibited the carrageenan-induced decrease in weight bearing (ED_{50} 0.2mgkg^{-1} p.o.; 100% @ 1mgkg^{-1} p.o.) and thermal paw withdrawal latency (ED_{50} 0.2mgkg^{-1} p.o.). GR190178 (1mgkg^{-1} p.o.) also reduced the associated paw oedema by 50%. GR190178 produced a dose-related inhibition of the FCA induced decrease in weight bearing after 6h (ED_{50} 0.5mgkg^{-1} p.o.; 100% @ 3mgkg^{-1} p.o.). In a model of established inflammatory hypersensitivity, GR190178 produced a dose-related reversal of the FCA induced decrease in weight bearing (ED_{50} 0.6mgkg^{-1} p.o.; 97% @ 3mgkg^{-1} p.o.). GR190178 (3mgkg^{-1} p.o.) increased normal mechanical and thermal nociceptive thresholds by 30% and 40% respectively. GR190178 (3mgkg^{-1} p.o.) reduced normal LMA by 40% so the TI with respect to analgesic activity in the carrageenan model was calculated as >15. In conclusion, these studies demonstrate that the low-efficacy A1 agonist GR190178 has a similar ceiling of efficacy as the full agonist GR79236 but with a greater TI. Therefore it may offer added benefit over full A1 agonists in the treatment of inflammatory pain states in the clinic.

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50P PROSTAGLANDINS INDUCE CALCITONIN GENE-RELATED PEPTIDE RELEASE FROM ADULT RAT TRIGEMINAL NEURONES

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Prostaglandins and calcitonin gene-related peptide (CGRP) have both been implicated in the pathogenesis of migraine. The pain of migraine is transmitted by the trigeminal Vth cranial nerve, which has its cell bodies located in the trigeminal ganglia. However, although prostaglandin E_2 (PGE_2) can cause migraine-like symptoms when administered to volunteers, trigeminal neurones have remained poorly characterized with respect to prostaglandin receptor distribution and function. In this study, we have used RT-PCR to identify mRNA for the EP & IP receptor types in whole trigeminal ganglia. In addition, we have also examined the effects of PGE_2 and carbaprostacyclin ($cPGI_2$) on CGRP release from cultured trigeminal neurones.

For RT-PCR, RNA was isolated from trigeminal ganglia from male Wistar rats (175-225g) and amplified using primers specific to EP_{1-4} and IP receptors as previously described (EP: Caggiano & Kraig, 1999; IP: Numaguchi *et al.*, 1999). Primary cultures were derived from adult rat trigeminal ganglia according to the method of Eckert *et al.*, (1997). Neurones were grown for 4-6 days in the presence of nerve growth factor (50 ng ml^{-1}) and cytosine-β-D-arabinofuranoside (20 µM). CGRP levels were determined over a 30 min period using an enzyme immunometric assay kit (SPIbio, Massy, France) and quantified in pg ml^{-1}. To account for differences in neuronal numbers between preparations, CGRP levels following exposure to drug or vehicle (DMSO, 0.001%) were compared to the baseline value (100%) obtained over the 30 min period immediately before stimulation in the same test well. Data are mean±s.e.m. from 3-9 experiments unless otherwise stated. Statistical comparison was by one-way analysis of variance followed by the Tukey test.

RT-PCR revealed specific bands of the expected size for EP_{1-4} and IP receptors. Their identity was confirmed by sub-cloning into pCR2.1 (Invitrogen) and subsequent sequencing. Incubation of trigeminal cultures with release buffer (Hingten *et al.*, 1995), containing 10 µM indomethacin, revealed an average baseline CGRP concentration of 54±11 pg ml^{-1} that varied between 28 and 126 pg ml^{-1} between preparations. Exposure to 1 µM PGE_2, $cPGI_2$ or iloprost for 30 min resulted in a significant increase in CGRP release of 260±23%, 267±17% and 268±26% ($P<0.001$), respectively. The release to PGE_2 and $cPGI_2$ was also observed to be both time- and concentration- dependent. No increase in CGRP release was seen in vehicle control wells (104±29%) or to $PGF_{2\alpha}$ (103±26%), GR63799 (95±13%), sulprostone (87±6%) or U46619 (76%; n=2).

Taken together, these data show, for the first time, the presence of transcripts for EP_{1-4} and IP receptors in adult rat trigeminal ganglia. In addition, we have demonstrated that, in cultured neurones, PGE_2, $cPGI_2$ and iloprost can cause CGRP release.

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51P ANTI-HYPERALGESIC EFFECTS OF LORNOXICAM, PIROXICAM, MELOXICAM, KETOROLAC, AND ASPIRIN IN RATS

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used as analgesics. The classical preclinical models do not allow a clear distinction to be made between the analgesic properties of agents belonging to this family. Clinical pain is characterised by the hyperalgesia, a substantial component of which is generated within the spinal cord (Woolf, 1994). Experimental models of hyperalgesia might be useful in order to differentiate NSAIDs and to evaluate their analgesic efficacy. We therefore studied the effects of NSAIDs with different selectivities for cyclo-oxygenase (COX)-1 and COX-2 in a simple behavioural model of spinally-mediated hyperalgesia (Bianchi & Panerai, 1997).

Male Sprague-Dawley CD rats were used (N=6/group). We assessed the effects of pre-administered (30 min) lornoxicam (L) , piroxicam (P), meloxicam (M), ketorolac (K), and aspirin (ASA) on the reduction of hindpaw nociceptive thresholds to thermal stimulation (hyperalgesia) produced by the injection of 0,1 ml 10% formaldehyde (F) in the tail. The Plantar test (Hargreaves *et al.*, 1988) was used to measure the hindpaw withdrawal latencies in basal conditions and 30, 60, 90, and 120 min after F injection. Hyperalgesic state was assessed by delta reaction time (basal latency – test latency). Each drug was administered intraperitoneally (i.p.) at its previously defined ED_{50} for the anti-inflammatory effect in the rat (i.e. the inhibition of carrageenan-induced hindpaw oedema measured by plethysmometry). The statistical evaluation of behavioural data was performed by two-way ANOVA followed by Bonferroni's t-test.

As expected, L (1.3 mg/kg), P (1.0 mg/kg), M (5.8 mg/kg), ASA (78 mg/kg) and K (1.1 mg/kg) did not modify thermal nociceptive thresholds. The effects of lornoxicam on hyperalgesia are described in Table 1:

	30'	60'	120'	180'
V+F	2.6 ± 0.2	3.2 ± 0.3	1.9± 0.7	1.2 ± 0.2
L+F	-0.4± 0.5	1.1 ± 0.2	0.5± 0.2	0.4 ± 0.2
CTR	0.03± 0.2	0.6 ± 0.4	0.8± 0.3	0.4 ± 0.2

Values are seconds, means ± S.E.M. of delta reaction time
V=Vehicle , CTR = controls

L completely ($P < 0.05$ vs F-injected rats, N.S. vs controls) inhibited the development of the hyperalgesic state (Table 1). P, M, and K significantly but not completely ($P < 0.05$ vs F-injected rats, and vs controls) reduced the hyperalgesia (data not shown). ASA was ineffective (N.S. vs F-injected rats).

The use of this experimental model permits the detection of different anti-hyperalgesic properties of NSAIDs. The anty-hyperalgesic efficacy does not seem to be strictly related to the selectivity for COX-1 or COX-2. As it is clearly effective against central sensitization in rats, lornoxicam might represent a valid analgesic agent in humans.

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52P EFFECTS OF TOPICAL BACLOFEN ON EVOKED-RESPONSES OF DORSAL HORN NEURONES IN CONTROL, SHAM-OPERATED AND SPINAL NERVE LIGATED RATS *IN VIVO*

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GABA is the major inhibitory transmitter in the spinal cord and plays a role in normal nociceptive transmission and may be subject to plasticity in persistent pain states. Here, the effects of intrathecal baclofen on evoked spinal neuronal responses in anaesthetised neuropathic, sham and control rats were assessed.

Spinal nerve ligated (SNL) male Sprague-Dawley rats (120-140g) were prepared under halothane anaesthesia as previously described by Chapman *et al.* (1998). Sham-operated rats and rats receiving no surgical intervention were also studied. Development of allodynia was assessed with von Frey hair (10g) stimulation to measure the sensitivity of the ipsi- and contra-lateral hindpaws. Extracellular single-unit recordings of convergent dorsal horn neurones (L4-L6), ipsilateral to SNL or sham procedure were made in anaesthetised (1% halothane in 2:1 N_2O:O_2) rats two weeks post-surgery. Electrically-evoked (16 stimuli @ 0.5Hz, x3 C-fibre threshold, 2ms pulse width) responses of neurones to receptive field stimulation were recorded every 10mins and were classified as: Aβ 0-20ms; Aδ 20-90ms; C 90-300ms, post-discharge (PD) 300-800ms post-stimulus. Following stable control recordings r-baclofen, 0.1μg/50μl-30μl/50μl in saline (≡9.4μM-2.8mM), was applied onto the spinal cord and the effect of each dose was followed for 50mins. Data are presented as mean±S.E.M. Statistical analysis was assessed using one-way analysis of variance with a Tukey's or Dunnett's post-hoc test as appropriate.

SNL, but not sham, rats developed ipsilateral mechanical allodynia. Control evoked neuronal responses of spinal neurones (Table 1) and neurone depths (826.1±40.5μm) were similar for the three groups.

	Control evoked-responses			
	Aβ	Aδ	C	PD
Control	97±18	82±24	389±98	212±47
Sham	127±23	56±21	363±48	235±41.8
SNL	125±23	81±30	326±61	283±76

Table 1. Mean electrically evoked responses of neurones in control (n=6), sham (n=6) and SNL (n=6) rats. Values are mean number of action potentials±S.E.M.

In control, sham and SNL rats baclofen significantly reduced Aβ fibre evoked responses (30μg/50μl: 24.7±23.9% $P<0.05$; 28.0±10.8% $P<0.05$; 46.4±20.3% $P<0.001$ respectively). Baclofen significantly ($P<0.001$) reduced Aδ fibre evoked responses in control, sham and SNL rats, (30μg/50μl: 1.3±1.3%; 7.0±6.2%; 30.8±29.8% respectively). C fibre evoked responses of neurones were significantly ($P<0.001$) reduced in control, sham and SNL rats (30μg/50μl: 0.0±0.0%; 6.7±6.4%; 8.5±6.0% respectively). PD was also significantly ($P<0.001$) reduced in control, sham and SNL rats (30μg/50μl: 0.0±0.0%; 7.2±6.5%; 1.7±1.29% respectively). There was no difference in the degree of reduction of firing by r-baclofen between the three groups.

The inhibitory effects of baclofen demonstrated here corroborate previous behavioural studies in neuropathic rats (Hwang & Yaksh, 1997). The data suggest that there is no change in the functional role of spinal $GABA_B$ receptors following peripheral nerve injury.

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53P NEUROSTEROID MODULATION OF NEURONAL EXCITABILITY IN THE PERIAQUEDUCTAL GREY MATTER INRATS: CARDIOVASCULAR AND ELECTROPHYSIOLOGICAL STUDIES WITH ORG20599

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The dorsal half of the periaqueductal grey matter (dPAG) constitutes a midbrain aversive system. Stimulation of the rat dPAG evokes escape behaviour, hyperpnoea and a pattern of sympathoactivation in which a raised cardiac output is diverted to skeletal muscle at the expense of the cutaneous and and visceral circulations (Lovick, 1996 for a review). The excitability of the dPAG can be modulated by changes in circulating levels of the progesterone metabolite allopregnanolone (ALLO; Lovick, 2000). In the present study ORG20599, a water soluble analogue of ALLO (Hill-Venning *et al*, 1996), has been used to investigate whether neuroactive steroids act directly within the PAG to modulate neuronal excitability.

In male Wistar rats (300-380 g) anaesthetised with urethane (1.5 g. Kg^{-1} i.p.), multibarrel micropipettes were used to make extracellular recordings from neurones in the dPAG. Ongoing activity was raised to 8-12 Hz by continuous iontophoretic ejection (0-10 nA) of D,L-homocysteic acid in order to facilitate the study of inhibitory events. Intravenous injection of ORG20599 (1 mg.Kg^{-1}) produced a 72-100% inhibition of firing which was maximal after 72-210 s and recovered after 4.5 - 6 min (n=3). GABA, applied iontophoretically (0-10 nA for 10 s) silenced the ongoing activity of 10/10 cells, whilst bicuculline methiodide (BIC, 10 - 20 nA) produced an increase in the firing rate, reflecting withdrawal of inhibitory GABAergic tone. BIC also abolished the GABA-evoked inhibition of these neurones.

In 8/8 GABA-sensitive neurones, iontophoresis of ORG20599 (25 - 40 nA for 20 s) produced a 80 - 100% inhibition of firing which developed slowly and lasted 1 - 4 min. The inhibition was reduced by 70 - 100% during application of BIC at a dose which blocked the effect of GABA on the same cells (4/4).

For functional experiments, rats were instrumented to record blood pressure, heart rate, femoral arterial blood flow and tracheal air flow. Electrical stimulation in the dPAG via a cannula electrode (10 s trains of 1 ms pulses, 80 Hz, 35-75 mA) evoked a pressor response (33 - 35 mmHg), tachycardia (20 - 61 beats min^{-1}, vasodilatation in the hindlimb (19 - 69% increase in conductance) and tachypnoea (29 - 78 breaths min^{-1}). The same pattern of response was reproduced by microinjection of BIC (5 nmol in 250 nl, n=7) into the dPAG. In contrast, microinjection of ORG20599 through the cannula electrode (374 pmol in 200 nl, n=3) had no effect on resting cardiorespiratory values but produced a 25-100%, mean 53.0 ± 8.5% reduction in the amplitude of each component of the electrical-evoked cardiorespiratory response. This effect was maximal after 5 min and recovered after 50 - 60 min.

The results suggest that a tonic inhibitory GABAergic influence normally controls the functional excitability of neuronal assemblies in the dPAG which intitiate the symapthexcitation associated with aversive behaviour. In turn, the GABA tone may be subject to modulation by neuroactive steroids acting at $GABA_A$ receptors.

Supported by the MRC and The Wellcome Trust.

RG20599 was a gift from Organon Ltd.

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54P THE ADENOSINE A1 RECEPTOR AGONIST GR79236 INHIBITS EVOKED FIRING OF MEDULLARY DORSAL HORN NEURONES IN THE ANAESTHETISED RAT

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The selective A1 receptor agonist GR79236 (Gurden et al.,1993) exerts potent analgesic activity in a rat model of neuropathic hypersensitivity (Collins et al., 1999). Since adenosine A1 receptors are localised on trigeminal neurones, we have studied the effect of GR79236 in an anaesthetised rat model of trigeminovascular signalling relevant to the study of pain transmission in headache.

Male Sprague Dawley rats (300-375 g) were anaesthetised (induction, isoflurane 5 % in O_2; maintenance, pentobarbitone 30 $mgkg^{-1}h^{-1}$ i.v.) and the jugular vein, carotid artery and trachea cannulated. Recording electrodes were positioned within the trigeminal nucleus caudalis (TNC) for identification of single units responding with short latency (<20 ms) to electrical stimulation (1 Hz, 100-500 μs, 0.4-4.0 mA) of the middle meningeal artery (MMA), or to non-noxious stimulation of the whiskers by pulsed air jet. Drug effects were determined on neuronal responses to trains of electrical stimuli applied every 3-5 min, or to trains of air pulses applied every 60 s. Data were analysed by ANOVA followed by Dunnett's *post hoc* test or Student's t-test as appropriate. A probability (P) value <0.05 was taken as significant.

Bolus injection of GR79236 (10 and 30 μgkg^{-1} i.v.) reduced firing of TNC neurones evoked by electrical stimulation of the MMA, with a median time to peak effect of 15 min (range 9-24 min) and 12 min (range 12-18 min), respectively (both n=5). At these times of peak effect, evoked TNC firing was inhibited by 40.2 ± 12.0 % (10 μgkg^{-1}; $p<0.05$, n=5) and 82.4 ± 9.3 % (30 μgkg^{-1}; $p<0.001$, n=5), when compared with paired saline control responses. Neuronal inhibition following the higher dose of GR79236 persisted for >25 min.

In separate experiments the inhibitory effect of GR79236 (30 μgkg^{-1} i.v.) was reversed by the adenosine A1 receptor antagonist, DPCPX (3$mgkg^{-1}$ i.v.) (Fredholm et al.,1998). Thus, in control (GR79236 + vehicle) and antagonist-treated (GR79236 + DPCPX) animals, inhibition of evoked TNC neuronal firing after 30 min was 68.4 ± 13.4 % (n=3) and 4.4 ± 7.1 % (n=4), respectively ($P<0.05$). DPCPX itself had no effect on evoked neuronal firing (n=2).

GR79236 (30 μgkg^{-1} i.v.) did not inhibit TNC neurones activated by non-noxious stimulation of the whiskers (i.e. which did not receive convergent input from the MMA). Thus, evoked neuronal firing at 12 and 15 min post GR79236 administration represented, respectively, 85.8 ± 2.0 % and 77.1 ± 4.8 % of maximal pretreatment responses (both n=3, $P>0.05$).

We conclude that GR79236 inhibits TNC firing evoked by electrical stimulation of the MMA, but not by innocuous whisker stimulation. This effect is likely mediated by activity at central adenosine A1 receptors. These results, together with data showing that GR79236 inhibits neurogenic dilatation of the MMA in the rat (Honey et al., 2000), indicate that A1 receptor agonists may represent a novel class of migraine treatment in the clinic.

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55P HYPOFUNCTIONALITY OF Gi PROTEINS AS AETIOPATHOGENETIC MECHANISM FOR MIGRAINE AND CLUSTER HEADACHE.

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Headache is the most common pain syndrome. It is also the most frequent symptom in neurology, where it may be a disease in itself (primary headache) or indicate an underlying local or systemic disease (secondary headache) (Raskin, 1994). In the complete absence of physical and laboratory alterations, diagnosis of primary headache remains purely clinical, based on the detailed description of symptoms by the patients. The involvement of Gi proteins in the modulation of pain perception has been widely established and mutations in G-proteins have already been identified as the aetiopathological cause of human diseases (Spiegel, 1996). The aim of the present study was to determine whether a deficiency or an hypofunctionality of the Gi proteins occurred in primary headache.

The functionality, evaluated as the capability to inhibit adenylate cyclase activity by the method of Brodde *et al.* (1985), and the level of expression, evaluated by the method of Laemmli (1970), of Gi proteins were investigated in peripheral blood lymphocytes from migraine without aura (1M/9F; age=43.5±3.8), migraine with aura (2M/3F; age=37.4±8.3) and cluster headache (5M/1F; age=36.3±3.2) sufferers in comparison with healthy subjects (9M/9F; age=38.5±2.7) and with patients suffering from painful diseases (3M/9F; age=69.0±2.5) such as neuropathic pain, rheumatoid arthritis and osteoarthritis.

In healthy subjects, the activation of Gi proteins by Gpp(NH)p (10^{-5} M), a non-hydrolysable analogue of GTP, produced a reduction of the forskolin-stimulated adenylyl cyclase activity. The cAMP levels were reduced from the value of 16.3±1.1 to the value of 10.3±1.1 pmol/10^6 lymphocytes. The capability to inhibit forskolin-stimulated adenylyl cyclase activity in headache patients was abolished (16.0±0.9; 17.0±1.5). This hypofunctionality of Gi proteins was similar in subjects suffering from migraine without aura, migraine with aura and cluster headache. By contrast, unaltered Gi protein functionality was observed in patients suffering from painful diseases. Migraine patients also showed basal cAMP levels about 3.5 times higher than controls (2.1±0.3; 6.8±0.6). The reduced activity of Gi proteins seems not to be related to a reduction of protein levels since no significant reduction of the Giα subunits was observed.

These results indicate the Gi protein hypofunctionality as an etiopathogenetic mechanism in migraine and cluster headache. Furthermore, unlike the laboratory tests available to date, the determination of an increase in cAMP basal levels in lymphocytes, together with the assessment of a Gi protein hypofunctionality, can make it possible to biochemically distinguish headache patients from patients suffering from other painful diseases.

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56P PHARMACOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF A_3 ADENOSINE RECEPTORS IN JURKAT T CELLS

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Adenosine exerts a number of physiological functions through activation of four cell membrane receptors classified as A_1, A_{2A}, A_{2B} and A_3. Earlier pharmacological evidence showed the existence of A_{2B} and A_{2A} adenosine receptors in Jurkat cells, whilst ruling out the expression of A_3 subtypes (Mirabet *et al.*, 1997). In the past the lack of high affinity and selective A_3 radioligands prevented the study of this receptor. The newly developed A_3 adenosine receptor antagonist, 5-N-(4-methoxyphenyl-carbamoyl)amino-8-propyl-2-(2-furyl)-pyrazolo-[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine [^{3}H]MRE 3008F20, represents a key advance towards the characterization of A_3 receptors on cells possessing multiple subtypes of adenosine receptors (Varani *et al.*, 2000).

In Jurkat cells, A_3 receptors were found first by means of RT-PCR experiments and were then characterized by performing binding studies using [^{3}H]MRE 3008F20 (for methods see Varani *et al.*, 2000). Saturation experiments, carried out by incubating different concentrations of radioligand in the range 0.2-20 nM, revealed a single high affinity binding site with K_d of 1.9 ± 0.2 nM and B_{max} of 1.3 ± 0.1 pmol/mg of protein. Competition experiments of [^{3}H]MRE 3008F20 binding (2 nM) to A_3 receptors in Jurkat cells were performed using a series of typical adenosine receptor ligands which displayed a rank order of potency consistent with that typically found for interactions with the A_3 receptors. Agonist competition curves yielded both high (30%) and low-affinity states of A_3 receptors. Cl-IB-MECA and IB-MECA were the most potent compounds with K_H of 1.6, 2.7 nM and K_L of 83, 150 nM, respectively. The addition of GTP converted the curves of agonists from biphasic to monophasic. On the contrary, competition binding curves with antagonists were monophasic and did not change by addition of GTP.

Thermodynamic parameters obtained from the van t'Hoff plot indicate that [^{3}H]MRE 3008F20 binding to A_3 adenosine receptors is enthalpy- and entropy-driven ($\Delta H°$= -27.97 ± 3.18 kJmol^{-1}, $\Delta S°$= 64.89 ± 6.35 J mol^{-1} K^{-1}) in agreement with data obtained in CHO cells expressing human A_3 receptors (Varani *et al.*, 2000). In functional assays Cl-IBMECA and IBMECA, the most A_3 selective agonists, were able to inhibit cAMP accumulations with EC_{50} values of 3.5 ± 0.3 and 12 ± 0.1 nM, respectively. The same agonists, at a concentration of 30 μM, stimulated Ca^{2+} release from intracellular Ca^{2+} pools followed by Ca^{2+} influx, suggesting the presence of phospholipase C-coupled receptors (basal level 100-150 nM, stimulated level 250-300 nM). By means of the first antagonist radioligand [^{3}H]MRE 3008F20 we could demonstrate functional A_3 receptors on Jurkat cells. We also found that adenosine, via the activation of A_3 receptors, inhibits cAMP levels and stimulate Ca^{2+} release, suggesting that further elucidation of the physiological significance of these mechanisms is likely to shed new light on the role of adenosine in leukemic T cells.

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57P LIPIDS EFFECTS ON RESPONSES AT $P2X_7$ RECEPTORS

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The functional properties of $P2X_7$ receptors can change in a number of ways after persistent or prolonged activation. First, the ionic-selectivity of the channel changes during prolonged activation (Surprenant et al., 1996). Second, repeated agonist application increases the magnitude of inward currents and slows the deactivation rate at rat $P2X_7$ receptors (Hibell *et al.*, 2000). Third, agonist potency increases after repeated agonist application (Hibell *et al.*, 2000). In this study we show that several phosphocholine (PC) containing lipids increase agonist potency at $P2X_7$ receptors.

Receptor function was assessed by measuring ATP or 2'-and 3'-O-(benzoylbenzoyl)-ATP (BzATP) stimulated ethidium accumulation in HEK293 cells transfected with rat, human or mouse $P2X_7$ receptors (Chessell *et al.*, 1998; Michel et al., 2000). Studies were performed at 22°C in 96 well plates in pH 7.4 buffer containing (mM), 10 Hepes, 10 glucose, 5 KCl, 0.5 $CaCl_2$ and either 280mM sucrose (sucrose buffer) or 140mM NaCl (NaCl buffer). Data are the mean±s.e.mean of 4-8 experiments. The significance of changes in potency was determined using a 1 way ANOVA and Tukey's test.

Pre-incubation of cells for 20mins in sucrose buffer with L-α-lysophosphatidyl (LP) choline (LPC, C16:0) increased BzATP potency (4±0.6fold at 30μM. $P<0.05$). Increases in BzATP potency were also observed with 30μM platelet activating factor (PAF: 3±0.3 fold) or Lyso-PAF (L-PAF; 4±0.7 fold). LP-ethanolamine (C16:0), LP-serine (C18:0) or phosphatidylcholine (C16:0) had no effect at 30μM. BzATP potency was also increased by 30μM of oleyloxyethyl-PC (OPC; 6±1.2 fold, $P<0.05$), hexadecyl-PC (HPC; 12±2.6fold, $P<0.05$) or sphingosylphosphorylcholine (SPC; 4±1.1 fold, $P<0.05$). LPC (30μM) increased the potency of BzATP (3±0.5 fold $P<0.05$) and ATP (4±0.6fold $P<0.05$) in NaCl buffer.

The lipids did not cause cell lysis at 30μM. However, at 100μM, LPC, SPC and HPC caused some cell lysis (5-20%) as assessed by release of lactate dehydrogenase and increases in the basal accumulation of ethidium. Digitonin did not affect BzATP potency at sub-lytic concentrations up to 0.0003% w/v. LPC, SPC, L-PAF and PAF increased ($P<0.05$) BzATP potency 18±1, 10±2, 11±2 and 27±7 fold, respectively, at mouse $P2X_7$ receptors (30μM, 60min preincubation, sucrose buffer). In contrast LPC or SPC (1-30μM) did not affect ATP-stimulated ^{45}Ca accumulation at rat $P2X_2$ or mouse $P2X_4$ receptors.

LPC can be produced by the action of phospholipase A2 (PLA2) on membrane phospholipids. In sucrose buffer, exogenous PLA2 (*Naja mossambica*, $100mU.ml^{-1}$) increased BzATP potency at human (3±0.4fold, $P<0.05$, 20min incubation) and mouse (2.3±0.4fold, $P<0.05$, 60min incubation) $P2X_7$ receptors.

In conclusion, lipid products of PLA2 and exogenous PLA2 increase agonist potency at human and mouse $P2X_7$ receptors. This effect is not observed at $P2X_2$ or $P2X_4$ receptors and so may be selective for $P2X_7$ receptors. Only phosphocholine containing lipids, including the anti-cancer agent, HPC (Muschiol et al., 1987), were effective. Since PLA2 activity is increased under inflammatory conditions, endogenous LPC may increase $P2X_7$ receptor function under these conditions. Furthermore, since $P2X_7$ receptor activation increases PLA2 activity (Alzola *et al.*, 1998), the LPC produced by this enzyme may exert a positive feedback effect on the $P2X_7$ receptor.

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58P SHORT TERM DESENSITIZATION AND INTERNALIZATION OF THE RAT A_{2B} ADENOSINE RECEPTOR IS MEDIATED BY ITS DISTAL C-TERMINUS TAIL

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In previous work we have established that rapid agonist-induced desensitization of endogenous A_{2A} and A_{2B} adenosine receptors is mediated by G-protein-coupled receptor kinase 2 (GRK2; Mundell *et* al. 1997, 1998). To investigate the role of the A_{2B} adenosine receptor cytoplasmic tail in mediating agonist-dependent desensitization and internalization responses, the A_{2B} adenosine receptor cDNA was cloned from rat brain and epitope-tagged with the Haemagglutinin tag (HA-tag) at the N-terminus. A C-terminus tail truncated mutant was produced by a Polymerase Chain Reaction method, in which the last 7 amino acid residues were truncated (Q325stop). Chinese Hamster Ovary cells were stably transfected with Wild Type (WT) or Q325stop cDNA, subcloned in the mammalian expression vector pcDNA3.

Cell lines expressing the truncated mutant displayed strikingly different properties from the WT receptor; although both receptors supported adenosine agonist-induced cAMP formation, short-term agonist-induced desensitization and internalization was effectively abolished in cells expressing the Q325stop receptor. Desensitization was measured in intact cells cultured in 24-well dishes. Cells were pretreated with the adenosine receptor agonist NECA (10μM) for different times, washed with PBS to remove the agonist and rechallenged with 10μM NECA for 20 minutes, in the presence of 100 μM Ro201724 as phosphodiesterase inhibitor. The amount of cAMP accumulated during the agonist challenge was measured (pmoles cAMP/mg total protein), and the difference in cAMP produced with and without agonist pretreatment used as a measure of functional desensitization. Values quoted are means ± s.e.mean. In WT cells, NECA pretreatment led to a 54.3 ± 6 % reduction in subsequent A_{2B} adenosine receptor responsiveness as compared to WT cells not pretreated with NECA (n=4). In complete contrast in Q325stop cells a similar pretreatment with NECA for 1 h did not lead to any observable desensitization in subsequent Q325stop receptor responsiveness (-1.9 ± 2 % reduction, n=4).

Internalization of the receptor was quantified by an immunosorbent assay (ELISA; Daunt *et al.* 1997), making use of the HA-epitope tag. After 1 h exposure to 10 μM NECA, 47.1 ± 3 % receptor internalization was observed in WT cells, whereas only 16.2 ± 2 % receptor internalization was observed in Q325stop cells (n=5). Imaging of WT and Q325stop receptors by confocal microscopy using an anti-HA primary antibody and rhodamine-linked secondary antibody fully supported this finding, indicating a lack of short-term agonist-promoted internalization for the truncated mutant. In addition, arrestin-2-Green Fluorescent Protein was transiently transfected into WT and Q325stop stable cell lines. Marked translocation of arrestin-2-Green Fluorescent Protein from cytosol to membrane was observable within 1 min of 10 μM NECA addition to WT cells, but did not occur in cells expressing the Q325stop mutant for up to 4 min after agonist addition.

These experiments demonstrate that the distal C-terminus tail of the A_{2B} adenosine receptor is required for short-term agonist-induced desensitization and internalization. In turn, the inability of the Q325stop mutant to undergo rapid desensitization and internalization may be due to the loss of arrestin interaction.

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59P FUNCTIONAL SIGNIFICANCE OF SEQUENCE VARIANTS IN THE C-TERMINAL TAIL OF THE HUMAN ADENOSINE A_{2A} RECEPTOR

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The cDNA sequence of the human adenosine A_{2A} receptor gene was first reported by Furlong *et al* (1992). Subsequently, however, an alternative sequence has twice been reported (Genbank X68486 and M97370). This varies from the first sequence by a one base-pair substitution of A by G at nucleotide position 1174, which changes the amino acid sequence from an arginine residue to a glycine residue at position 392, near the C-terminal tail of the receptor. The pharmacological properties of the two sequence variants were examined functionally by expressing them in yeast and Chinese hamster ovary (CHO) cells.

The yeast assay was agonist-dependent induction of FUS1-lacZ (which encodes for the enzyme beta-galactosidase), causing the cleavage of a substrate to fluorescein, which was measured fluorimetrically (Brown *et al* 2000). A similar cAMP-dependent reporter gene assay was performed in CHO cells (McDonnell *et al*, 1998). Competition binding was performed on CHO membranes using the A_{2A} antagonist radioligand [^{3}H]ZM241385 (Tocris), and Western blotting was carried out on yeast membranes using an A_{2A} antibody (Alexis Biochemicals). Drugs used (Gurden *et al*, 1993; Hannon *et al*, 1998) were the non-selective agonist N^6-ethylcarboxamidoadenosine (NECA); IB-MECA (A_3-selective agonist); GR79236 (A_1-selective agonist); CGS21680 and 1-[2-[(4-aminocyclohexyl)amino]-6-[(2,2-diphenylethyl)amino]-9H-purin-9-yl]-1-deoxy-N-ethyl-β-D-ribofuranuronamide (GR163819; both A_{2A}-selective agonists); and ZM241385 (A_{2A}-selective antagonist).

In both yeast and mammalian cell functional assays the order of potency of the adenosine receptor agonists for activating both sequence variants was GR163819>NECA>CGS21680>IB-MECA >GR79236. The A_{2A}-selective antagonist ZM241385 competitively antagonised the response to NECA in yeast with a pK_B value of 9.0 for both sequence variants. Thus, the general pharmacological properties of the variants were similar. However, a highly significant decrease in constitutive activity was found for 392G when compared to 392R in yeast (mean constitutive activity ±standard error of 392G was 27000 ± 2200 fluorescence units vs 61000 ± 6600 for 392R; n=12 independent transformants; $p<0.01$). Furthermore, the mean pEC_{50} value (with 95% confidence limits) for NECA in CHO cells was highly significantly more potent at the 392R variant than 392G (8.8 [8.7–8.8] vs 7.6 [7.0–8.1]; n=12; $p< 0.01$). Using membranes from CHO cells expressing the 392R variant, binding of [^{3}H]ZM241385 was observed which could be displaced by all of the agonists tested with a rank order of potency equal to that in the functional assays and by ZM241385 with a pK_i value of 9.0. With 392G membranes, however, no significant displaceable binding could be detected. Western blotting confirmed that the A_{2A} receptor expression levels were greater for 392R than for 392G in the yeast cells.

Taken together, the functional and binding data indicates that the 392G variant is expressed at a lower level than 392R in both expression systems used in this study. It is still unknown whether the two sequences represent genuine polymorphisms. If so, and if the above pharmacological characteristics are maintained, then A_{2A} agonists could be more potent in man in the 392R population.

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60P DECREASES IN Na^+ TRANSEPITHELIAL FLUX BY DOPAMINE IS PRIMARILY DEPENDENT ON INHIBITION OF THE APICAL Na^+/H^+ EXCHANGER

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The present study was aimed to evaluate whether inhibition of Na^+ transepithelial flux by dopamine is primarily dependent on inhibition of the apical Na^+/H^+ exchanger, inhibition of the basolateral Na^+,K^+ ATPase or both. For this purpose we used Opossum kidney (OK) cells which are known to express several transport systems characteristic of proximal tubular cells, namely the Na^+/H^+ exchanger (Azarani et al., 1995), the Na^+-independent HCO_3^- transport system (Pastoriza-Munoz et al., 1992), and the Na^+,K^+-ATPase that is responsible for the maintenance of the driving force for vectorial Na^+ transport from the apical to the basolateral membrane (Pedemonte et al., 1997). OK cells are endowed with D_1- and D_2-like receptors, the activation of the former, but not the later, being accompanied with stimulation of adenylyl cyclase (Gomes et al., 2000). Results are arithmetic means with s.e.mean, n=4-8. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. Intracellular pH was measured with the pH sensitive fluorescence indicator BCECF. Dopamine produced intracellular acidification with an EC_{50} of 84±2 nM; maximal acidification (decrease in pH_i by 0.082±0.012 units) was attained at 1 μM dopamine. The effect of 0.3 μM dopamine on pH_i was significantly antagonised by 1 μM SKF 83566, a D_1–like receptor antagonist, but not by 1 μM (S)-sulpiride, a D_2–like receptor antagonist (Jose et al., 1992). Because dopamine is known to inhibit Na^+-K^+ ATPase activity leading to decreases in sodium transepithelial flux, the next series of experiments were aimed to evaluate if the acidifying effect of dopamine depended on the inhibition of Na^+-K^+ ATPase activity. For this purpose, the effect of dopamine (1 μM) was tested in the presence of the Na^+-K^+ ATPase inhibitor ouabain (100 μM). Ouabain (100 μM) alone produced a marked acidification effect (from pH 7.39±0.01 to pH 7.22±0.03). However, pretreatment with ouabain (100 μM) failed to prevent the acidification induced by 1 μM dopamine; pH_i decreased by 0.082±0.012 and 0.063±0.007 units in vehicle and ouabain-treated cells, respectively. The inhibitory effect of dopamine on Na^+/H^+ exchange (68±12 % of control values) was completely reverted by SKF 83566 (1 μM), but not (S)-sulpiride (1 μM). Dopamine (1 μM) was devoid of effect on the Na^+-independent HCO_3^- transport system. To study Na^+-K^+ ATPase activity in OK cells, it was decided to use an eletrophysiological method in which cell monolayers were continuously monitored for changes in I_{sc} after the addition of amphotericin B to the apical cell side, to increase the sodium delivered to Na^+-K^+ ATPase to the saturating level. Pretreatment with dopamine applied from the apical cell side significantly reduced the effect of 1.0 μg ml^{-1} amphotericin B upon I_{sc}, this being prevented by the D_1–like receptor antagonist SKF 83566 (1 μM) (71±5 % of control values). The D_1–like receptor agonist SKF 38393 (1 μM) was also found to attenuate, in a concentration dependent manner, the effect of amphotericin B on I_{sc} (63±12 % of control values). The data presented here shows that dopamine produces marked intracellular acidification and attenuation of amphotericin B-induced decreases in I_{sc}, without affecting the pH_i recovery after CO_2 removal. These results agree with view that dopamine through the activation of D_1- but not D_2-like receptors inhibits both the Na^+/H^+ exchanger and Na^+,K^+-ATPase, without interfering with the Na^+-independent HCO_3^- transporter. Because the dopamine-induced decrease in pH_i was insensitive to ouabain, it is suggested that the acidifying effect of dopamine results primarily from inhibition of the Na^+/H^+ exchanger.

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Supported by grant 35747/99 from FCT.

61P THE FUNCTIONAL RESPONSE OF TRACAZOLATE IS DEPENDENT ON THE SUBUNITS PRESENT WITHIN THE $GABA_A$ RECEPTOR

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The pyrazolopyridines, of which tracazolate is an example, were first synthesised nearly 30 years ago. Behavioural studies have shown that these compounds are anxiolytic; however, functionally they potentiate $GABA_A$ receptors, antagonise adenosine receptors and inhibit phosphodiesterase. No work on recombinant receptors has been reported. The aim of this study was to examine the effects of tracazolate on recombinant human $GABA_A$ receptors expressed in *Xenopus* oocytes, using the conventional two-electrode voltage-clamp technique. All data are expressed as mean ± s.e. mean, n=4-6 and statistical differences assessed by Student's *t*-test.

Concentration-response curves (CRC) examining the modulation of a control GABA EC_{20} response by tracazolate were constructed for a variety of $GABA_A$ receptor subtypes. Tracazolate potentiated $\alpha1\beta3$, $\alpha1\beta3\gamma2s$ and $\alpha6\beta3\gamma2s$ receptors with similar pEC_{50} (ranging from -5.58 to -5.95) maximum (224 to 363%) and Hill coefficients (1.24 to 1.42). Similar to a number of $GABA_A$ receptors modulators eg loreclezole, selectivity for $\alpha1\beta3\gamma2s$ receptors over $\alpha1\beta1\gamma2s$ receptors was observed (pEC_{50}=-5.83±0.10 and -4.88±0.11 respectively). Like loreclezole, Asn265 within TM2 of the β3 subunit was shown to be responsible for this increased sensitivity. Replacement of the γ2s subunit with the ε subunit reversed the functional response of tracazolate, as the GABA EC_{20} response was inhibited. The pIC_{50} for inhibition of $\alpha1\beta3\varepsilon$ receptors (-5.94±0.10) was similar to the pEC_{50} for potentiation of $\alpha1\beta3\gamma2s$ receptors. Selectivity for β3 over β1 was retained with $\alpha1\beta1\varepsilon$ receptors, being 4-fold less sensitive than $\alpha1\beta3\varepsilon$ receptors (pIC_{50}=-5.40±0.17). A small degree of potentiation with tracazolate was observed on $\alpha1\beta1\gamma1$and $\alpha1\beta1\gamma3$ receptors (21.0±5.9 and 34.7±10.1 respectively) while concentrations greater than 10μM elicited inhibition. Like $\alpha1\beta1\gamma2s$ receptors, $\alpha1\beta1\delta$ receptors were potentiated by tracazolate with similar pEC_{50} (-4.88±0.11 vs -4.78±0.06) and Hill coefficients (1.54 vs 1.38). The maximum potentiation, however, was significantly greater ($P<0.05$) for $\alpha1\beta1\delta$ receptors (1368±377%) vs $\alpha1\beta1\gamma2s$ receptors (168±16%). Unusually this maximum potentiation was approximately 3 times greater than the current elicited by a maximum GABA concentration.

Further studies on $\alpha1\beta3\gamma2s$, $\alpha1\beta3\varepsilon$ and $\alpha1\beta1\delta$ receptors examined the effect of single concentrations of tracazolate on the GABA CRC. On $\alpha1\beta3\gamma2s$ receptors, 1μM tracazolate produced a significant ($P<0.01$) 2.5±0.3-fold shift to the left of the GABA pEC_{50} with no significant effect on the maximum response or Hill coefficient. Higher concentrations of tracazolate (10μM and 30μM) further increased this leftward shift of the GABA concentration-response curve (21.6±5.5 and 39.3±15.8-fold respectively). In addition, the maximum response of GABA in the presence of 10μM and 30μM tracazolate was significantly ($P<0.01$) reduced compared to that obtained for the control GABA CRC (67.6±4 and 40.0±2.9% respectively). Reductions in the peak GABA response were also observed for $\alpha1\beta3\varepsilon$ receptors in the presence of 1 and 3μM tracazolate (46.5±1.1 and 17.5±2.7% respectively); however, no differences in the pEC_{50} or Hill coefficients were observed. Similar to $\alpha1\beta3\gamma2s$ receptors, CRC to GABA on $\alpha1\beta1\delta$ receptors were shifted to the left by 10μM and 30μM tracazolate. However, unlike $\alpha1\beta1\gamma2s$ the maximum response to GABA in the presence of 10 and 30μM tracazolate was significantly greater (203±28 and 305±34% respectively).

In summary, tracazolate modulates recombinant human $GABA_A$ receptors in a unique manner, the functional response and apparent pEC_{50}/IC_{50} being dependent on the receptor subtype.

62P EVIDENCE THAT PKA AND PKC REGULATE ENDOGENOUS SECRETIN RECEPTOR RESPONSIVENESS

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The G_s-coupled secretin receptor endogenously expressed in NG108-15 mouse neuroblastoma x rat glioma cells undergoes robust agonist-induced desensitization (Mundell *et al.* 1997). In the present study, we investigated the roles of PKA and PKC in the desensitization of secretin receptor responsiveness.

Wild type NG108-15 cells, cultured as previously described (Mundell *et al.*, 1997), were seeded into 24 well plates. The PKA inhibitor H-89 (10 μM), the PKC activator phorbol 12-myristate 13-acetate (PMA, 1 μM) or the PKC inhibitor GF109203X (2 μM) were added to the wells 15-30 min prior to agonist addition. The phosphodiesterase inhibitor Ro201724 (100 μM) was also added to each well. With the exception of basal, cells were challenged with either secretin (100 nM), the adenosine receptor agonist NECA (10 μM), the IP-prostanoid receptor agonist iloprost (1 μM), or the adenylyl cyclase activator forskolin (10 μM). Whole cell cAMP accumulation was subsequently determined by a protein binding assay (Mundell *et al.* 1997). Data are expressed as means ± s.e.mean. Pretreatment of cells with H-89 resulted in an increase in secretin-stimulated cAMP accumulation (cAMP accumulation after 60 min of stimulation in the presence of H-89 was 176 ± 21% that of the control secretin response; n=5). There was no difference in the EC_{50} value for secretin-mediated cAMP accumulation in the presence of H-89 (mean EC_{50} values for control- and H-89- treated curves were 29.6 ± 21 nM and 7.2 ± 4 nM, respectively; n=3). cAMP accumulation stimulated by forskolin, iloprost and NECA did not appear to be affected by H-89 treatment (cAMP accumulation after 60 min of agonist stimulation in the presence of H-89 for forskolin, iloprost and NECA was 104 ± 18%, 118 ± 32% and 128 ± 15% that of the control agonist response, respectively; n=5).

Pretreatment of cells with PMA attenuated both secretin- and forskolin- stimulated cAMP accumulation to 66 ± 9% and 44 ± 2% that of the control agonist response in the absence of PMA, respectively (n=3). However, unexpectedly, cAMP accumulation stimulated by NECA and iloprost was unaffected by PMA pretreatment (cAMP accumulation values were 106 ± 24% and 107 ± 7% compared to the control agonist reponse, respectively). The PMA- induced desensitization of the secretin- and forskolin- mediated responses was effectively reversed by co-addition of the PKC inhibitor GF109203X.

The results suggest that PKA selectively regulates secretin receptor responsiveness at the level of the receptor itself since adenylyl cyclase responses to other endogenous receptors, or directly through forskolin, were unaffected by H-89 treatment. However, the site of PMA's action is less clear, since although PKC activation inhibited secretin and forskolin responsiveness, it did not affect responsiveness to A_2 and IP-prostanoid receptor activation.

Mundell, S.J. *et al.* (1997) Mol. Pharmacol., 51, 991-998.

63P STUDIES ON THE PHARMACOLOGY OF PROSTAMIDE $F_{2\alpha}$, A NATURALLY OCCURRING SUBSTANCE

Chen, J., Krauss, A.H-P., Protzman, C.E., Gil, D.W., Usansky, H., Burk, R.M., Andrews, S.W. & Woodward, D.F. The Departments of Biological Sciences, Chemical Sciences, and Pharmacokinetics/ Drug Metabolism, Allergan, Irvine, USA.

Recent studies have demonstrated that prostamide $F_{2\alpha}$ (prostaglandin $F_{2\alpha}$ 1-ethanolamide) is a naturally occurring substance (Woodward *et al.*, 2000a). This study extends previous findings by demonstrating the presence of prostamide $F_{2\alpha}$ in ocular tissues and further characterization of its pharmacology.

Prostamide $F_{2\alpha}$ activity was investigated in a variety of prostaglandin $F_{2\alpha}$ sensitive preparations. These included the cat iris sphincter (n=6) (Coleman *et al.*, 1984), relaxation of the pre-contracted rabbit jugular vein (n=6) (Chen *et al.*, 1995) and Ca^{2+} signaling in human CRL 1497 cells (n=4). In addition, Ca^{2+} signaling studies (n=3) and radioligand binding competition studies (n=3) were performed in HEK-293 cells stably expressing either the cat or human recombinant FP receptor (Woodward *et al.*, 2000b). Prostamide $F_{2\alpha}$ activity was also studied on human recombinant $EP_{1\text{-}4}$ and TP receptors (n=3), and on human platelet DP and IP receptors (n=4). Analytical studies employed fresh rabbit ocular tissues (n=4), which were extracted with methanol and subjected to liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) for identification and quantitation of prostamide $F_{2\alpha}$.

Prostamide $F_{2\alpha}$ contracted the cat iris sphincter with potent activity (EC_{50} = 58 ± 9 nM, s.e. mean, n=6) that was not exhibited in the other $PGF_{2\alpha}$ sensitive preparations (EC_{50} > 1000 nM) investigated. Since prostamide $F_{2\alpha}$ preferentially stimulated cat iris sphincter smooth muscle (EC_{50} = 58 nM), the possibility that this activity was feline specific was examined by performing studies on the cat recombinant FP receptor. An EC_{50} value of 1458 ± 998 nM (s.e. mean, n=3) was obtained for a functional Ca^{2+} response to prostamide $F_{2\alpha}$ at the cat recombinant FP receptor. Prostamide $F_{2\alpha}$ had no pharmacological activity (inactive or EC_{50} > 10,000 nM) in preparations expressing other prostanoid receptors ($EP_{1\text{-}4}$, DP, IP, TP). Thus, the activity of prostamide $F_{2\alpha}$ is not consistent with stimulation of prostanoid receptors but provides further evidence for the presence of novel receptors that recognize prostamide $F_{2\alpha}$ and synthetic neutral $PGF_{2\alpha}$ analogs such as $PGF_{2\alpha}$ 1-OH and $PGF_{2\alpha}$ 1-OCH_3 (Woodward *et al.*, 2000b).

Prostamide $F_{2\alpha}$ was detected in a diverse variety of ocular tissues (values are mean ± s.e. mean). The highest level (1380 ± 280 ng g^{-1} tissue) was detected in the cornea, an avascular tissue. The iris-ciliary body contained 934 ± 227 ng g^{-1} tissue. The lowest concentration was in the lens (25.5 ± 1.9 ng g^{-1} tissue).

Chen, J., Champa-Rodriguez, M.L., and Woodward, D.F. (1995) Br. J. Pharmacol. **116** 3035-3041.

Coleman, R.A., Humphrey, P.P.A., Kennedy, I. *et al.* (1984) Trends Pharmacol. Sci **5** 303-306.

Woodward, D.F., Tang-Liu, D.D-S., Madhu, C. *et al.* (2000a) 11th International Conference on Advances in Prostaglandin and Leukotriene Research 27.

Woodward, D.F., Krauss, A.H-P., Chen, J. *et al.* (2000b) Br. J. Pharmacol. **130** 1933-1943.

64P ENDOGENOUSLY RELEASED ENDOTHELIN-1 FROM HUMAN PULMONARY ARTERY SMOOTH MUSCLE PROMOTES CELLULAR PROLIFERATION

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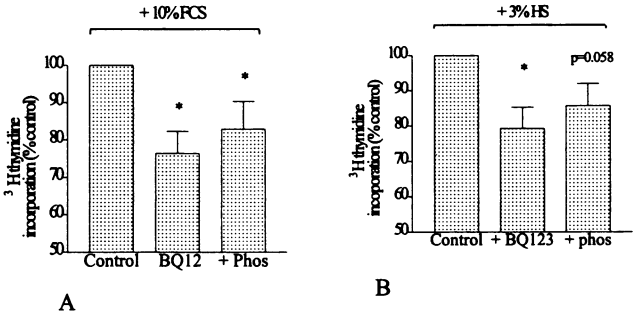
Endothelin-1 (ET-1) is a potent vasoconstrictor and co-mitogen for vascular smooth muscle (VSM). It has been implicated in vascular wall remodelling in pulmonary hypertension (PH). Although the endothelium is considered to be the main source of ET-1, the VSM can produce significant quantities of this peptide under inflammatory conditions (Woods *et al.*, 1999). We investigated the possibility that endogenously released ET-1 from human pulmonary artery smooth muscle (HPASM) cells, stimulated with serum, could act in an autocrine manner to promote proliferation.

Specimens of human pulmonary artery were obtained from pulmonary resection operations performed at the Royal Brompton hospital. HPASM cells were isolated by an explant method (Woods *et al.*, 1999). Smooth muscle (SM) phenotype was confirmed by morphology and staining with a SM specific α-actin antibody. After serum deprivation for 24 h, cells were incubated with foetal calf serum (FCS, 0-10%) for 24 h. ET-1 release was measured by ELISA (R&D systems). For proliferation experiments cells were serum deprived for 72 h before incubation with FCS (0-10%) for 24 and 72 h in the presence or absence of an endothelin-converting enzyme inhibitor, phosphoramidon (phos,50μM) or an ET-A receptor selective antagonist, BQ123 (10μM). Proliferation was determined by ^{3}H thymidine uptake during the final 6 h of incubation. In separate experiments human serum (HS) collected from healthy donors was used as a stimulus for proliferation.

FCS caused a concentration dependent increase in ET-1 release from HPASM cells at 24 hours; e.g. control (no serum) 31.6 ±4.8 pg/million cells, 10% FCS, 65.0 ±10.3 pg/million cells, $p<0.05$, n=8, one-way ANOVA. 3% HS (24 hrs) and 10% FCS (72 hrs) also increased thymidine uptake vs no serum (4858 ±1888 vs 333±73 and 7445±5689 vs 350±133 cpm, respectively). Phos and BQ123 inhibited thymidine uptake both in the presence of FCS at 72 h (Figure 1A, n=5) and HS at 24 h (Figure 1B, n=7).



ET-1 released endogenously from HPASM exposed to serum acts in an autocrine manner to promote proliferation. This has important implications for understanding the pathophysiology of PH and directing future therapies.

Woods *et al.* (1999). *Mol Pharmacol.* 55:502-9.

This work was funded by The Wellcome Trust and BHF.

65P EFFECTS OF ENDOTHELIN-1 AND ANGIOTENSIN-II ON THE CARDIAC CONNEXIN EXPRESSION

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Increased production of endothelin-1 (ET-1) and angiothensin-II (Ang-II) appears to play an important role in ventricular remodeling and cardiac hypertrophy induced by various diseases, e.g. chronic heart failure, renal diseases, hypertension, and after acute myocardial infarction (Dietz et.al., 1999; Miyauchi and Masaki, 1999). Regulation of the gap junction-mediated intercellular communication may influence heart function and its response to the cardiac injury. This study examined the effects of ET-1 and Ang-II on the major cardiac gap junction proteins connexin43 (Cx43) and connexin40 (Cx40) in primary cultures of neonatal rat ventricular cardiomyocytes (NRC). Cardiomyocytes were isolated from ventricles of Wistar newborn rats using the collagenase digestion method. NRC cultures were grown in M199 medium containing 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin and 1% FCS. The cells were exposed to different concentrations of either ET-1 or Ang-II in the cell culture medium for 24 hours. Changes in the expression of gap junction proteins were estimated by immunoblot and immunocytochemical analysis using specific antibodies. Concentration-response relationships were established for ET-1 and Ang-II by densitometric analysis of the protein signals. Data are expressed as mean ± S.E.M.
Both, ET-1 and Ang-II induced an increase in the level of expression, phosphorylation and membrane localisation of Cx43 in a dose-dependent manner (Table1). The observed effects could be blocked by MEK inhibitor PD98059 (25 µM) indicating an involvement of ERK1/2 in the regulation of cardiac Cx43 at the molecular level.

Table1.

	Total Cx43		Cx43-P : Cx43-NP	
	ET-1	Ang-II	ET-1	Ang-II
control	100%	100%	0.013±0.003	0.004±0.001
10 nM	123±20%*	127±4%*	0.50±0.06*	0.004±0.001
50 nM	154±48%*	178±41%*	0.75±0.11*	0.005±0.001
100 nM	138±12%*	340±42%*	0.75±0.09*	0.62±0.06*
500 nM	227±58%*	291±28%*	0.87±0.08*	1.00±0.14*
1 µM	271±80%*	304±35%*	0.99±0.07*	0.89±0.07*

denotes *P<0.05 compared to control (Student's *t*-test, n=3); Cx43-P - phosphorylated Cx43; Cx43-NP - non-phosphorylated Cx43

In contrast to Cx43, the level of the Cx40 expression did not significantly change neither in the ET-1 nor in the Ang-II treated cardiomyocyte cultures. This work demonstrates that ET-1 and Ang-II can selectively modulate the specific expression of cardiac Cx43 through a signal transduction pathway involving ERKs.

Dietz R., Osterziel K.J. et.al. (1999). *Thromb Haemost.*, 82 Suppl 1:73-5.
Miyauchi T. and Masaki T. (1999). *Annu Rev Physiol.*, 61:391-41

supported by the DFG (Dh 3/6-1, Dh 3/8-1)

66P INTERACTION BETWEEN OUABAIN AND ENDOTHELIN-1 IN BLOOD PRESSURE EFFECTS ELICITED FROM THE PERIAQUEDUCTAL GREY AREA OF RATS

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Endothelin-1 (ET-1) participates in the central control of the cardiovascular function with a modulatory role towards several mediators (Gardiner et al., 1990; D'Amico et al., 1995). However, the scenario is not complete yet. In fact, in spite of the wealth of publications on the central actions of ouabain (OUA) some of them argue a relationship between Na^+/K^+ ATPase pump inhibitor (OUA) and ET-1 in the central control of blood pressure (Shah & Jandhyala, 1993). Here we investigated this. We focussed on the periaqueductal grey area (PAG), an area notoriously involved in the central control of blood pressure. Intra-PAG microinjections of ET-1 and OUA were conducted in anaesthetised normotensive (Sprague-Dawley, SD) and hypertensive (DOCA-salt) rats. In each study, rats were allocated randomly to one of the following groups with n=5: vehicle (saline); ET (10 pmol); OUA (0.3-0.6-3 and 6 µg/rat); ET + OUA (6µg/rat); FR 139317 (ETA receptor antagonist, 5 nmol) + OUA 6 µg/rat; SB209670 (ETA/ETB receptor antagonist, 3 nmol) + OUA 6 µg/rat; BQ-788 (ETB receptor antagonist, 5 nmol) + OUA 6 µg/rat; KCl (0.75 and 1.25 µmoles/rat); ET + KCl 1.25 µmoles/rat; OUA 6 µg/rat + KCl 1.25 µmoles/rat. Statistical differences were assessed by ANOVA followed by Bonferroni test. The basal mean arterial blood pressure (MABP) of the rats was 114±3 mmHg. This was increased by ET-1 (10 pmol, 134±2 mmHg, p<0.01) or OUA (3µg, 122±2 mmHg, p<0.05; and 6µg, 139±3 mmHg, p<0.01) microinjected into the PAG area separately. Co-administration of ET-1 (10 pmol) + OUA (6µg) did not produce any adjunctive change in MABP with respect to OUA or ET-1 administered alone (data not shown). Increases in MABP induced by OUA were significantly (p<0.01) reduced by intra-PAG microinjections of the ET-antagonists, FR139317 and SB209670, but not by BQ-788. Pretreatment of the PAG area with OUA or ET-1 significantly (p<0.05) attenuated the centrally mediated hypotensive effects of KCl 1.25 µmoles/rat (e.g. KCl, 88±5 mmHg; ET+KCl, 108±7 mmHg). The basal mean arterial blood pressure of the DOCA-salt rats was 169±8 mmHg. This was paralleled by significant increase in ET-1 levels in whole brain as assessed by specific EIA (Alexis, San Diego, CA) (SD, 5.8±0.6 pg/mg; DOCA, 11.6±1.28 pg/mg, n=6, p<0.05). In contrast, a 40% reduction of ETA mRNA levels into the brain were detected by RT-PCR. The basal MABP of DOCA rats was not significantly changed by ET-1 (177±4 mmHg) or OUA (6µg, 172±3 mmHg) microinjected into the PAG area separately. Similarly, co-administration of ET-1+OUA (6µg) did not produce any significant change in MABP. PAG injection of FR139317 or SB209670, but not BQ-788 caused a significant (p<0.05) decrease of the basal blood pressure.

Together these observations indicate that a) ET-1 and ouabain interact within the PAG area both in normotensive and hypertensive DOCA rats; b) ET-1 may participate in the regulation of the blood pressure from the PAG area also through an ouabain-like mechanism on the Na^+/K^+ pump.

Gardiner SM. et al., Br. J. Pharmacol. (1990) 101, 632-639.
D'Amico M. et al., Hypertension (1995) 25, 507-510.
Shah J. & Jandhyala BS. J. Cardiovasc. Pharmacol. (1993) 22, S13-S15.

67P ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR IN HUMAN PRESSURISED SMALL SUBCUTANEOUS RESISTANCE ARTERIES MAY BE A PRODUCT OF PHOSPHOLIPASE A_2 AND CYTOCHROME P450 METABOLISM

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Endothelium-derived hyperpolarizing factor has been identified as the cytochrome P450 enzyme product 11,12-epoxyeicosatrienoic acid, derived from the endothelium-dependent metabolism of arachidonic acid, in porcine coronary arteries (Fisslthaler et al. 1999). We therefore aimed to test this hypothesis in human resistance subcutaneous arteries.

Subcutaneous arteries (lumen diameter ~120μm, n=18) isolated from gluteal biopsies taken from volunteers (~60 years) with no history of hypertension or diabetes were studied on a pressure myograph in the continual presence of 100μM nitro L-arginine and 30μM indomethacin. Endothelium-dependent vasorelaxation was examined to acetylcholine before and after luminal incubation with cumulative concentrations of the specific phospholipase A_2 inhibitor oleyloxyethyl phosphorylcholine (OOPC, 30μM and 100μM) and the P450 enzyme inhibitor ketoconazole (1μM, 10μM, 30μM, and 100μM).

Endothelial specificity of these inhibitors was assessed by repeating response to sodium nitroprusside, the K_{ATP} channel opener pinacidil and the K_{Ca} opener 1-ethyl-2-benzimidazoline (EBIO) in the presence of the maximal concentrations of OOPC and ketoconazole (100μM)

OOPC reduced the sensitivity and maximum relaxation to acetylcholine in a concentration-dependent manner. (control: 84 ± 3%; 30μM: 49 ± 6%; 100μM: 20 ± 6%; $p<0.05$: 30μM versus control; 100μM versus 30μM; n=6).Luminal incubation with OOPC had no effect on the relaxation response to sodium nitroprusside, pinacidil and EBIO (n=3).

Ketoconazole also resulted in a concentration-dependent inhibition of the relaxation to acetylcholine (control: 79 ± 4%; 1μM: 51 ± 5%; 10μM: 24 ± 5%; 30μM: 7 ± 4%; 100μM: 7 ± 4%; $p<0.05$: 1μM versus control; 10μM versus 1μM; 30μM versus 10μM; n=6). Again, ketoconazole had no effect on the relaxation response to sodium nitroprusside, pinacidil and EBIO (n=3).

These results provide evidence that the endothelium-dependent relaxation to endothelium-derived hyperpolarizing factor is likely to be a product of phospholipase A_2/arachidonic acid/P450 enzyme metabolism in human subcutaneous resistance arteries.

Fisslthaler, B., Popp, R., Kiss, L. *et al.* (1999). *Nature.* **401**:493-497.

68P COMPONENTS OF THE BRADYKININ-INDUCED ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION OF PIG CORONARY ARTERY.

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The characteristic feature of the response to the endothelium-derived hyperpolarizing factor (EDHF) in most vascular preparations is its abolition by charybdotoxin + apamin but only slight inhibition by iberiotoxin + apamin. However, in the porcine coronary artery, there are conflicting reports on the sensitivity (Fisslthaler et al., 2000) or resistance (Edwards et al., 2000) of the EDHF response to inhibition by iberiotoxin. This study investigates further the endothelium-dependent hyperpolarization induced by bradykinin or by substance P.

Porcine left descending coronary arteries were dissected from pig hearts which were obtained from the local abattoir. Experiments were performed on endothelium-intact segments of artery incubated at 37°C in bicarbonate-buffered Krebs solution (K^+ concentration 4.6 mM), gassed with 95% O_2: 5% CO_2 (Edwards et al., 2000) or in oxygenated HEPES-buffered Tyrode solution (4.7 mM K^+, pH 7.4: Fisslthaler et al., 2000), each containing 10 μM indomethacin and 300 μM L-nitroarginine. Vessels were pinned open and smooth muscle cells were impaled via the intimal surface using micro-electrodes filled with 3M KCl (resistance 40-80 MΩ).

In fresh preparations (control : Krebs solution) or in arteries incubated for 18–24 h at 24°C in Krebs solution (18h Krebs) iberiotoxin had no effect on the hyperpolarizations induced by bradykinin or substance P (Table 1). However, after the arteries had been incubated for 18–24 h in HEPES-buffered Tyrode solution (18h HEPES) substance P had no effect and the bradykinin response was significantly reduced (comparison with controls or 18h Krebs, $P<0.05$: t-test).

	100 nM SP	100 nM BK
control	33.9 ± 1.0, n=5	32.3 ± 1.2, n=4
+IbTX	35.7 ± 0.9, n=5	29.9 ± 0.9, n=4
18h Krebs	27.1 ± 0.9, n=4	26.6 ± 0.5, n=4
+ IbTX	25.1, n=2	24.4 ± 2.4, n=4
18h HEPES	0.0 ± 0.0, n=4	14.8 ± 0.8, n=4
+ IbTX	–	0.5 ± 0.4, n=4

Table 1: Mean (± s.e.mean) hyperpolarizations (mV) induced by substance P (SP) or bradykinin (BK) in the presence or absence of iberiotoxin (IbTX).

We recently provided evidence of a role for gap junctions in the EDHF response in porcine coronary arteries (Edwards et al., 2000). HEPES inhibits homomeric or heteromeric gap junctions containing connexin 26 (Bevans & Harris, 1999). We propose that HEPES may also inhibit coronary artery gap junctions and that this results in the loss of the EDHF response after incubation in HEPES-buffered Tyrode solution. Under these conditions, bradykinin (but not substance P) is able to stimulate an endothelium-dependent hyperpolarization which, as suggested by Fisslthaler and coworkers (Fisslthaler et al., 2000) is probably due to 11,12-epoxyeicosatrienoic acid (11,12-EET). However, the present data do not support the view (Fisslthaler et al. 2000) that 11,12-EET is EDHF.

Bevans, C.G. & Harris, A.L. (1999) J. Biol. Chem. 274: 3711-3719.

Edwards, G., Thollon, C., Gardener, M.J., et al. (2000) Br. J. Pharmacol. 129:1145-1154.

Fisslthaler, B., Hinsch, N., Chataigneau, T., et al. (2000) Hypertension. 36:270-275.

69P PRE-TREATMENT WITH IL-1β INHIBITS SNP-INDUCED APOPTOSIS OF HUMAN VENOUS SMOOTH MUSCLE CELLS

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Apoptosis of vascular smooth muscle cells (SMCs) is both a physiological and a pathological process. In atherosclerosis SMC apoptosis is implicated in plaque rupure (Bennett *et al.*, 1995). Basal apoptosis of SMCs in culture is very low although apoptosis can be induced by a number of agents including nitric oxide (NO: Pollman *et al.*, 1996). Here, using human cultured venous SMCs, we confirm previous observations (Pollman *et al.*, 1996) that the NO donor sodium nitroprusside (SNP) induces vascular SMC apoptosis. We go on to investigate both the direct and indirect effects of IL-1β on SNP-induced apoptosis.

Saphenous vein samples were cultured as described previously (Stanford *et al.*, 2000). At the beginning of all experiments new supplemented medium was placed on confluent SMCs. After 24 hrs cells were treated with increasing concentrations of SNP ($1x10^{-4}$-$1x10^{-3}$M) ± IL-1β (1ng/ml). In some experiments cells were treated with IL-1β 24 hrs prior to treatment with SNP. SMCs were lysed and apoptosis assessed using an ELISA (Boehringer Mannheim) to determine levels of histone-associated mono- and oligonucleosomes as described previously (Fortenberry *et al.*, 1998).

SNP induced apoptosis of venous SMCs. IL-1β had no direct effect on basal or SNP-induced apoptosis. Pre-treatment of SMCs with IL-1β had no effect on basal apoptosis but significantly inhibited SNP-induced apoptosis (Figure 1).

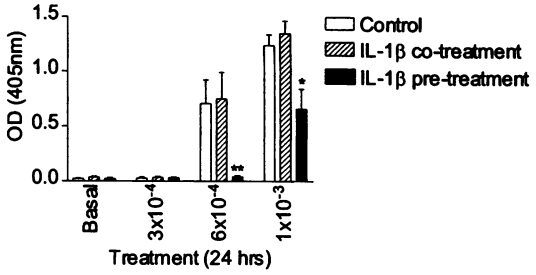

Figure 1 Effect of IL-1β (1ng/ml) or IL-1β pre-treatment (24 hours) on SNP ($3x10^{-4}$-$1x10^{-3}$M)-induced apoptosis of human venous SMCs. Data represents mean±sem, n=6 using venous cells cultured from 3 patients. One-way ANOVA vs. control (post-test Dunnett): *P<0.05, **P<0.01.

IL-1β, added as a co-treatment, has no effect on basal or SNP-induced apoptosis of human venous SMCs in culture. However pre-treatment of cells with IL-1β was able to inhibit subsequent SNP-induced apoptosis suggesting that stimulated SMCs cells release some, as yet unidentified, mediator which acts in a autocrine fashion to influence SMC survival. This may be of importance, for example, in the atherosclerotic plaque where both NO and cytokines are present.

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This work was funded by grants from the BHF and the Wellcome Trust.

70P CHARACTERISATION OF ENDOTHELIUM-DEPENDENT VASODILATOR RESPONSES IN THE BOVINE ISOLATED PERFUSED EYE

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The factors regulating vasomotor tone in the eye are poorly understood. The aim of this study was, therefore, to characterise the endothelium-dependent vasodilator responses to acetylcholine (ACh) in the bovine isolated perfused eye.

Bovine eyes were perfused according to the constant flow method described by Wilson *et al.* (1993). Briefly, eyes obtained from a local abattoir were cannulated through a long posterior ciliary artery and perfused at 2.5 ml min^{-1} at 37°C with Krebs solution gassed with O_2 containing 5% CO_2. After an equilibration period of at least 30 min, drugs were added either to the Krebs reservoir or as bolus doses injected immediately proximal to the cannula. To observe vasodilator responses, the perfusion pressure in control experiments was raised by 89.7 ± 9.0 mmHg using the thromboxaneA_2-mimetic, U46619 (200 nM). In some experiments, blocking drugs affected the perfusion pressure, and in these the concentration of U46619 was adjusted in order to ensure that the perfusion pressure attained was similar to control experiments. In some experiments the detergent, 3-[(3-cholamidopropyl) dimethyl-ammonio] 1-propanesulfonate (CHAPS, 0.3%), was infused for 2 min to selectively destroy the endothelium (Randall and Hiley, 1988). Vasodilator responses are given as % reduction (mean ± s.e.mean, n≥6) of the U46619-induced pressure. Differences were determined by one-way ANOVA with Bonferroni's post-test or by an unpaired t-test, as appropriate.

ACh (10 nmol) reduced the perfusion pressure by 51.7 ± 5.0 %. Infusion of CHAPS almost abolished this vasodilatation (5.4 ± 1.8 %, P<0.0001). Treatment with L-NAME (100 μM), an inhibitor of nitric oxide synthase, elevated perfusion pressure by 36.3 ± 7.4 mmHg, but had no effect on ACh-induced vasodilatation (46.3 ± 6.0 %). The cycloxygenase inhibitor, flurbiprofen (30 μM), also had no effect on ACh-induced vasodilatation. In the presence of 30 mM KCl, the ACh-induced vasodilatation was markedly inhibited (7.4 ± 2.6 %, P<0.0001). The effects of a number of K^+ channel blockers were examined on ACh-induced vasodilatation: the non-selective K^+ channel blocker, tetraethylammonium (TEA, 10 mM), inhibited vasodilatation (31.1 ± 2.0 %, P<0.005); the small conductance calcium-sensitive K^+ channel blocker, apamin (100 nM), and the large conductance calcium-sensitive K^+ channel blocker, charybdotoxin (10 nM), had no effect by themselves (45.8 ± 4.0 % and 52.4 ± 3.2 %, respectively), but the combination produced a significant inhibition (24.2 ± 2.4 %, P<0.001); and the ATP-sensitive K^+ channel blocker, glibenclamide (10 μM), had no effect (51 ± 2.1 %).

The data suggest that a tonic basal level of nitric oxide regulates perfusion pressure in the bovine isolated perfused eye, but nitric oxide does not appear to mediate the powerful ACh-induced vasodilatation seen in this preparation. This vasodilatation is also not mediated by a cycloxygenase product. The blockade by high potassium, by TEA, or by the combination of charybdotoxin and apamin suggests that ACh-induced vasodilatation may be mediated by an endothelium-derived hyperpolarizing factor (EDHF)-like substance (Feletou and Vanhoutte, 1999).

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71P OXIDATION OF NITROXYL ANION TO NITRIC OXIDE BY QUINONES

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Nitroxyl anion (NO^-) generators such as Angeli's salt are powerful vasodilators (Fukuto *et al.*, 1992). This action results from the rapid tissue-dependent oxidation of nitroxyl to nitric oxide but the pathway for conversion is unknown. We have recently shown that copper ions catalyse the oxidation of nitroxyl to nitric oxide (Nelli *et al.*, 2000), suggesting a possible role for endogenous copper containing enzymes. We describe here our findings with tyrosinase, a copper-containing enzyme that converts monophenols to diphenols and subsequently oxidises them to their unstable quinone (Jiménez & García, 2000).

Experiments were conducted in HEPES (5 mM, pH 7.4)-buffered Krebs solution at 22°C. Nitric oxide formation was detected using an ISO-NO Mark II meter (World Precision Instruments Ltd., UK). Nitric oxide signals (in pA) are expressed as mean ± s.e. mean of ≥ 5 observations, with differences determined by ANOVA followed by Bonferroni's post-test.

When either tyrosinase (250 u ml^{-1}) or its substrate L-tyrosine (100 μM) was added alone to the nitroxyl anion generator, Angeli's salt (10 μM), no nitric oxide was produced. In contrast, if both were present together with Angeli's salt, a powerful nitric oxide signal was detected (7,419 ± 380 pA, P<0.001). L-DOPA (100 μM), the primary product of the reaction between tyrosinase and L-tyrosine, also failed by itself to generate nitric oxide from Angeli's salt, but together with tyrosinase produced a nitric oxide signal (11,431 ± 257 pA, P<0.001). Two other substrates for tyrosinase, i.e. catechol and tyramine (each at 100 μM), when present with the enzyme also produced nitric oxide signals from Angeli's salt (7,175 ± 322 and 8,041 ± 466 pA, respectively, both P<0.001). It was therefore likely that the respective quinone produced in each case was responsible for the oxidation of nitroxyl to nitric oxide but this could not be tested directly because of their unstable nature. The more stable analogue, 1,4-benzoquinone (100 μM), did, however, produce a nitric oxide signal (11,533 ± 2,213, pA, P<0.001) from Angeli's salt in the absence of tyrosinase. Coenzyme Q_0 (1 mM), a water soluble analogue of endogenous coenzyme Q (ubiquinone), alone failed to generate nitric oxide from Angeli's salt, but generated a large signal (11,837 ± 740, pA, P<0.001) in the presence of its mitochondrial complex III cofactor, ferricytochrome c (100 μM).

In conclusion, our findings show that quinones can catalyse the one-electron oxidation of nitroxyl to nitric oxide. The possibility that endogenous quinones participate in this reaction and so underlie the vasodilator actions of nitroxyl generators such as Angeli's salt is therefore worthy of investigation.

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72P *IN UTERO* EXPOSURE TO CARBON MONOXIDE ALTERS THE ELECTROPHYSIOLOGICAL MATURATION OF NEONATAL RAT VENTRICULAR CARDIOMYOCYTES

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In vivo carbon monoxide (CO) exposure is a well-documented model of tissue hypoxia. Prenatal chronic exposure to low CO concentrations (150-200 ppm) produces various developmental alterations in the rat, including neurobehavioral modifications (Carratù *et al.*, 1993) and changes in the electrical properties of rat skeletal muscle fibres (De Luca *et al.*, 1996). These modifications suggest that prenatal exposure to CO may hinder postnatal development in rat.

In neonatal rat hearts, the physiological cell growth is accompanied by a significant increase in the density of the transient outward current I_{to}, responsible for the reduction of the action potential duration (APD) (Guo *et al.*, 1996), and a decrease of the pacemaker current (I_f) expression, likely associated with the disappearance of spontaneous activity (Cerbai *et al.*, 1999). No data are available concerning the effects of prenatal CO exposure on cardiac development. The aim of this study was to evaluate and compare postnatal electrophysiological changes in ventricular myocytes isolated from male Wistar rats born from mothers exposed to 0 (CTR) or 150 ppm CO during pregnancy.

Myocytes were isolated from rat hearts at different ages after birth (1 to 5, 8 to 15, 20 to 30 days) (Cerbai, *et al.*, 1999). Patch-clamped cells were superfused with Tyrode's solutions pre-warmed to 35°C, appropriately modified to measure action potentials, I_{to} and I_f. Data are expressed as mean±s.e.m. Statistics was performed by means of ANOVA followed by the Student-Neuman-Keuls test. Probability of less than 0.05 was considered significant.

Postnatal increase in membrane capacitance, an index of cell size, was similar in both groups: from 14±1 pF (n=38) to 60±5 pF (n=16) in CTR and from 15±1 pF (n=45) to 59±5 pF (n=26) in CO. While all measured values were not different in the two groups early after birth, differences were observed during growth. APD, measured at −50 mV, progressively decreased in CTR from 131±24 ms at 5 days (n=14) to 76±13 ms at 28 days (n=9) but not in CO rats, where it remained significantly prolonged (157±28 ms at 28 days, n=11, p<0.05 vs CTR). Similarly, I_{to} density, which significantly increased in CTR from 4.1±0.7 pA/pF (n=22) to 11.8±3.4 pA/pF (n=10) (p<0.01), remained low in CO rats (5.8±0.9 pA/pF at 28 days, n=17, p<0.01 vs CTR). The density of the pacemaker current I_f decreased by 75% from 1 to 28 days in CTR, but remained high in CO rats (at 28 days: 2.1 pS/pF (n=10) vs 0.7 pS/pF (n=7) in age-matched CTR, p<0.05).

In conclusion, exposure to CO during the fetal life affects the normal process of electrophysiological maturation of the cardiac ventricular myocyte.

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73P INHIBITORS OF POLY(ADP-RIBOSE) POLYMERASE (PARP) ARE NEUROPROTECTIVE AGAINST NECROTIC BUT NOT APOPTOTIC POST-ISCHEMIC CELL DEATH.

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An excessive activation of poly(ADP-ribose) polymerase (PARP) with the subsequent depletion of cellular NAD and ATP has been proposed to play a key role in the mechanisms leading to post-ischemic neuronal death (Szabò & Dawson, 1998; Ha & Snyder, 2000). We examined the neuroprotective effects of the PARP inhibitors benzamide, 6(5H)-phenanthridinone (PND) and 3,4-dihydro-5-[4-1(1-piperidinyl) buthoxy]-1(2H)-isoquinolinone (DPQ) in three different models of cerebral ischemia: murine cortical cell or rat organotypic cultures exposed to oxygen-glucose deprivation (OGD) *in vitro*, and mongolian gerbils subjected to transient global ischemia *in vivo*.

The addition to the incubation medium of benzamide, PND, and DPQ at increasing concentrations attenuated neuronal injury induced by 60 min OGD in cortical cell cultures. The release of LDH into the medium decreased from 310 ± 25 units/L (OGD) to 42 ± 3 (OGD + 1 mM benzamide), 126 ± 10 (OGD + 100 μM PND) and 44 ± 2 (OGD + 10 μM DPQ) units/L. However, the three PARP inhibitors were unable to reduce CA1 pyramidal cell loss in hippocampal slices exposed to 30 min OGD or in gerbils following 5 min bilateral carotid occlusion. In order to understand whether there was a correlation between these results and the pattern of cell death in our ischemic models, we examined the necrotic and apoptotic features of OGD-induced neurodegeneration in cortical cells and hippocampal slices using biochemical and morphological approaches.

In cultured cortical cells, the time-dependent release of LDH into the medium at various time points after OGD was associated with the appearance of ultrastructural features of necrotic cell death, including nuclear and plasma membrane breaks, large vacuoles and swollen organelles in a dispersed cytoplasm, and irregularly clumped chromatin. On the contrary, no caspase-3 activation nor morphological characteristics of apoptosis were observed at any time point after OGD.

In organotypic hippocampal slices, 30 min exposure to OGD led to to a time-dependent increase in caspase-3 activity that was 2.6-fold higher than controls 24 h following OGD. In addition, the density of apoptotic cells in the CA1 region of hippocampal slices, as detected by a fluorescent DNA fragmentation detection kit, increased as a function of the duration of the recovery period. Electron microscopy revealed typical characteristics of apoptotic neuronal death in hippocampal slices exposed to OGD, including clumped chromatin, darkened cytoplasm with vacuoles, membrane preservation, and apoptotic bodies.

These findings suggest that PARP overactivation may be an important mechanism leading to post-ischemic neurodegeneration of the necrotic but not of the apoptotic type, and that PARP inhibitors may therefore be of therapeutic importance in brain pathologies were necrosis predominates.

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74P ANTAGONISTS OF METABOTROPIC GLUTAMATE 1 RECEPTORS REDUCE POST-ISCHEMIC NEURONAL DEATH

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The role of metabotropic glutamate 1 (mGlu1) receptors in post-ischemic neuronal death is unclear. We examined whether the recently characterized and selective mGlu1 receptor antagonists 1-aminoindan-1,5-dicarboxylic acid (AIDA) (Moroni et al., 1997) and (S)-(+)-2-(3'-carboxybicyclo[1.1.1] pentyl)-glycine (CBPG) (Mannaioni et al., 1999) could reduce neuronal injury *in vitro* and *in vivo*.

In mixed cortical cell cultures exposed to oxygen-glucose deprivation (OGD) for 60 min, AIDA (300-1000 μM) and CBPG (300-1000 μM) reduced neuronal loss (by 85% and 75%, respectively) when added during OGD and the subsequent 24 h recovery period. Similarly, AIDA (300-1000 μM) and CBPG (300-1000 μM) reduced post-ischemic pyramidal cell loss (by 70% and 50%, respectively) in the CA1 area of organotypic hippocampal slices exposed to 30 min OGD. Neuroprotection was observed also when mGluR1 antagonists were added at 300 μM up to 60 min (in cortical neurons) or 30 min (in hippocampal slices) after OGD. Immunocytochemistry using an mGluR1a antibody revealed that the receptor was expressed in vulnerable cortical cultured cells but not in CA1 pyramidal cells.

In gerbils, i.c.v. administration of AIDA (300 mol X 2) reduced CA1 pyramidal cell injury by 90% following 5 min bilateral carotid occlusion, a model of transient global ischemia. Neuroprotection was also observed when 3 mM AIDA was added to the hippocampal perfusion fluid in microdialysis experiments, and this effect was associated with a 6-fold potentiation of the ischemic output of GABA.

We then characterized a new series of compounds, among which the most interesting molecule was 2-(3'-methyl-5'-carboxythien-2-yl)-glycine (3-MATIDA). In mammalian cells transfected with specific mGlu receptors, 3-MATIDA displayed a selective antagonist activity on mGlu1 (IC_{50} = 6.3 μM) when compared with mGlu5, mGlu2 and mGlu4 (IC_{50}s > 300 μM) receptors. When tested as an antagonist on NMDA and AMPA receptors in mouse cortical wedges, its IC_{50} was > 250 μM. 3-MATIDA displayed no agonist activity on any of these receptor subtypes. 3-MATIDA reduced by 70% the extent of cultured cortical cell death following OGD as well as the volume of the infarct (by 40%) in a rat model of focal cerebral ischemia (permanent occlusion of the middle cerebral artery).

In conclusion, we have identified three compounds (AIDA, CBPG and 3-MATIDA) with selective antagonist activity on mGlu1 receptors and neuroprotective properties in ischemic models *in vitro* and *in vivo*. In addition, our results demonstrate that AIDA and CBPG are neuroprotective even when administered after the ischemic insult and suggest that different mechanisms could mediate their effects following blockade of mGluR1 in cortical and hippocampal neurons.

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75P EXTRACELLULAR AMINO ACIDS IN SEVERELY HEAD-INJURED CHILDREN: A MICRODIALYSIS STUDY

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Severe head injury is a significant cause of mortality in children. Previous microdialysis studies in adult head-injured patients have demonstrated marked increases in the extracellular levels of the excitotoxic amino acids, glutamate (GLU) and aspartate (ASP). However, a high correlation with levels of structural amino acids suggested that these changes arose from non-specific destructive processes rather than neuronal events (Bullock et al., 1998). In view of differences in the patho-physiology of brain trauma between adults and children, including delayed increases in intracranial pressure (ICP) and brain oedema in the latter, we have used microdialysis to measure amino acids in head-injured children.

As part of routine neuromonitoring, and following Ethics Committee approval, a microdialysis probe was placed into the cortex of seven children (ages 1 to 15) with severe closed head injury (GCS < 8), at the time of ICP bolt insertion and at a site adjacent to, but not within, the traumatised region. Probes were perfused with sterile artificial CSF, initially at 15 µl/min for 6 min to remove air bubbles and substances arising from implantation damage. Flow was then reduced to 0.3 µl/min, and samples collected at hourly intervals for up to 1 week post-trauma prior to amino acid analysis by HPLC.

There was marked variation in GLU levels between patients, with peak values ranging from 0.92 to 89.3 µM. In 4 patients, elevated initial levels declined with time. In 2 patients, GLU levels continued to increase for 3 to 7 hours post-implantation, before beginning to fall. In the final patient, initial GLU levels were below 1 µM and remained at that level throughout. This, together with differences in the rate of decline of GLU levels between patients, suggests that the increased levels observed arose from brain trauma rather than implantation damage. GLU levels were highly correlated to ASP ($r=0.87 \pm 0.05$, mean ± s.e.mean, $p<0.01$) at each time point within each patient, and to a lesser extent with other neuroactive amino acids such as GABA ($r=0.49 \pm 0.22$, mean ± s.e.mean, $p<0.05$) and citrulline (CIT) ($r=0.37 \pm 0.12$, mean ± s.e.mean, $p<0.05$). However, in contrast to adults (Bullock et al., 1998), there was no correlation with levels of structural amino acids (e.g. tyrosine, threonine), suggesting that changes observed reflected neuronal events rather than non-specific destructive processes. In contrast to GLU, extracellular levels of CIT varied little between patients (2.48 ± 0.18 µM, mean ± s.e.mean, n=7). As a by-product of nitric oxide (NO) synthesis, this would suggest that NO metabolism does not mirror the pattern of GLU release. Glutamine (GLN) participates in the replenishment of transmitter stores of GLU. In most patients, extracellular GLN far exceeded GLU (10 to 500-fold) and the GLN/GLU ratio continued to increase with time. However in the one patient where GLU levels were high and sustained (remaining above 20 µM throughout), the GLN/GLU ratio was around unity and tended to decrease with time. This may reflect imbalances in GLU uptake and metabolism. Finally, there was a significant correlation ($r=0.814$, $n=7$, $p<0.05$) between the mean GLU level throughout the recording period and the mean ICP over the same period.

In conclusion, results obtained so far suggest that patterns of amino acid release in severe head injury may differ between children and adults and that prolonged increases in ICP may be associated with high extracellular GLU.

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76P EXTRACELLULAR CONCENTRATIONS OF ACETYLCHOLINE, GABA, GLUTAMATE AND ASPARTATE IN THE RAT VENTRAL HIPPOCAMPUS DURING EXPLORATORY ACTIVITY IN AN OPEN FIELD.

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Open field exploration and its habituation are known to be closely related to the activity of the cholinergic septohippocampal pathway. A modulatory activity on this pathway may be exerted by the GABAergic and glutamatergic systems (Giovannini *et al.*, 1997). This study investigated the activation of the septohippocampal cholinergic system, during the exploratory activty induced by expo-sure to an open field, and the possible involvement of hippocampal GABAergic and glutamatergic systems, using vertical microdialysis in the hippocampus of freely moving rats.

Male adult Wistar rats (250-260 g) were anaesthetized with chloral hydrate (400 mg/kg b.w., i.p.) and the microdialysis probe was inserted stereotaxically in the ventral hippocampus (AP: –5.5; L: -4.8; V: -7.8). After 24 h recovery, the probe was perfused (flow-rate 4 µl/min) with artificial CSF containing 7 µM physostigmine. After a 90 min stabilisation period, samples were collected at 10 min intervals. The first four samples were collected to detect basal release, the rats were then transferred to an open field (78 x 69 x 36 cm arena) and allowed to explore it for 60 min, and then returned to their home cage.

Total motor activity (s) was recorded by a microwave sensor at 10 min intervals. The extracellular concentrations of ACh, GABA, aspartate and glutamate were measured by HPLC, as previously described (Giovannini *et al.*, 1997; Bianchi *et al.*, 1999) and were expressed as nM (mean ± s.e.m. n=5). The area under the concentration-time curve (AUC), calculated before and during exploratory activity, was used for statistical analysis (ANOVA, with Newman-Keuls post-hoc test).

During the first 10 min of arena exploration, hippocampal ACh and motor activity increased from a basal level of 8.6 ± 2.3 nM and 32 ± 10 s (as observed 20-60 min), respectively, to maxima of 22.9 ± 6.0 nM ($P<0.01$) and 249 ± 22 s ($P<0.001$), respectively. Hippocampal aspartate and, to a lesser extent, glutamate levels were also maximally increased in the 10 min fraction, from basal levels of 54 ± 5 and 135 ± 45 nM, respectively, to maxima of 140 ± 34 ($P<0.01$) and 196 ± 65 nM ($P<0.05$), respectively. From the second 10 min fractions onwards ACh, aspartate and glutamate levels gradually declined towards basal levels, whereas motor activity declined immediately. However, GABA levels showed a 10 min delay in reaching their maximum, 7.4 ± 1.2 ($P<0.05$), from a basal level of 4.5 ± 0.6. 3 nM TTX abolished ($P<0.01$) the exploration-induced increase of the four neurotransmitters and significantly reduced (-32%, $P<0.05$) the motor activity in the arena.

The present data indicate that the septohippocampal cholinergic pathway is activated during exploration of a novel environment, probably due to increased arousal and attention. However, after the first 10 min of exploration, habituation takes place, as demonstrated by the immediate decrease of motor activity and the gradual return of Ach, aspartate and glutamate concentration to basal levels. The hippocampal GABAergic system which is maximally activated during the second 10 min of exploration, seems to act as an inhibitory brake on the cholinergic and glutamatergic system during habituation.

Work supported by MURST, National Project, Rome, Italy & COST D8

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77P Edg4 RECEPTOR DISTRIBUTION AND FUNCTIONALITY

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The Edg4 (endothelial differentiation gene) receptor, recently identified by An *et al.*, (1998) is one of a family of eight GPCRs. *In situ* hybridisation studies in adult monkey brain indicate a discrete localisation of the Edg4 receptor within the dentate gyrus (Beer *et al.*, 2000). Here we have determined the distribution of the receptor protein in adult rat brain. In addition Edg4 receptor activation by the endogenous ligand lysophosphatidic acid (LPA) and several structural analogues has been investigated.

Immunohistochemistry in adult rat brain was carried out using a rabbit polyclonal antibody raised to amino acids 353-369 of the rat Edg4 receptor sequence. 6 micron paraffin wax fixed sections were rehydrated to water. Non-specific binding was blocked by incubation with 5% normal goat serum for 1hr. This was replaced with serum (1:10000) and incubated overnight at 4°C. Antibody binding was demonstrated using an Optimax™ immunostainer (Menarini) in combination with the ABC Elite™ system (Vector), diaminobenzidine as the chromogenic substrate and counterstained in Mayers haematoxylin (Biomen). Sections were dehydrated and mounted with DPX for light microscopical examination.

Immunoreactivity, which was abolished in the presence of excess Edg4 receptor peptide, was observed in select cell bodies in the dentate gyrus, specifically in the subgranular zone, and in the ventricular zone surrounding the lateral ventricle. This distribution pattern is reminiscent of that observed for adult mouse brain progenitor cells seen with BrdU labelling (Kuhn *et al.*, 1996) and also in transgenic mice expressing green flourescent protein under the control of a neuronal progenitor marker gene (Yamaguchi *et al.* 1999).

Agonist-induced [^{35}S]GTPgS binding was carried out on membranes prepared from HEK239 cells transiently expressing the hEdg4 receptor as previously described (Beer *et al.*, 2000). The effects of oleoyl LPA, glycerol phosphatidic acid (LPG), ethanolamine phosphatidic acid (LPE) and choline phosphatidic acid (LPC) were investigated.

LPA was the most potent and efficacious compound tested in the *in vitro* functional assay yielding a pEC50 value of 7.14 ± 0.19. LPG was equally efficacious yielding a pEC50 value of 5.56 ± 0.11. LPC was a weak partial agonist giving 18% of the response seen with LPA. LPE yielded no intrinsic activity.

Hence immunoreactivity is consistent with earlier *in situ* hybridization studies indicating a discrete localisation in the dentate gyrus as well as the ventricular zone surrounding the lateral ventricle of the adult brain. These areas correlate specifically with regions of known adult neurogenic activity. In addition the limited number of compounds tested indicate that attachments to the phosphate group of LPA are tolerated for Edg4 receptor activation.

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78P ACTH 4-10 DECREASES PREFERENCE OF C57 STRAIN MICE FOR ALCOHOL

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The inbred C57 strain of mice has been used for many years for studies on their high voluntary alcohol consumption. We recently found that, given a free choice between 8% ethanol or water, these mice vary considerably in their alcohol intake, showing a biphasic distribution with primarily either high preferring (ratio of 8% ethanol to total fluid 0.7 and over) or low preferring (ratio below 0.4) (Little et al., 1999). The experiments presented here are part of an ongoing study to investigate the effects of hormones of the hypothalamo-pituitary-adrenal (HPA) axis on the alcohol consumption of high alcohol preferring mice.

The mice (25-30g) were male C57/Bl0 strain from an in-house breeding programme, housed singly, with ad lib access to food. Mice were first identified as high or low preference mice on the basis of a screening procedure in which the ratio of 8% v/v ethanol to water consumed was monitored 3 days per week for 3 weeks. Mice with a high ratio (0.7 and above) in the last 3 measurements were used for the subsequent experiments.

In these studies, mice had 24h access to a free choice between tap water and 8% v/v ethanol. In the first experiment a single i.c.v injection (150 µg/mouse in 2 µL, of either a type I (spironolactone) or a type II (RU38486) corticosterone receptor antagonists was given and fluid consumption measured six hours later. In the next experiment the corticosterone synthesis inhibitor, metyrapone, was injected i.p. at 100 mg/kg twice daily, for seven days. Fluid consumption was measured daily. In the final experiment, ACTH fragment 4-10 (which does not possess adrenocorticotrophic activity, Greven & de Wied 1967) was injected once at 5 p.m., and fluid consumption was measured the next morning at 10 am and again 24 hours later.

Neither corticosterone antagonist altered alcohol preference. Metyrapone significantly decreased the ethanol preference and ethanol consumption of the mice (Table 1). The total fluid intake was unchanged. ACTH had a large and significant ($P<0.001$) effect in decreasing the alcohol preference and consumption (Table 2) with no change in total fluid intake. By the following day alcohol consumption had returned to baseline values.

The results indicate that the antagonists had no effect on alcohol intake but inhibition of corticosterone synthesis significantly lowered the consumption. However the effect of ACTH administration was considerably greater. It is possible the effect of metyrapone was due to the resultant increase in circulating ACTH owing to decreased feedback. The lack of adrenocorticotrophic action of ACTH4-10 suggests that the effect of this peptide may be due to neuronal actions.

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Table 1: Ratio of alcohol to total fluid consumed mean ± s.e.m., before and each day after metyrapone (Met) or saline (Sal) *P< 0.05. Measure = mean of two days measurements

	Baseline	Measure 1	Measure 2	Measure 3
Sal	0.77±0.04	0.78±0.02	0.85±0.03	0.79±0.01
Met	0.76±0.05	0.68±0.02*	0.62±0.06*	0.69±0.02*

Table 2: Ratio of alcohol to total fluid consumed, mean ± s.e.m. before (0 hour) and after (17 hours & 36 hours) ACTH(4-10). administration. *P< 0.001

Treatment	0 hour	17 hours	36 hours
Saline	0.79±0.02	0.77±0.02	0.76±0.04
ACTH	0.79±0.03	0.39±0.06*	0.73±0.02

79P THE BETA CARBOLINES NORHARMANE AND HARMALINE BUT NOT THE SPECIFIC MAO-B INHIBITOR RO16-6491 SUBSTITUTE FOR THE IMIDAZOLINE$_2$ LIGAND 2-BFI IN RAT DRUG DISCRIMINATION

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The imidazoline site has been separated into three classes, the clonidine preferring - I_1 site, the idazoxan preferring - I_2 site, and the pancreatic methoxy-idazoxan preferring - I_3 site (Eglen *et al*, 1998). The specific imidazoline I_2 site ligand 2-(-2-benzofuranyl)-2-imidazoline (2-BFI) generates a 'cue' in rat drug discrimination (Jordan *et al.*, 1996) and structurally related compounds dose dependently substitute for 2-BFI (MacInnes & Handley, 1999). We have reported the ability of some endogenous (e.g. harmane) and non-endogenous (e.g. ibogaine) beta carbolines, agmatine and the specific MAO-A (Monoamine oxidase A) inhibitor RO41-1049 to substitute significantly for 2-BFI (MacInnes & Handley, 2000). To complement these findings the present study examines the ability of structurally related beta carbolines and the specific MAO-B inhibitor RO16-6491 to substitute for 2-BFI.

Drugs were tested in one of 2 groups of Hooded Lister rats, (n=7, n=8) taught to discriminate 2-BFI (7mgkg^{-1} i.p. -20 min) from saline vehicle, in two lever skinner boxes with condensed-milk reward (Jordan *et al.*, 1996). Consistent with our previous findings the beta carbolines tested showed significantly elevated 2-BFI-appropriate responding when compared to saline control (see fig 1): norharmane; 3mgkg^{-1} - 34.7 ± 13.8 (M ± SEM), 6mgkg^{-1}- 48.6 ± 10.9, 9mgkg^{-1}- 78.6 ± 9.2: harmaline; 1.5mgkg^{-1} - 54.9 ± 12.4, 3mgkg^{-1}- 76.8 ± 11.6, 6mgkg^{-1}- 95.9 ± 2.9. All doses were significantly different from their corresponding saline controls ($P<0.05$, Dunnet's test) except 3 mgkg^{-1} norharmane. The specific MAO-B inhibitor RO16-6491 failed to substitute significantly ($p>0.05$) at any dose; 3mgkg^{-1} - 30.8 ± 13.3 (M ± SEM), 6mgkg^{-1}- 37.0 ± 13.3, 9mgkg^{-1}- 44.9 ± 13.9.

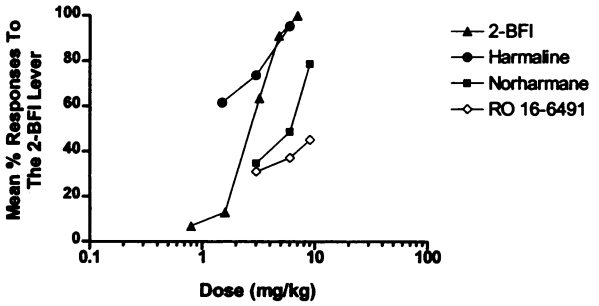

Fig. 1. Ability of I_2 associated ligands to substitute for 2-BFI.

These findings confirm the potency of the beta carbolines for the I_2 site *in vivo*. Although I_2 distribution closely matches MAO-B rather than MAO-A (Saura *et al*, 1992; Lione et al, 1998) RO16-6491 failed to substitute significantly.

This work was supported by the BBSRC. 2-BFI was kindly donated by Pierre Fabre.

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80P PROLONGED ELEVATION OF PPE-A AND PPT MRNA FOLLOWING CHRONIC L-DOPA TREATMENT OF 6-OHDA LESIONED RATS

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L-DOPA remains the most commonly used drug for the treatment of Parkinson's disease (PD), but its actions are not fully understood. In particular, maximal activity is not observed for up to one week when starting treatment and a sustained effect can be observed for some weeks following cessation of therapy (Muenter & Tyce 1971, Hauser et al. 2000). We have attempted to mimic these actions of L-DOPA in 6-OHDA lesioned rats and the role played by the indirect and direct striatal output pathways.

Unilateral 6-OHDA lesioned (8µg free base/4µl saline) male Wistar rats (200g) were treated for 14 days with L-DOPA methyl ester (25mg/kg free base or vehicle i.p., b.i.d.) with each treatment 6 hours apart. All animals received carbidopa (12.5mg/kg) both i.p. b.i.d. and in their drinking water. Rotational behaviour was assessed twice daily for up to day 36. A group of animals (n=6) were acutely challenged with L-DOPA methyl ester (25mg/kg free base i.p.) plus carbidopa on days 21, 28, and 35. Remaining animals (n=3-6 per group, per time point) were either killed 3 hours after the final dose of L-DOPA or vehicle, or at day 17, 21, 28 and 56. Brains were snap frozen in isopentane at -50°C and contiguous transverse sections (12µm) cut through the striatum. Preproenkephalin-A (PPE-A) and preprotachykinin (PPT) mRNA expression was studied by in situ hybridisation and quantitative densitometry (Zeng et al. 1995).

The rotational response to L-DOPA increased to a maximal effect on day 7 which was sustained until day 14 ($p<0.05$, two-way ANOVA with Mann-Whitney U test). Carbidopa treatment alone produced no rotational behaviour. On cessation of L-DOPA treatment, rotational behaviour immediately returned to control levels. L-DOPA challenge following cessation of chronic L-DOPA showed a sustained behavioural response equivalent to that seen on day 14, but which declined over the following 3 weeks. The behavioural response to L-DOPA was mirrored by persistent elevation in PPE-A mRNA expression and reversal of the 6-OHDA induced reduction of PPT mRNA expression (Figure 1).

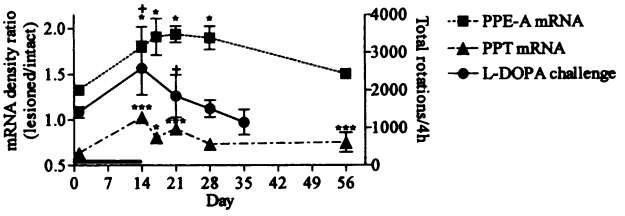

*Figure 1: Rotational behaviour following acute L-DOPA treatment, and PPE-A and PPT mRNA expression following chronic L-DOPA treatment (▬). Data are expressed as mean ±s.e.m.. +$p<0.05$ compared to day 1, Kruskal-Wallis with Mann Whitney U test for behaviour data *$p<0.05$, ***$p<0.001$ compared to day 1, one-way ANOVA with Newman Keuls test for in situ hybridisation data.*

Administration of L-DOPA to 6-OHDA lesioned rats shows the slow development of L-DOPA response seen in man, but not the persistence of the effect on drug withdrawal. However an increased response to L-DOPA is observed and this correlates with persistent changes in both the direct and indirect striatal output pathways. These changes may be related to the long-duration response to L-DOPA and to the development of dyskinesia (Hurtig et al. 1997).

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81P TOPOGRAPHICAL ASSESSMENT OF BEHAVIOURAL PHENOTYPE IN CONGENIC DOPAMINE D_{1A} RECEPTOR 'KNOCKOUT' MICE

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Delineating the behavioural role of the D_{1A} dopamine receptor has proved difficult in the absence of selective agonists and antagonists. D_{1A} 'knockout' constitutes an alternative approach, the phenotype of which continues to be examined (Drago et al., 1994; Clifford et al., 1998, 1999). However, the interpretation of these studies may be confounded by possible effects of the mixed genetic background [129/Sv x C57BL/6] on which the 'knockout' was constructed. One approach toward clarifying this issue is through analysis of the mutation on a more homogeneous genetic background. In this study we have sought to provide the first systematic examination of phenotype at the level of spontaneous behaviour using congenic D_{1A} 'knockouts'.

The D_{1A} 'knockout' was constructed on a mixed 129/Sv x C57BL/6 background as described previously (Drago et al., 1994). Mice were then backcrossed over 14 generations into C57BL/6. Animals were assessed topographically using an ethologically based rapid time-sampling behavioural checklist technique, for 10 min periods over 370 min (n=39 wildtypes, n=39 'knockouts') (Clifford et al., 1998). Statistical analysis was by analysis of variance followed by Student's t-test or Mann-Whitney U-test. Relative to wildtypes, congenic D_{1A} 'knockouts' evidenced over the initial 1hr exploratory period: a marked increase in locomotion [+79% to 39 ± 0.7 $p< 0.001$] with subsequent failure to habituate; abolition of rearing free [$p< 0.001$]; a marked reduction in sifting [-83% to 0.1 ± 0.04 $p< 0.001$]. Sniffing 6.9 ± 0.7, total rearing 4.7 ± 0.6, rearing seated 3.4 ± 0.5 and rearing to wall 1.3 ± 0.3 counts at final time interval, subsequently occurred to excess after exploratory phase [$p< 0.001$] due to failure to habituate. Grooming behaviours were not altered substantively.

These results define the phenotype of congenic D_{1A} 'knockout' mice. However, several differences are apparent vis-à-vis our previous findings on a mixed genetic background using similar techniques (Clifford et al., 1998). In congenics, increased locomotion was much more prominent, which may have disrupted elevations in grooming noted previously. Also, several topographies of behaviour evidenced a much more prominent failure to habituate. These results indicate that the D_{1A} 'knockout' phenotype varies with the genetic background on which the mutation is present.

These studies were supported by RCSI, the Stanley Foundation, IBRF-Galen and HEA.

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82P CHARACTERISATION OF NATIVE TISSUE 5-HT RECEPTORS IN THE HUMAN DORSAL RAPHE NUCLEUS (DRN) BY [^{35}S]GTPγS AUTORADIOGRAPHY

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5-HT receptors (with the exception of 5-HT_3) belong to the superfamily of G-protein-coupled receptors. Activation of these receptors can be detected in tissue sections by measuring changes in [^{35}S]GTPγS binding using autoradiography (Sim *et al.*, 1997; Day *et al.*, 1999). In the present study, we have used [^{35}S]GTPγS autoradiography to analyse activation of native tissue 5-HT receptors in the DRN using a range of 5-HT receptor ligands.

Sections of DRN were pre-incubated in 50mM Tris-HCl (pH7.4), 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl, 0.6mM GDP, 1mM dithiothreitol, 10μM pargyline, 0.5mM ascorbic acid, ± antagonists for 30min, followed by incubation in the presence of 0.1nM [^{35}S]GTPγS and test compounds for 2h. Sections were then washed, dried and apposed to Kodak BMR film. Grey level measurements were made in the DRN using NIH Image software. The data were analysed using GraphPad Prism software.

In the first study, the 5-HT receptor subtype in the DRN was characterised by studying antagonism of 5-HT-stimulated [^{35}S]GTPγS binding by the selective 5-HT_{1A} receptor antagonist, WAY100635. The non-selective agonist, 5-HT, caused a maximum stimulation of [^{35}S]GTPγS binding of ~2.5 fold over basal levels, resulting in a mean pEC_{50} of 7.0 ± 0.1 (n=3 donors). WAY100635 (30nM) antagonized the response to 5-HT with an apparent pK_B of 9.0 ± 0.2 (n=3 donors). This is consistent with the reported affinity of this ligand for human recombinant 5-HT_{1A} receptors (Fletcher *et al.*, 1996) and suggests that 5-HT receptor stimulation of [^{35}S]GTPγS binding in the DRN is mediated almost exclusively by 5-HT_{1A} receptors.

In the second study, 5-HT receptors in the DRN were characterised using five 5-HT receptor ligands: 5-HT, (+)8-OHDPAT (selective 5-HT_{1A} receptor agonist), buspirone (5-HT_{1A} receptor agonist), (±)pindolol (5-$HT_{1A/1B}$ receptor antagonist) and SB-272183 (5-chloro-2,3-dihydro-6-[4-methylpiperazin-1-yl]-1-[4-[pyridin-4-yl]napth-1-ylaminocarbonyl]-1H-indole) (5-$HT_{1A/1B/1D}$ receptor antagonist; Roberts *et al.*, this meeting). The resulting pEC_{50} values for the agonists are shown in Table 1. In this experiment, 5-HT stimulated [^{35}S]GTPγS binding to ~2.4 fold over basal levels, while (+)8-OHDPAT and buspirone acted as partial agonists, stimulating [^{35}S]GTPγS binding to ~1.9 and ~0.2 fold over basal levels respectively. (±)Pindolol and SB-272183 did not stimulate [^{35}S]GTPγS binding up to 100μM. Furthermore, SB-272183 at 1μM fully antagonised the increase in [^{35}S]GTPγS binding produced by 500nM 5-HT. These data are consistent with the presence of 5-HT_{1A} receptors in the DRN.

Table 1. pEC_{50} values and max stimulation (% of basal; in brackets)

	5-HT	(+)8-OHDPAT	Buspirone
Donor 1	7.1 (367)	7.1 (284)	6.1 (120)
Donor 2	7.2 (318)	7.0 (297)	6.4 (124)

This study is the first to demonstrate functional 5-HT_{1A} receptors in the human DRN. In addition, we have demonstrated the use of [^{35}S]GTPγS autoradiography to pharmacologically characterize G-protein-coupled receptors and their ligands in discrete regions of the human brain.

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83P COMPARISON OF FUNCTIONAL EFFICACY MEASURED AT HUMAN RECOMBINANT AND NATIVE TISSUE 5-HT_{1A} RECEPTORS

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Human(h) recombinant receptors are routinely used in pharmacological assays to assess functional efficacy of test compounds. One of the major issues arising from the use of cloned receptors is whether the information obtained represents efficacy at native tissue receptors. In this study we have used [^{35}S]GTPγS binding and measurement of cell firing to compare the functional efficacy of compounds at human recombinant 5-HT_{1A} receptors and at rat and human native tissue 5-HT_{1A} receptors in the dorsal raphe nucleus (DRN).

[^{35}S]GTPγS binding studies in HEK293 cells expressing h5-HT_{1A} receptors were carried out as described by Watson *et al.* (2000). [^{35}S]GTPγS autoradiographic studies on rat and human brain slices were carried out as described by Sim *et al.* (1996) and Day *et al.* (this meeting) respectively. Measurement of cell firing in rat DRN was performed as described by Corradetti *et al.* (1996).

The 5-HT_{1A} receptor agonist (+)8-OH-DPAT acted as a partial agonist in all of the [^{35}S]GTPγS assays tested and inhibited raphe firing through 5-HT_{1A} receptor activation (Table 1). (±)Pindolol and SB-272183 (5-Chloro-2,3-dihydro-6-[4-methylpiperazin-1-yl]-1-[4-[pyridin-4-yl]napth-1ylamino-carbonyl]-1H-indole) (Roberts *et al.*, this meeting) stimulated [^{35}S]GTPγS binding in h5-HT_{1A} receptors to give an intrinsic activity of 0.4 but did not show any significant functional activity at native tissue 5-HT_{1A} receptors. In [^{35}S]GTPγS binding studies, buspirone displayed high intrinsic activity in h5-HT_{1A}/HEK receptors but showed weaker activity in native tissues. In cell firing studies, buspirone fully inhibited firing.

Table 1. Comparison of Efficacies Using Different Assay Systems

Intrinsic Activity at 5-HT_{1A} Receptors Compared to 5-HT				
Compound	recombinant (GTPγS)	human native (GTPγS)	rat native (GTPγS)	rat native (firing)
5-HT	1.0	1.0	1.0	1.0
8-OH-DPAT	0.8 ± 0.1	0.6	0.8 ± 0.1	1.0
buspirone	0.9 ± 0.1	0.2	0.5 ± 0.2	1.0
pindolol	0.4 ± 0.1	I.A.	0.1 ± 0.1	I.A.
SB-272183	0.4 ± 0.1	I.A.	I.A.	I.A.

Data represent mean of n=2 or more experiments.
I.A. – inactive up to 10μM.

These results suggest that compounds showing partial agonism at recombinant h5-HT_{1A} receptors do not necessarily activate 5-HT_{1A} receptors in the DRN. High receptor expression in the recombinant system probably generates receptor reserve and could account for these differences. This highlights the need for caution when predicting the functional efficacy of compounds at native tissue 5-HT_{1A} receptors, when the primary test is in a recombinant system.

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84P THE EFFECT OF PHENYLARSINE ON NIK-DEPENDENT IKK/ NFκB SIGNALLING IN LPS-STIMULATED RASMC

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The transcription factor nuclear factor kappa B (NFκB) plays a key role in the regulation of a number of inflammatory genes and is central to the onset of sepsis (Fujii *et al.*, 2000). We have previously shown that in rat aortic smooth muscle cells (RASMC), lipopolysaccharide (LPS) stimulates NF-κB activation, preceeded by degradation of the inhibitory κB (IκB) isoforms and the activation of IκB kinases (IKKs) (manuscript in preparation). Pharmacological evidence is now accumulating for a role of a tyrosine phosphatase in the regulation of NFκB activation (Paul, Torrie & Plevin, 1999) however the site of regulation is unknown. NFκB-inducing kinase, (NIK), a member of the MAP3K family of proteins, is believed to be upstream of IKK in some cell types (Regnier *et al.*, 1997). Therefore we sought to determine i) whether NIK was involved in IKK-dependent NFκB activation in response to LPS and ii) if NIK was the site of action of the protein tyrosine phosphatase (PTPase) inhibitor phenylarsine oxide (PAO).

IKK- and NIK-dependent NFκB activation in RASMCs were determined by luciferase reporter assay following co-transfection of cells with an NFκB-dependent reporter plasmid (0.5μg) and either dominant negative (DN) IKKs or DN-NIK constructs (1-10μg). NF-κB DNA-binding activity was detected by electrophoretic mobility shift assay (EMSA) whilst IκB protein expression levels were determined by Western blotting. IKK activities were measured by immunocomplex-kinase assay *in vitro*. NIK was immunoprecipitated from RASMC using specific anti-NIK antibodies. All values represent the mean ± s.e.m. from at least 3 experiments.

Transfection of RASMC with either DN-IKKα or DN-IKKβ (10μg) reduced LPS-stimulated NFκB-dependant reporter activity (100μgml^{-1}) (reporter activity as fold stim.; control=1±0.1, control+DN-IKKα=0.1±0.01, control+DN-IKKβ=0.1±0.07, LPS=18.7±6.6, LPS+DN-IKKα=0.5±0.2, LPS+DN-IKKβ=2.1±1.1). Reporter activity was also inhibited by DN-NIK (10μg) (reporter activity as fold stim; control=1±0, LPS=10.39±1.34, LPS+DN-NIK=2.62±0.35). These effects occurred in a concentration-dependent manner. Preincubation of RASMC with PAO (2.5μM) for 30 min abrogated LPS-stimulated NF-κB-DNA binding, IκB-α, -β and -ε degradation and IKK activity (IKK-α activity as fold stim; control=1±0.2, LPS=8.23±0.37, LPS+PAO=1±0.41). However, there was no change in the phosphotyrosine content of NIK immunoprecipitated from RASMC following LPS or PAO pretreatment.

This study strongly suggests that in RASMC I) LPS stimulates IKK/NFκB signalling through a NIK-dependent pathway II) NIK is unlikely to be a direct target for regulation by a PTPase.

This work was supported by the British Pharmacological Society and The British Heart Foundation. L.J.T. is an A.J. Clark student.

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85P THE EFFECT OF *E.COLI* 0157:H7 AND ITS ASSOCIATED VEROTOXINS ON THE STRESS-ACTIVATED PROTEIN KINASE AND NFκB PATHWAYS IN VERO CELLS

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Escherichia coli O157:H7 (Vero-toxigenic *E. coli* - VTEC) infection can lead to diseases such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS), mediated in part via the production and release of toxins, termed verotoxins (VTs). The VTs exert a profound cytotoxic effect on vascular endothelium and African green monkey kidney cells (Vero), and are known protein synthesis inhibitors and inducers of apoptosis (Taguhi *et al.*, 1998). Recently, evidence has suggested that the stress-activated protein kinase (SAP kinase), consisting of homologues of C-Jun N-terminal kinase (JNK) and p38 MAP kinases and NF-κB pathways are important in cell death in response to cellular stress and inflammatory cytokines (Shifrin & Anderson, 1999). Therefore, the aim of the present study was to examine whether supernatant from *E. coli* O157:H7 or VT1 & 2 could activate SAP kinase and NF-κB pathways in Vero cells.

JNK activity was measured by *in vitro* kinase assay. NF-κB activity was detected by electrophoretic mobility shift assay (EMSA). IκBα expression was assessed by Western Blotting. All values represent the mean ± s.e.m. of at least three individual experiments.

In Vero cells, supernatant from *E. coli* O157:H7, VT1 and VT2 (2 μgml^{-1}) activated JNK in a time-dependant manner reaching a maximum by 60 min and remaining sustained for up to 5 h (fold stim. = 20.. ± 2.6 at 60 min and 18.2 ± 2.1 at 5 h). Pre-treatment of the cells with anti-VT1 and/or anti-VT2 antibodies 5 μgml^{-1}) decreased the O157:H7-induced JNK activity (O157:H7 fold stim. = 10.25 ± 0.55, O157:H7 + anti-VT1 = 9.25 ± 0.13, O157:H7 + anti-VT2 = 5.2 ± 0.2 and O157:H7 + anti-VT1&2 = 1.1 ± 0.4). A non-VTEC serotype, O166:H27, transiently stimulated JNK activity which was maximal at 30 min and had returned to basal levels by 2 h (fold stim. = 18.23 ± 0.45 at 30 min and 1.3 ± 0.9 at 2 h). O157:H7 supernatant also stimulated a sustained degradation of IκBα whilst VT1 & 2 were without effect. However, O166:H27 induced a rapid but transient degradation of IκBα yet showed sustained degradation of IκBα up to 4 h in the presence of VT1 (2 μgml^{-1}). Pre-treatment of Vero cells with anti-VT1 and/or anti-VT2 antibodies (10 μgml^{-1}) inhibited the O157:H7 supernatant induced-IκBα degradation at 4 h. O157:H7 induced NF-κB-DNA binding in a time-dependant manner, reaching a maximum by 60 min and remaining sustained for up to 4 h, whilst O166:H27 transiently induced NF-κB-DNA binding which was sustained up to 4 h when co-stimulated with VT1 (2μgml^{-1}). Pre-treatment of Vero cells with anti-VT1 and anti-VT2 antibodies (10 μgml^{-1}) partially inhibited the O157:H7 supernatant induced-NF-κB-DNA binding at 4 h.

These data strongly suggest VT mediates sustained JNK activity in Vero cells, and prolongs IκBα degradation and NF-κB activation in response to *E. coli* O157:H7 supernatant. However, an additional component in the supernatant of *E. coli* O157:H7 and another serotype, seems to be responsible for transient activation of JNK and initial stimulation of NF-κB.

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86P THE NF κB PATHWAY PARTICIPATES IN THE INCREASE IN IRF-1 EXPRESSION IN HUMAN ENDOTHELIAL CELLS STIMULATED BY LIPOPOLYSACCHARIDE AND TUMOUR NECROSIS FACTOR- α

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Interferon regulatory factor (IRF-1) is an inducible 48kDa protein that plays a key role in the regulation of immune cell function (Kamijo *et al.*, 1994). The Janus kinases/signal transducers and activators of transcription (JAK/STAT) and nuclear factor kappa B (NFκB) are strongly implicated in the regulation of IRF-1 induction through binding to the γ-activated sequence/factor (GAS/GAF) and NFκB response elements within the IRF-1 promoter. Previously, we found that in RAW 264.7 macrophages, despite rapid stimulation of NFκB and delayed activation of GAS/GAF DNA binding, lipopolysaccharide (LPS) stimulated a very modest increase in IRF-1 expression (Liu, Paul & Plevin, 1999). We therefore sought to examine whether the characteristics of LPS induction of IRF-1 expression were comparable in human umbilical vein endothelial cells (HUVECs).

IRF-1 expression in whole cell extracts was assessed in cultured HUVECs by Western blotting using anti-IRF-1 antibodies. NFκB and GAS/GAF DNA-binding activities were assessed in nuclear extracts by electrophoretic-mobility shift assay (EMSA). Results are expressed in density units mean±s.e.m. of at least three experiments.

In HUVECs, LPS (10μgml^{-1}) stimulated a substantial time-dependent increase in the expression of IRF-1 which was maximal at 2-4 h before returning to near basal values by 8h (expression in density units; control=0.02±0.003, LPS (4h)=0.30±0.04, LPS (8h)=0.09±0.01). Tumour necrosis factor (TNF)-α also stimulated a similar increase in the expression of IRF-1 with comparable kinetics. As expected both LPS and TNFα-stimulated a substantial increase in NFκB DNA-binding activity however, both agonists also provoked a rapid increase in GAS/GAF-DNA-binding activity. Preincubation of HUVECs with the NFκB inhibitor pyrrolidine dithiocarbamate (PDTC), selectively inhibited LPS-stimulated IRF-1 induction although another NFκB inhibitor, tosyl-lys-chloromethylketone (TLCK), inhibited the responses to both LPS and TNFα (control=0.02±0.00, LPS=0.65±0.07, LPS+PDTC (1mM)=0.01±0.00, LPS+TLCK (100μM)=0.03±0.01). The agonist-dependent sensitivity to PDTC but not TLCK was reflected in the inhibition of NFκB-DNA binding. PDTC abolished LPS but not TNFα stimulation whilst TLCK was equally effective against both agonists. A similar profile of inhibition of agonist stimulated GAS/GAF-DNA binding was also observed using PDTC and TLCK

These data suggest that NFκB is likely to play a more pronounced role in the regulation of IRF-1 expression in HUVECs and further highlights the agonist/cellular selectivity of PDTC as a NFκB inhibitor. Finally, LPS and TNF-stimulated GAS/GAF activity may also be dependent upon prior NFκB activation.

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87P PROSTACYCLIN RECEPTOR-INDEPENDENT EFFECTS OF NON-PROSTANOID PROSTACYCLIN MIMETICS

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In Chinese hamster ovary (CHO) cells transfected with mouse prostacyclin (mIP) receptor cDNA, stimulation by IP agonists such as iloprost can increase both cyclic AMP and inositol phosphate production by coupling to G_s and G_q proteins, respectively (Namba *et al.*, 1994). When testing a range of IP agonists for activation of the phospholipase C pathway, we noted that octimibate and related non-prostanoid prostacyclin mimetics were unexpectedly inactive at concentrations from 100 nM to 30 μM in CHO cells transiently expressing the mIP receptor. Preliminary experiments suggested that these compounds could inhibit iloprost-stimulated [^{3}H]-inositol phosphate production in a non-competitive manner. This present study was designed to examine further the inhibitory activity of these non-prostanoid prostacyclin mimetics.

Wild-type CHO cells, which do not express detectable IP receptors, were assayed at >80% confluency in 12-well plates following labelling with [^{3}H]-*myo*-inositol (2 μCi/well) for 48 h (Conklin *et al.*, 1992). Cells were incubated in HEPES-buffered saline (plus 20 mM LiCl and 3 μM indomethacin) with prostanoid receptor agonists for 10 min, followed by a further 60 min with 100 μM ATP to activate the endogenous purinergic P2Y receptors. The present results (Table 1) suggest that only the non-prostanoid drugs with specificity for the IP receptor can inhibit ATP-stimulated [^{3}H]-inositol phosphate production in an IP receptor-independent manner.

Table 1. Effect of prostanoid receptor agonists on ATP-stimulated [^{3}H]-inositol phosphate production in CHO cells.

Agonist (receptor specificity)	Responses (%control) Agonist (1 μM)	Agonist (30 μM)
Prostanoids		
Cicaprost (IP)	103 ± 13	97 ± 4
Iloprost (IP)	93 ± 11	98 ± 6
Carbacyclin (IP)	102 ± 6	86 ± 10
PGE_1 (IP/EP)	111 ± 18	116 ± 13
PGD_2 (DP)	114 ± 8	114 ± 8
PGE_2 (EP)	97 ± 8	125 ± 8
$PGF_{2\alpha}$ (FP)	111 ± 8	93 ± 6
U46619 (TP)	104 ± 12	96 ± 5
Non-prostanoids		
Octimibate (IP)	44 ± 4*	13 ± 2*
BMY 42393 (IP)	83 ± 11	2 ± 2*
BMY 45778 (IP)	92 ± 12	69 ± 3*
ONO-1301 (IP)	71 ± 2*	21 ± 2*
ONO-AP-324 (EP_3)	116 ± 9	100 ± 6

Results are mean ± s.e.mean of 3-4 experiments. Solvent controls (DMSO or ethanol) were inactive. *P<0.01 compared with control response to 100 μM ATP (ANOVA followed by Dunnett's).

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This work was supported by the Research Grants Council of Hong Kong.

88P Ca^{2+} SIGNALLING BY RECOMBINANT CXCR2 CHEMOKINE RECEPTORS IS POTENTIATED BY P2Y2 PURINOCEPTORS IN HUMAN EMBRYONIC KIDNEY (HEK) CELLS

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In mammalian cells G protein-coupled receptor crosstalk may couple a receptor to an atypical signalling pathway. For example, stimulation of a $G\alpha_q$-coupled receptor potentiates calcium signalling *via* a co-expressed $G\alpha_i$-coupled receptor (Okajima *et al*, 1992). The aim of this study was to determine whether endogenous purinergic P2Y receptors ($G\alpha_q$-coupled) were able to promote intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) signalling *via* recombinant CXCR2 ($G\alpha_i$-coupled).

HEK cells expressing recombinant human CXCR2 (Murphy & Tiffany, 1991) were grown to confluence and loaded with Fluo-3-acetoxymethylester (5μM in balanced salts solution (BSS) for 1hr at room temperature). $[Ca^{2+}]_i$ was measured by Fluorescence Imaging Plate Reader (FLIPR), at 37°C, in response to either a purinergic agonist or vehicle (BSS) followed 150s later by interleukin-8 (IL-8) in the continued presence of the initial agonist. Increases in $[Ca^{2+}]_i$ are expressed as a percentage of the maximal response to ATP (1mM). Data are mean ± SEM ($n \geq 3$). Statistical significance was assessed by unpaired Student's t-test.

Agonist pharmacology of the initial purinergic response was consistent with the presence of both P2Y1 and P2Y2 receptors (Schachter *et al*, 1997) and is summarised in Table 1.

Table 1: Agonist pharmacology of the initial purinergic $[Ca^{2+}]_i$ response

	ATP	UTP	2MeSADP
pEC_{50} (M)	5.9±0.1	5.7±0.1	8.0±0.1
Slope	1.3±0.2	1.1±0.04	0.9±0.1
Emax (%)	100	88±3	99±4

Initial experiments demonstrated that stimulation with ATP (1mM) caused a marked increase in both the potency and E_{max} of a subsequent concentration-response curve to IL-8 (vehicle pEC_{50} = 8.8±0.2M, E_{max} = 29±7%, ATP pEC_{50} = 9.9±0.1M, E_{max} = 78±9%; P:<0.001).

2MeSADP (P2Y1-selective) and UTP (P2Y2-selective) were then used to investigate the pharmacology of this potentiation. When compared to vehicle, UTP caused a concentration-related increase in the response to a single concentration of IL-8 (1nM) (pEC_{50} = 5.0±0.3M, E_{max} = 66±2%; P:<0.001). 2MeSADP did not significantly increase the response to IL-8 (max. response = 22±1% at 10μM), suggesting that potentiation is mediated by P2Y2, but not P2Y1 receptors. This was supported by the observation that at concentrations of 2MeSADP and UTP that were maximal and equi-effective on $[Ca^{2+}]_i$ elevation, (1μM and 100μM, respectively), UTP caused a significant increase in the potency and E_{max} of the subsequent curve to IL-8 (P:<0.001) whereas 2MeSADP did not (P:>0.05). See Table 2.

Table 2: Effect of vehicle, UTP and 2MeSADP on concentration-response curves to IL-8

	Vehicle	UTP	2MeSADP
pEC_{50} (M)	8.1±0.1	9.8±0.1	8.7±0.2
Slope	2.7±0.5	1.9±0.4	1.1±0.1
Emax (%)	22±2	51±3	33±4

We conclude that purinergic pre-stimulation of HEK CXCR2 cells potentiates a subsequent $[Ca^{2+}]_i$ response to IL-8. In this experimental paradigm, this phenomenon appears to be specific to the P2Y2 purinoceptor subtype, although the mechanism(s) underlying this phenomenon remains to be established.

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