1P DEVELOPMENT OF TOLERANCE TO THE ANXIOGENIC EFFECT OF NICOTINE IS MEDIATED BY THE DORSAL HIPPOCAMPUS

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In the social interaction (SI) test of anxiety, systemic administration of nicotine has anxiolytic and anxiogenic effects that are both dose and time-dependent (File et al., 1998; Irvine et al., 1999). After 7 days of nicotine treatment, tolerance develops to both effects. The dorsal hippocampus is one site mediating the anxiogenic effect of nicotine in the SI test (File et al., 1998), and this can be reversed by co-administering the 5-HT_{1A} receptor antagonist WAY 100,635 (Kenny et al., 2000a). Furthermore, nicotine increases basal [^{3}H]-5-HT from dorsal hippocampal slices in a concentration dependent manner (Kenny et al., 2000b). The purpose of the present experiment was to explore the role of the dorsal hippocampus in mediating the tolerance that occurs to the anxiogenic effect of 0.1 mg/kg nicotine and to determine whether tolerance develops to the effects of nicotine on [^{3}H]-5-HT release. Thus, rats were treated for 6 days with nicotine (0.1 mg/kg, s.c.) or vehicle and then either tested in the SI test after bilateral administration of nicotine (5ng and 1μg) into the dorsal hippocampus or [^{3}H]-5-HT release was measured in superfused dorsal hippocampal slices taken from these animals.

Nicotine (1μg) significantly reduced social interaction in the vehicle pre-treated rats($p<0.01$), and tolerance to this effect was seen in the rats pre-treated for 6 days with s.c. nicotine, see Table 1. Nicotine was without effects on locomotor activity, see Table 1.

In the vehicle pre-treated rats, nicotine (50-200μM), stimulated [^{3}H]-5-HT release from dorsal hippocampal slices. This stimulation was significantly higher than that seen in nicotine pre-treated animals ($p<0.01$), indicating the development of tolerance to the effects of nicotine on 5-HT release, see Table 1.

	Social Interaction	Locomotor Activity		% [^{3}H]5-HT Release
Vehicle			Vehicle	
aCSF (9)	76.9±8.8	298.3±20.5	50μM (6)	33.3±2.3†
5ng (7)	70.0±13.8	279.6±33.8	100μM (6)	46.3±1.7††
1μg (7)	19.6±10.3**+	233.9±25.6	200μM (6)	55.1±2.7††
Nicotine			Nicotine	
aCSF (9)	75.4±7.5	301.4±26.9	50μM (6)	16.4±1.2§
5ng (9)	101.0±10.9	298.3±28.3	100μM (6)	22.7±0.7§§
1μg (8)	50.6±8.4	299.7±30.7	200μM (6)	22.0±0.1§§

Table 1. Mean (± sem) time spent in social interaction (s), locomotor activity (beam breaks) and % increase in basal [^{3}H]-5-HT release evoked by nicotine, in animals pre-treated with either vehicle or nicotine (0.1 mg/kg, s.c.). **p<0.01 compared with the vehicle control and +p<0.05 compared with the animals treated with nicotine for 6 days and then challenged with 1μg nicotine in the dorsal hippocampus. †p<0.05 and ††p<0.01 compared with the baseline release, and §p<0.05 and §§p<0.01 compared with the baseline release and the nicotine evoked release in vehicle treated animals. Numbers in parentheses indicate group size. The scores from the behavioural studies and the release were analysed by two-way analyses of variance and comparisons with individual groups were then made with least significance difference post-hoc tests. The increase from the baseline of the nicotine evoked release was analysed by single factor repeated measures for each dose of nicotine.

We can conclude that the dorsal hippocampus is crucially involved in mediating the development of tolerance to nicotine's anxiogenic effect, and could be mediated by a decreased effect of nicotine on the release of 5-HT from terminals in this region.

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2P BIA 3-202, A LONG-ACTING CATECHOL-*O*-METHYLTRANSFERASE INHIBITOR WITH LIMITED BRAIN ACCESS

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In recent years, the development of new inhibitors of catechol-*O*-methyltransferase (COMT) has been accelerated by the hypothesis that inhibition of this enzyme may provide significant clinical improvements in patients with Parkinson's disease undergoing treatment with L-DOPA plus a peripheral aromatic L-amino acid decarboxylase inhibitor. The experiments reported here were designed to evaluate the oral bioavailability and effects upon brain and liver COMT of BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone), a new COMT inhibitor. BIA 3-202 and reference compounds (entacapone and tolcapone) were given by gastric tube (30 mg kg^{-1} in 0.5% carboxymethylcellulose) to overnight fasted male Wistar rats (240-260 g; Harlan, U.K.). Thereafter, at defined intervals, animals were killed by decapitation and organs removed and used to determine COMT activity. COMT activity was evaluated by the ability to methylate adrenaline to metanephrine, as previously described (Vieira-Coelho & Soares-da-Silva, 1999). Results are arithmetic means with s.e.mean. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. A saturating concentration of adrenaline (liver, 1000 μM; brain, 100 μM) was chosen to use in inhibition studies (Vieira-Coelho & Soares-da-Silva, 1999). BIA 3-202 was found to be a potent inhibitor of both brain and liver COMT, the maximal inhibitory effect being achieved within 30 min after their oral administration (Table 1). When investigating the duration of inhibitory effect upon liver COMT it became evident that BIA 3-202 was a particularly long acting compound. Notably, inhibition of liver COMT by BIA 3-202 and tolcapone at 9 h after oral administration was approximately 70%, whereas entacapone was almost devoid of COMT inhibitory properties. BIA 3-202 and entacapone were much less potent than tolcapone at inhibiting brain COMT (Table 1). The potency of BIA 3-202, tolcapone and entacapone at inhibiting brain and liver COMT was evaluated in experiments in which rats were given increasing doses of the test compounds (0.3 to 30 mg kg^{-1}) by gastric tube. In these experiments rats were killed 1 h after the administration of the compounds (at t_{max}) and COMT activity determined as described above. The results obtained indicate that BIA 3-202 and tolcapone were equally potent at inhibiting liver COMT with ED_{50}'s (in mg kg^{-1}) of 0.7±1.1 and 0.7±0.1, respectively; entacapone was slightly less potent with a ED_{50} value of 1.9±0.2. However, BIA 3-202 was less potent than tolcapone at inhibiting brain COMT with ED_{50}'s of 5.3±1.1 and 1.6±0.1, respectively. At the highest dose tested (30 mg kg^{-1}), entacapone failed to reach the 50% inhibition level. In conclusion, BIA 3-202 is a long acting COMT inhibitor with limited access to the brain.

Table 1. Percent inhibition (%) of COMT activity by BIA 3-202, entacapone and tolcapone in homogenates of rat brain and liver, determined at 0.5, 1, 3, 6 and 9 h after their administration by gastric tube. Results are means ± s.e.mean of 4 experiments per group.

	Time (h)				
Brain	0.5	1	3	6	9
BIA 3-202	83.6±1.3	80.9±2.7	65.0±3.9	31.5±3.2	21.9±2.7
Entacapone	71.7±7.0	44.8±7.0	30.1±6.4	19.9±7.1	22.8±3.4
Tolcapone	98.9±0.1	98.7±0.2	97.0±0.5	85.8±8.2	77.5±1.8
Liver					
BIA 3-202	98.6±0.4	96.7±1.7	96.2±0.8	75.9±4.2	69.8±3.6
Entacapone	98.2±0.3	96.2±1.1	85.9±2.2	73.6±5.4	24.7±7.9
Tolcapone	100.0±0.0	99.9±0.1	98.0±0.7	94.1±0.3	67.0±4.0

Vieira-Coelho, M.A. & Soares-da-Silva, P. (1999). *Brain Res.*, **821**, 69-78.

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3P BIA 3-202 ENHANCES THE AVAILABILITY OF L-DOPA TO THE BRAIN AND REDUCES ITS *O*-METHYLATION

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BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone) is a new long acting COMT inhibitor with limited access to the brain. The present study evaluated the interference of BIA 3-202 upon levels of L-DOPA and metabolites in plasma (3-*O*-methyl-L-DOPA) and brain (3-*O*-methyl-L-DOPA and dopamine) in rats orally treated with L-DOPA plus benserazide. Two groups of male Wistar rats (200-250 g; Harlan, U.K.) fasted overnight were administered orally with BIA 3-202 (0, 3, 10 and 30 mg kg^{-1}, n=12), suspended in 0.5% carboxymethylcellulose (4 ml kg^{-1}). Thirty min later, the first group was administered orally with L-DOPA (20 mg kg^{-1}) plus benserazide (30 mg kg^{-1}) and the second group with vehicle (0.5% carboxymethylcellulose, 4 ml kg^{-1}). Six hours after the administration of the COMT inhibitor, animals were anaesthetised with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and blood samples taken by heart puncture. The right striatum was quickly dissected out and stored in perchloric acid 0.2 M. Blood samples were centrifuged for 15 min at 3,000 *g* (4°C) and the plasma samples were stored at -80°C till the assay of L-DOPA and 3-*O*-methyl-L-DOPA. Assay of catecholamines was carried out by means of h.p.l.c. with electrochemical detection (Vieira-Coelho & Soares-da-Silva, 1996). Results are arithmetic means with s.e.mean, n=4. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. Levels of L-DOPA and 3-*O*-methyl-L-DOPA in the striatum and plasma of animals given the vehicle were below detection limit. As shown in table 1, levels of dopamine in the striatum in L-DOPA plus benserazide treated rats were higher than in vehicle treated rats. However, this increase in striatal dopamine was higher ($P<0.05$) in rats given BIA 3-202. This effect was accompanied by a marked decrease in 3-*O*-methyl-L-DOPA levels in the striatum of L-DOPA plus benserazide treated rats. Table 1 also shows that administration of BIA 3-202 was accompanied by increases in levels L-DOPA and decreases in 3-*O*-mehtyl-L-DOPA levels in plasma. BIA 3-202 did not significantly affect levels of DOPAC and HVA in the striatum. It is concluded that administration of BIA 3-202 enhances the availability of L-DOPA to the brain by reducing its *O*-methylation, which may prove beneficial in parkinsonian patients treated with L-DOPA plus an aromatic amino acid decarboxylase inhibitor.

Table 1. Levels of L-DOPA, 3-O-methyl-L-DOPA (3-OM-L-DOPA) and dopamine in the striatum and plasma of vehicle and BIA 3-202 treated rats 6 h after the administration of vehicle or L-DOPA (20 mg kg^{-1}) plus benserazide (30 mg kg^{-1}). Results are means ± s.e.mean (n=4); n.d., not detectable.

	Striatum (in ng mg^{-1})			
	Vehicle	BIA 3-202 (3 mg/kg)	BIA 3-202 (10 mg/kg)	BIA 3-202 (30 mg/kg)
	Vehicle			
Dopamine	9.5±0.9 #	8.1±0.8 #	11.2±0.7 #	14.1±0.5 #,*
	L-DOPA + benserazide			
L-DOPA	0.4±0.4	0.1±0.0	0.5±0.3	1.3±0.4
3-OM-L-DOPA	12.8±0.7	6.6±0.6 *	2.8±0.3 *	1.1±0.3 *
Dopamine	12.3±0.4	14.0±1.4	15.3±1.6	19.0±1.5 *
	Plasma (µmol l^{-1})			
	L-DOPA + benserazide			
L-DOPA	1.3±0.3	14.7±2.5 *	39.7±2.1 *	76.7±3.3 *
3-OM-L-DOPA	48.9±2.3	29.3±4.1 *	6.2±0.5 *	2.0±0.3 *

Significantly different from vehicle treated rats (* $P<0.05$) and rats treated with L-DOPA plus benserazide (# $P<0.05$)

Vieira-Coelho, M.A. & Soares-da-Silva, P. (1996). *British J. Pharmacol.*, 117, 516-520

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4P TOPOGRAPHICALLY BASED SPECIFICATION OF BEHAVIOURAL PHENOTYPE IN CONGENIC MICE WITH TARGETED GENE DELETION OF THE D_3 DOPAMINE RECEPTOR

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The behavioural role of the D_3 dopamine receptor remains uncertain. It may exert an inhibitory role, either presynaptically or at a postsynaptic location; alternatively, these functions may involve D_2 receptors. 'Knockout' mice with deletion of the D_3 receptor offer a new route to clarifying these issues, but on a mixed genetic background the phenotype appears variable (Accili et al., 1996; Xu et al., 1997). We have applied topographically-based ethological assessment to congenic D_3 'knockout' mice.

D_3 'knockouts' were constructed as described previously (Accili et al., 1996), backcrossed 14 times into C57BL/6 mice, and genotyped using PCR. Animals were assessed using an ethologically-based rapid time-sampling behavioural checklist technique, for 10 min periods over 370 min (n=20 males, 20 females per group) for spontaneous behaviour, or over 60 min (n=8 females per group) following challenge with the selective D_2-like agonist RU 24213 (Clifford et al., 2000). Statistical analysis was by analysis of variance followed by Student's t-test or Mann-Whitney U-test. Relative to wildtypes, D_3 'knockouts' evidenced an overall increase in rearing, which occured primarily due to delayed habituation among females over 80-170min [+80%, $P<0.05$ at 170 min]; there were no significant effects of genotype for sniffing, locomotion, grooming, sifting or chewing. Following challenge with RU 24213 (0.016-0.25 mg/kg s.c.), locomotion [$D_3^{+/+}$, -72%; $D_3^{-/-}$, -77%], rearing [$D_3^{+/+}$, -56%; $D_3^{-/-}$, -51%] and sifting [$D_3^{+/+}$, -63%; $D_3^{-/-}$, -79%] decreased [$P<0.01$] in a dose-dependent manner in animals of each genotype.

Congenic mice with targeted gene deletion of the D_3 receptor were characterised by delayed habituation of rearing, primarily among females, in the absence of any other prominent phenotypic differences at the level of spontaneous behaviour; however, as appears to be the case for D_2 'knockouts' (Clifford et al., 2000), a role for compensatory processes can not be excluded. Exploratory behaviours were decreased by low doses of the selective D_2-like agonist RU 24213 to similar extents in each genotype, indicating conservation of this effect in the absence of the D_3 receptor and thereby implicating an important role for the D_2 receptor.

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5P INCREASE IN EFFICACY AND POTENCY OF DOPAMINE D_{2short} RECEPTOR AGONISTS FOLLOWING SODIUM BUTYRATE TREATMENT

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Sodium butyrate treatment of recombinant cell lines results in increased receptor expression levels enabling agonist and antagonist properties to be examined in systems with varying receptor density (Lesage *et al.*, 1998, Gazi *et al.*, 1999). It has been shown previously that sodium butyrate treatment of a recombinant $CHOD_{2S}$ cell line increases dopamine D_{2S} receptor density 2.5 fold (Marston & Strange, 2000). In the present study, a series of dopamine D_{2S} receptor partial agonists has been examined using stimulation of [^{35}S]GTPγS binding. Agonist efficacy and potency values have been compared at two different receptor expression levels (untreated and sodium butyrate treated).

Human dopamine D_{2S} receptors were stably expressed in Chinese hamster ovary (CHO) cells and incubated with 5mM sodium butyrate for 18 hours before preparation of the cell membranes. The [^{35}S]GTPγS binding assay was carried out essentially as described by Gardner *et al.* (1996). Cell membranes (20μg) were incubated in buffer (20mM HEPES, 6mM $MgCl_2$ and 100mM NaCl, pH 7.4) with 1μM GDP and drugs at 30°C. After 30 minutes, [^{35}S]GTPγS (100pM) was added and incubated for a further 30 minutes before termination of the assay.

A range of partial agonists was tested for their ability to stimulate [^{35}S]GTPγS binding to membranes from untreated and sodium butyrate treated $CHOD_{2S}$ cells. The maximal responses for S-(-)-3-PPP, *p*-tyramine, β-phenylethylamine and *m*-tyramine (in order of increasing efficacy) were all less than for the full agonist dopamine (Table 1). Following sodium butyrate treatment the maximal [^{35}S]GTPγS stimulation by dopamine increased 1.8 ± 0.1 fold. The relative efficacies (% of dopamine) of S-(-)-3-PPP, *p*-tyramine and β-phenylethylamine were significantly increased compared with values obtained in untreated cells. The pEC_{50} values were significantly different for *m*-tyramine and dopamine in membranes at the two different expression levels but were not significantly different for the lower efficacy compounds (Table 2).

Table 1. Increase in efficacy following sodium butyrate treatment. *p<0.05 versus corresponding untreated value, unpaired student's *t*-test.

Compound (n=5-10)	Efficacy (% of dopamine) mean ± s.e.mean	
	Untreated	Treated
S-(-)-3-PPP	23.6 ± 4.1	48.1 ± 6.4*
p-tyramine	54.3 ± 1.8	75.9 ± 3.0*
β-phenylethylamine	58.6 ± 5.1	76.8 ± 3.0*
m-tyramine	82.8 ± 4.6	86.4 ± 8.2
dopamine	100	100

Table 2. Increase in potency following sodium butyrate treatment. *p<0.05 versus corresponding untreated value, unpaired student's *t*-test.

Compound (n=5-10)	pEC_{50} (mean ± s.e.mean)	
	Untreated	Treated
S-(-)-3-PPP	-7.22 ± 0.22	-6.97 ± 0.18
p-tyramine	-4.42 ± 0.06	-4.57 ± 0.08
β-phenylethylamine	-4.88 ± 0.08	-5.19 ± 0.13
m-tyramine	-5.55 ± 0.14	-6.26 ± 0.15*
dopamine	-6.74 ± 0.07	-7.11 ± 0.07*

In conclusion, compounds with lower maximal responses in untreated cells displayed an increased relative efficacy at a higher receptor density. The partial agonist *m*-tyramine gave a maximal response that was not significantly different between the two expression levels but did exhibit an increased potency at a higher receptor density. These data do not appear to fit current models of drug-receptor action.

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6P KINETICS OF NATIVE AND A RECOMBINANT FORM OF RAT SOLUBLE CATECHOL-*O*-METHYLTRANSFERASE

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Catechol-O-methyltransferase (COMT) catalyses the transfer of a methyl group from S-adenosyl-methionine to a catechol substrate. The soluble (S) and membrane-bound (MB) forms of COMT are encoded by a single gene using two separate promoters. The aim of this work was to produce a recombinant rat liver S-COMT and evaluate its kinetic profile. For that purpose, the coding region of S-COMT, obtained from rat liver total RNA by RT-PCR with specific primers for rat COMT, was introduced into the pCAL-n-FLAG® vector using the Affinity LIC cloning kit (Stratagene) and expressed in the *E. coli* BL21(DE3) strain. S-COMT was synthesised as a fusion protein with a calmodulin binding peptide tag on its amino terminal. Upon isopropyl-1-thio-β-$_D$-galactopyranoside induction of the bacterial culture, the recombinant protein was detected in both the soluble and insoluble cellular fractions by denaturing gel electrophoresis (SDS-PAGE). Recombinant COMT was purified to homogeneity (as ascertained by SDS-PAGE) from the soluble fraction by a calmodulin affinity cromatography followed by gel filtration. Up to 12 mg of pure recombinant protein were recovered per liter of culture. Rat liver S-COMT (native S-COMT) was obtained by the method of Nissinen *et al* (1988). A partially-purified preparation of rat liver S-COMT (Part. purif. S-COMT) was obtained from rat livers by a modification of the procedure described by Tilgmann & Kalkkinen (1990). COMT activity was evaluated at pH 7.2 by the ability to methylate adrenaline (0.025 to 2000 μM) to metanephrine as previously described (Vieira-Coelho & Soares-da-Silva, 1999). Some kinetic parameters for the *O*-methylation of adrenaline were then determined in the bacterial soluble fraction (BL21-COMT) and compared to native S-COMT. The catalytic number (K_{cat}), which is a measure of the methylation efficiency, was determined from Ackermann-Potter plots, where the initial enzyme rates were plotted against enzyme concentrations at different concentrations of a tight-binding inhibitor (3,5-dinitrocatechol). From these plots it is also possible to know the actual enzyme concentrations through the molar equivalency value (Meq). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=3-4. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

Table 1. Kinetic parameters of BL21-S-COMT and Native S-COMT

	K_m, μM	K_{cat}, min^{-1}	Meq, nM
BL21-COMT	418 (301; 536)	10.1 ± 1.5	7.2 ± 1.0
Native S-COMT	530 (329; 732)	8.3 ± 0.3	0.087 ± 0.001

The affinity for the substrate and the methylation efficiency of the recombinant enzyme did not differ from the native enzyme, as evidenced by K_m and K_{cat} values (Table 1). As expected, the molar equivalency of BL21-COMT is much higher than that for the native S-COMT. The kinetic characteristics of the purified recombinant enzyme (pure rec. COMT) were also determined and compared to those for the part. purif. S-COMT (Table 2).

Table 2. Kinetic parameters of pure rec. COMT and part. purif. S-COMT

	K_m, μM	IC50, nM
Pure recombinant COMT	336 (75; 596)	455 (266, 777)
Part. purif. S-COMT	439 (168; 711)	454 (199, 1037)

Again no differences were observed in the affinity for the substrate (K_m) or in the inhibitory potency of 3,5-dinitrocatechol (IC_{50}) by the two enzymes. In conclusion, a recombinant form of S-COMT kinetically identical to the native enzyme was produced in large amounts and can now be used for various studies.

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7P NITRIC OXIDE IS A MORE EFFECTIVE PHYSIOLOGICAL ANTAGONIST OF ENDOTHELIN-1- THAN U46619-MEDIATED CONSTRICTIONS IN HUMAN CORONARY ARTERY

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The potent vasoconstrictors endothelin–1 (ET-1) and thromboxane have both been shown to be up-regulated in coronary artery disease (Bacon *et al*, 1996; Halishka & Lefer, 1987). Impaired release of nitric oxide (NO) from the endothelium is also associated with cardiovascular disorders (Drexler, 1999). Our aim was to determine the ability of NO to reverse constrictions mediated by ET-1 and the thromboxane mimetic U46619 in human coronary arteries using the NO-donor diethylamine NONOate (DEA/NO; Morely & Keefer, 1993).

Rings of coronary artery (4mm; n=6 individuals) were dissected from explanted hearts, denuded of their endothelium and mounted in organ baths for the measurement of isometric tension (37°C; bathed in oxygenated Krebs' solution). Initial responses were obtained with 100mM KCl. Constrictions were subsequently induced with either 10nM ET-1 or 100nM U46619, both sub-maximal concentrations which gave similar amplitudes of response (ET-1: 7.6 ± 1.9g weight, U46619: 8.7 ± 2.8g weight). Once the responses had reached a plateau three-fold concentration response curves to DEA/NO (10nM-30μM) were constructed. Results were expressed as % constrictor response ± s.e.mean and EC_{50} values were determined using the iterative curve-fitting software Fig P (Biosoft, Cambs, UK) and expressed as geometric means with 95% CI.

DEA/NO was found to have a significantly greater effect on constrictions mediated by ET-1 compared to U46619 (Figure 1; $P<0.05$, Student's *t*-test; n=6). DEA/NO was found to partially reverse the constriction mediated by U46619 (maximum relaxation; 82.3 ± 6.3%), whereas the ET-1 mediated constriction was fully reversed with additional relaxation of original basal tone (maximal relaxation; 124.8 ± 14.8%). EC_{50} values for DEA/NO did not differ significantly between the two groups.

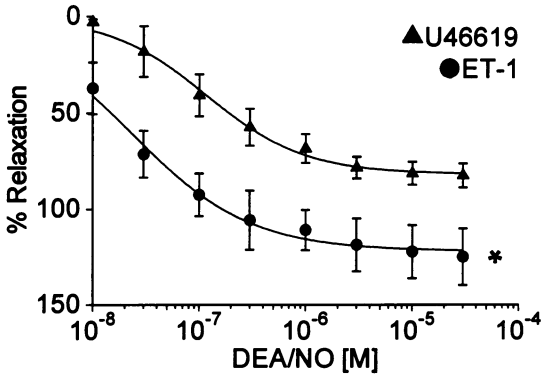

Figure 1 Cumulative concentration-response curves to DEA/NO, reversing constrictions induced by ET-1 and U46619 (*P <0.05, Student's *t*-test; n=6 individuals).

These data suggest that NO can counteract both ET-1-and U46619-mediated constrictions in human coronary artery. The combination of a loss in NO production with increased plasma levels of potent vasoconstrictors such as ET-1 and thromboxane in coronary artery disease may shift the balance even further towards heightened tone.

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8P HOMOCYSTEINE FURTHER AUGMENTS IMPAIRED ACETYLCHOLINE-STIMULATED RELAXATION AND CYCLIC GMP FORMATION IN AORTAE FROM DIABETIC RABBITS

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In patients with diabetes mellitus (DM), angiopathy is augmented by elevated blood levels of homocysteine (HC) (Emsley *et al.*, 1998). In turn, both DM and HC are associated with impaired vascular endothelial function, including reduced NO formation (Emsley *et al.*, 1998; Jeremy *et al.*, 1999). In order to investigate this area further, the effect of HC on endothelium-dependent relaxation and cGMP formation in aortae from diabetic rabbits was investigated.

New Zealand White rabbits (2 kg) were rendered diabetic with alloxan (65 mg / kg, i.v.). After 6 months rabbits were killed by cervical dislocation, aortae excised and 2 mm rings prepared. Rings were mounted under isometric tension in an organ bath and relaxation elicited with acetylcholine (Ach) following precontraction to maximal with phenylephrine (5-10 μM). Cyclic GMP formation was assessed using radioimmunoassay. The effect of preincubation for 30 min with HC +/- superoxide dismutase (SOD) on these parameters was then investigated.

All rabbits studied in the diabetic group had a plasma glucose concentration of > 25 mmol/l. Ach-stimulated relaxation and cGMP formation were significantly impaired in aortae from diabetic rabbits compared to controls an effect reversed with 300 U / ml SOD (tables 1 and 2). HC at 10 μM and 100 μM reduced Ach-stimulated relaxation and cGMP formation in aortae from diabetic rabbits, an effect reversed with 300 U / ml SOD (tables 1 and 2). HC had no effect on relaxation or cGMP formation in control aortae (tables 1 and 2).

Table 1. Effect of HC (μM) ± SOD on maximal relaxation [mean ± S.E.M., n = 6] in aortae from diabetic (D) and control (C) rabbits. * p < 0.01 compared to zero values. ** p < 0.01 C vs D (ANOVA)

	zero	HC (10)	HC (100)	HC (10)+SOD	HC (100)+SOD
C	95 ± 8	96 ± 6	93 ± 7	90 ± 9	93 ± 7
D	38 ± 5**	12 ± 6*	8 ± 7*	86 ± 10	80 ± 8

Table 2. Effect of HC (μM) ± SOD on Ach-stimulated cGMP formation (fmoles / mg tissue / min) [mean ± s.e.m., n = 6] in aortae from diabetic (D) and control (C) rabbits. *p < 0.01 compared to zero values. **p < 0.01 C vs D

	zero	HC (10)	HC (100)	HC (10)+SOD	HC (100)+SOD
C	258±20	261±26	238±37	254±27	258±30
D	98±9**	30±6*	10±7*	210±30*	220±40*

These data confirm that superoxide release is increased in arterial tissue from diabetic animals. Since superoxide reacts with NO to form peroxynitrite (Jeremy *et al.*, 1999) this explains the reduced endothelium-dependent relaxation and cGMP formation observed in the aortae from diabetic animals. HC augmented the formation of superoxide in arteries from diabetic rabbits but not control animals. Thus, the increased incidence of angiopathy in patients with DM and elevated levels of HC may be due, in part to increased superoxide and diminished NO formation. In turn, these data indicate that the use of antioxidants may be beneficial in treating cardiovascular disease in patients with both DM and elevated levels of HC.

Supported by the British Heart Foundation and Garfield Weston Trust.

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9P VASODILATOR EFFECTS OF QUERCETIN AND ITS METABOLITES, ISORHAMNETIN AND TAMARIXETIN, IN RAT ISOLATED VESSELS

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Flavonoids are secondary plant metabolites which are present in several foods (the estimated average human daily intake is 23 mg). Several epidemiological studies have shown an inverse association between dietary flavonoid intake and long term mortality from coronary heart disease (Yochun et al., 1999). Absorbed quercetin, the most abundant flavonoid, is rapidly metabolised into isorhamnetin and tamarixetin (3′- and 4′-methylquercetin, respectively) which show a long lasting presence in plasma. We have analysed the vasodilator effects of quercetin and its metabolites in rat conductance and resistance arteries.

Isolated rings (2-3 mm in length) with or without endothelium from the thoracic (TA) or abdominal aorta (AA), iliac (IA) or main mesenteric arteries (MA) from male Wistar rats (250-300 g) were mounted for isometric tension recording in Krebs solution (Pérez-Vizcaíno et al., 1999). The isolated rat mesenteric bed (MB) was perfused at constant flow (2 ml min^{-1}, Pérez-Vizcaíno et al., 1995). Endothelium removal was performed in the MB by perfusing 0.3% sodium deoxycolate for 30 s. Preparations were stimulated with 10^{-6}M noradrenaline and cumulative concentration-response curves to the flavonoids were constructed. Inhibitors were added 15 min before noradrenaline. Differences between means were compared with an ANOVA followed by a Newman Keuls′ test.

The three flavonoids induced a concentration-dependent relaxation in all vessels pre-contracted by NA (Table 1). There was a good inverse correlation between the potency (pIC_{50}) of the flavonoids vs the vessel diameter ($r^2 > 0.95$, $p < 0.02$ for the three drugs), indicating the selectivity towards the resistance arteries. The vasodilator responses of quercetin and isorhamnetin were not modified after removal of the endothelium in TA (pIC_{50} = 4.78 ± 0.08 and 4.54 ± 0.10, respectively) or MB (5.10 ± 0.14 and 5.81 ± 0.09, respectively). In the MB, quercetin- but not isorhamnetin-induced vasodilation was enhanced by 10^{-6}M indomethacin (pIC_{50} =5.80 ± 0.11, n = 6, $P < 0.05$), whereas the guanylate cyclase inhibitor ODQ (10^{-6}M), KCl (40 mM) or ouabain (10^{-3}M) had no effect.

In conclusion, quercetin metabolites produce endothelium-independent relaxation, isorhamnetin being more potent than the parent compound in the AA, IA and MB. The three flavonoids showed selectivity for the resistance vessels.

Table 1. Potency (pIC_{50}) of quercetin and its metabolites in isolated endothelium-intact vascular preparations.

	Quercetin	Isorhamnetin	Tamarixetin
TA	4.68 ± 0.08 (6)	4.61 ± 0.08 (6)	4.73 ± 0.11(6)
AA	4.70 ± 0.07 (7)	5.02 ± 0.10 (8)*	4.82 ± 0.05(4)
MA	4.92 ± 0.10 (7)	4.97 ± 0.08 (6)	nd
IA	4.98 ± 0.06 (7)	5.27 ± 0.05 (8)*	5.16 ± 0.07(4)
MB	5.35 ± 0.15 (6)	5.89 ± 0.11 (5)*	5.34 ± 0.10(4)

Means ± SEM (n). nd = not determined. * $P < 0.05$ *vs* quercetin.

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10P A STUDY OF THE EFFECT OF METHYL-β-CYCLODEXTRIN ON CHOLESTEROL CONTENT AND VASCULAR RESPONSES OF THE PORCINE ISOLATED CORONARY ARTERY

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Cholesterol is an essential constituent of the plasma membranes and has been shown to exert important modulatory function on responses mediated by G protein-coupled receptors (Gimpl *et al.*, 1997). In human coronary arteries ß-adrenoceptor-mediated relaxation is reduced in atherosclerotic vessels, suggesting that elevated smooth muscle cholesterol selectively impairs receptor function (Berkenboom *et al*, 1989). We have examined the effect of short-term exposure to methyl-ß-cyclodextrin (MßCD), a sequestrator of cholesterol (Gimpl *et al.*, 1997), on isoprenaline- and forskolin-mediated relaxations of the porcine isolated coronary artery.

Segments (4 mm wide) of the porcine isolated coronary artery were prepared for isometric tension recording as previously described (Lawrence *et al*, 1999), following overnight storage at 37°C in Krebs-Henseleit solution (containing an antibiotic, antimycotic mixture) in the presence or absence of 3mM MßCD. Following the attainment of reproducible responses to 60mM KCl, preparations were exposed to increasing concentrations of either KCl or the thromboxane-mimetic U46619. In a seperate series of experiments, vasoconstrictor tone was induced by U46619 (approximately 60% of the response to 60mM KCl) and the effect of isoprenaline (1nM-1μM) and forskolin (10nM-1μM) examined. With the exception of responses to KCl, the potency of each agent was determined as the negative logarithm of the concentration producing 50% of the maximum response (-log EC_{50}). At the end of the study each segment was blotted, weighed and frozen at -20°C prior to lipid extraction and enzymic determination of cholesterol content. Differences between mean values were compared by Students' test ($p<0.05$).

Overnight exposure to 3mM MßCD significantly ($p<0.0001$) reduced the cholesterol content of segments from 1.28±0.04 to 0.63±0.02 $\mu g mg^{-1}$, n=24), but contractions to 60mM KCl in control (9.03±0.7 g, n=19) and MßCD-treated (8.93±0.86 g, n=19) segments were not different. As shown in Table 1, while the potency of KCl was not affected by pre-treatment with MßCD, the concentration response curve for U46619 was shifted rightward (4-fold) with no change in the maximum response. Forskolin and isoprenaline caused concentration-dependent inhibition of U46619-induced contractions (to 100%). While the effect of forskolin was unaltered by MßCD, the potency of isoprenaline was increased 3-fold (Table 1).

Table 1: Mean EC_{50} and -log EC_{50} values for contractile and relaxant agents in the porcine isolated coronary artery (n=8-10)

	KCl (mM)	U46619	Forskolin	Isoprenaline
Control	29.4±1.7	7.72±0.08	7.11±0.16	7.33±0.16
MßCD	26.6±1.4	7.10±0.06*	7.06±0.16	7.88±0.07*

* - statistically significant difference ($p<0.05$) from control.

The results of this study show that overnight pre-treatment with MßCD can significantly reduce the cholesterol content of isolated coronary arteries. The finding that isoprenaline-induced relaxations were enhanced by MßCD pretreatment, while responses to KCl and forskolin were unaffected, is consistent with observations in human coronary arteries (Berkenboom *et al.*, 1989) and a modulatory effect of cholesterol on plasma membrane G protein function (Gimpl *et al.*, 1997).

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11P α_2–ADRENOCEPTOR-MEDIATED VASOCONSTRICTION: INVOLVEMENT OF MAP KINASE SIGNAL TRANSDUCTION CASCADE AND CALCIUM INFLUX

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Introduced by D. A. Kendall.

Direct α2-adrenoceptor-induced vasoconstriction is mediated through an unknown signal transduction pathway which is independent of a decrease in cyclic AMP levels (an adenylyl cyclase-independent pathway) (Wright *et al.*, 1995; Roberts *et al.*, 1998). Recently, the MAP kinase signal transduction cascade has been shown to mediate vasoconstriction (Florian & Watts, 1998). Therefore, the aim of this present study was to determine whether this signalling pathway is involved in α2-adrenoceptor-mediated, adenylyl cyclase-independent vasoconstriction.

Porcine palmar lateral veins were dissected into 5 mm segments, and mounted in a tissue bath containing Krebs-Henseleit buffer maintained at 37°C, and gassed with 95% O_2/ 5% CO_2. Contractions were measured using a force transducer linked to a MacLab data acquisition system. After reproducible responses to 60 mM KCl were obtained, cumulative concentration-response curves to the selective α2-adrenoceptor agonist UK14304 were obtained 1 h after the addition of various inhibitors, or vehicle control (0.1 % DMSO for PD98059, genistein and daidzein; 0.1% ethanol for nifedipine).

Both the MAP kinase kinase inhibitor PD98059 and the L-type calcium channel blocker nifedipine caused concentration-dependent reductions in the α2-adrenoceptor-mediated vasoconstriction (figure 1). Removal of extracellular calcium virtually abolished the contraction (figure 1). The protein tyrosine kinase inhibitor genistein (10 μM) also reduced the maximum response to UK14304 (33.0 ± 6.8 % of the 60 mM KCl response compared to 77.0 ± 7.8 % (vehicle control), n=9). However, there was also a significant inhibition in the presence of the inactive analogue of genistein, daidzein (10 μM; 49.1 ± 7.3 % of the 60 mM KCl response, n=9). These data suggest that both the MAP kinase signal transduction cascade, and influx of extracellular calcium are major mediators of α2-adrenoceptor-induced vasoconstriction.

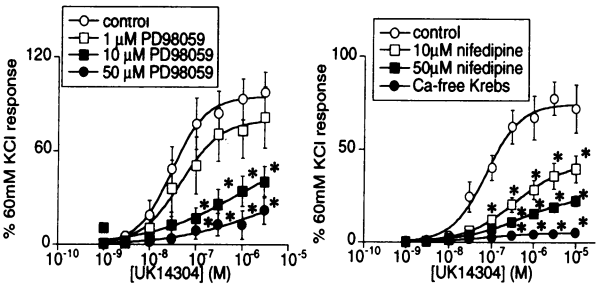

Fig. 1. Effect of various inhibitors on UK14304 responses in the porcine palmar lateral vein. Contractions to UK14304 are expressed as a % of the response to 60 mM KCl (mean ± s. e. mean, n=6-8). * indicates significant difference ($p<0.05$) from control values, ANOVA followed by a Bonferroni test.

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Supported by the Wellcome Trust

12P INFUSION OF β_2–ADRENOCEPTOR ANTAGONISTS AND INVERSE AGONISTS RESTORES HISTAMINE RESPONSES IN TRANSGENIC MICE WITH CARDIAC OVEREXPRESSION OF THE β_2–ADRENOCEPTOR

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Antagonists and inverse agonists can produce different cellular responses. The effects of three ligands, alprenolol, carvedilol and ICI-118,551 were examined in left atria and homogenized heart cytosolic fractions from wild type (WT) and transgenic (TG35) mice with cardiac-specific overexpression of the human β_2-adrenoceptor (β_2-AR) (Milano *et al.*, 1994). Specifically, we examined the ability of these three ligands to cross-regulate another cardiac G_s-coupled receptor, the histamine receptor. These ligands have been shown to vary in their negative efficacy at the human β_2-AR; two behaving as inverse agonists, carvedilol and ICI-118,551, and one as an antagonist, alprenolol (Nagaraja *et al.*, 1999; Bond *et al.*, 1995). The TG35 mice exhibit signs of chronic cardiac ß-AR activation. For example, TG35 mice have elevated G_i protein and G protein receptor kinase-2 protein levels (Nagaraja *et al.*, 1999). These proteins are elevated in other models of chronic ß-AR activation, such as congestive heart failure (CHF).

In 2-4 month old, TG35 and litter mate non-transgenic (WT) mice, drug treatments were performed using subcutaneous osmotic minipumps for 14-day infusion: alprenolol (ALP), 1.2 $mgkg^{-1}h^{-1}$; carvedilol (CARV), 0.4 $mgkg^{-1}h^{-1}$; and ICI-118,551 (ICI), 0.7 $mgkg^{-1}h^{-1}$. The mice were killed during the last hour of pump infusion and left atria were isolated for isometric tension recordings as described by Bond *et al.*, 1995. Sodium pentobarbital (40 $mgkg^{-1}$) was used as an anaesthetic. The right atria and ventricles were homogenized and cytosolic fractions were used to determine protein kinase A (PKA) activity using a colometric assay kit (Pierce Inc., Rockford, IL).

The atria from TG35 mice exhibited a significantly impaired inotropic response to histamine relative to responses in atria from WT mice (Figure 1). Cardiac tissue from the TG35 mice exhibited significantly elevated levels of PKA activity relative to levels in WT mice (Figure 2). Fourteen-day infusions of the three ligands partially restored the impaired histamine response (Figure 1) and significantly lowered the elevated levels of PKA activity of the TG35 hearts to control levels (Figure 2). Data are expressed as mean ± s.e.mean.

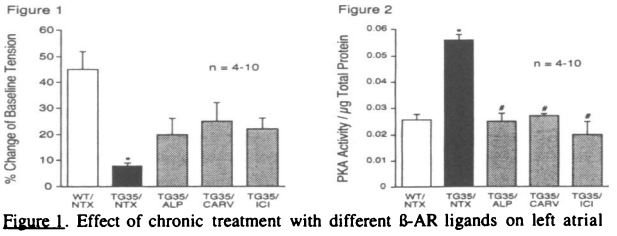

Figure 1. Effect of chronic treatment with different ß-AR ligands on left atrial isometric tension in TG35 mice. Figure 2. Effect of chronic treatment (14-day infusion) with different ß-AR ligands on PKA activity in TG35 mice. *$p < 0.05$ compared to WT/NTX (ANOVA); #$p < 0.05$ compared to TG35/NTX (ANOVA)

We conclude that restoration of the impaired histamine responses in the atria from TG35 mice can be observed following 14-day infusions of both, an antagonist and inverse agonists. The reversal of the effects of the transgene by both inverse agonists and an antagonist suggest that agonist occupancy, and not spontaneous activity, of the β_2-AR is producing the impaired histamine response and the elevated PKA levels. This restoration of contractile responses through another G_s protein-coupled receptor(s) may partly explain why some 'ß-blockers' improve left ventricular ejection fraction in patients with CHF.

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13P CHRONIC SWIM TRAINING POTENTIATES ACETYLCHOLINE HAEMODYNAMIC RESPONSES IN NORMOTENSIVE RATS

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The beneficial effects of physical exercise training against cardiovascular complications have been documented, although the mechanisms are not well understood (Tanaka *et al.*, 1997). In the present study, we investigated the effect of chronic swiming on haemodynamic responses to vasoactive drugs in normotensive rats.

Male Sprague Dawley rats (10 weeks of age) were randomly divided into two groups: Swimmers (SW, 60 min day^{-1} for 4 weeks), Sedentary (SED). Twenty four hours following the last training, rats were aneasthetized (sodium pentobarbital 60 mg kg-^{1}, i.p.) and body temperature was maintained at 36-37°C. Following tracheostomy, two polyethylene cannula were inserted into the left carotid artery and femoral vein for blood pressure (BP, mmHg) measurement and drugs administration respectively. After midline abdominal incision, a 1RB flow probe was implanted around the distal abdominal aorta for hindquarter blood flow (HQBF, ml min^{-1}) measurement. One hour after completion of surgery, baseline cardiovascular variables and changes in BP and hindquarter conductance (HQC, ml min^{-1}mmHg^{-1}) in response to acetylcholine (ACh, 0.001 to 1 μg kg^{-1}), S-nitroso-N-acetyl-penicillamine (SNAP, 10 to 30 μg kg^{-1}) and N^{G}-nitro-L-arginine methyl ester (L-NAME, 1 to 30 mg kg^{-1}) were measured.

Results are expressed as mean ± s.e.m. (* P<0.05 vs SED; ANOVA and Scheffe test, n = 10/group).

Basal BP was not different between the two groups (100±4 and 105±4 in SED and SW respectively), however, resting heart rate (bpm) was significantly (P<0.05) lower in SW (309±8) than that in SED (341±9). Although HQBF was higher (p<0.05) in SW (18.8±1.7) than in SED (14.4±1.2), HQC was similar in both groups (SED: 0.17±0.02; SW: 0.19±0.02).

Table 1. Maximal peak responses to ACh (I), SNAP (II) and L-NAME (III). Data are mean ± s.e.m. (* P<0.05 vs SED; ANOVA and Scheffe test) change from baseline

	SED (n = 10)		SW (n = 10)	
	Δ BP	Δ HQC	Δ BP	Δ HQC
(I)	-13.4±3.6	+0.05±0.02	-33.8±4.9*	+0.18±0.05*
(II)	-54.2±6.1	+0.19±0.14	-52.7±5.8	+0.24±0.11
(III)	+48.3±5.6	-0.14±0.01	+67.7±8.9*	-0.20±0.02*

Endothelium-dependent ACh-induced hypotensive responses and hindquarters vasodilations were augmented in SW. Responses to the nitric oxide (NO) donor, SNAP were similar in both groups. NO synthesis inhibition with L-NAME induced pressor and constrictor responses that were augmented in SW.

Our results indicate that swim training improves endothelial function likely through an increase in NO release.

Supported by the Heart and Stroke Foundation of Canada.

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14P LACK OF TORSADE DE POINTES WITH TERFENADINE COMPARED TO CLOFILIUM IN AN *IN VIVO* MODEL

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There is increasing awareness that a variety of drugs can prolong the QT interval of the ECG and that this predisposes towards the development of torsade de pointes. However, it is difficult to predict which drugs will cause torsade de pointes based solely on the extent of QT prolongation (Hii *et al.*, 1992). We have modified an *in vivo* model originally described by Carlsson *et al.*,(1990) and have compared the proarrhythmic effects of the antihistamine terfenadine and the Class III antiarrhythmic clofilium.

Male New Zealand White rabbits (2.5 to 3.5 kg) were anaesthetized with sodium pentobarbitone (~30 mg kg^{-1} i.v.), and the trachea cannulated to permit ventilation with room air. A midline thoracotomy was performed and an incision made in the pericardium. Arterial blood pressure, heart rate, limb lead ECGs and bipolar endocardial and epicardial monophasic action potentials were recorded. Animals were assigned randomly to receive clofilium, (20, 60 and 200 nmol kg^{-1} min^{-1} n=8), terfenadine (75, 250 and 750 nmol kg^{-1} min^{-1} n=7), or vehicle (n=5). Each dose was infused i.v. for 19 min. Phenylephrine infusion (75 nmol kg^{-1} min^{-1}) commenced 5 min prior to infusion of each dose of the drug of interest and the rate of phenylephrine infusion was increased to 150, 225 and 300 nmol kg^{-1} min^{-1} after 15, 18 and 21 min. There was a 10 min drug free interval between each 24 min dosing cycle.

Some ventricular premature beats (VPBs) were observed with phenylephrine in the vehicle group, but no ventricular tachycardia (VT) or torsade de pointes was seen. Terfenadine and clofilium both prolonged the rate corrected QT (QTc) interval, but only clofilium induced monomorphic VT and torsade de pointes. The total number of VPBs was also significantly greater in the rabbits which received clofilium (Table 1).

Table 1. Incidence of VT and torsade de pointes (TdP), and mean ± s.e. mean values for the total number of VPBs during the whole experiment and the QTc intervals 10 min after commencing infusion of the second dose of drug.

	No. of VPBs	VT	TdP	QTc (ms)
Vehicle	215 ± 91	0/5	0/5	370 ± 17
Terfenadine	455 ± 184	0/7	0/7	454 ± 28*
Clofilium	1742 ± 44*	5/8#	6/8#	470 ± 16*

*P<0.05 Kruskal-Wallis test, #P<0.05 Fisher's exact test, compared with vehicle.

Although terfenadine did not cause torsade de pointes, cardiac contractile failure occurred with the highest dose in all of the rabbits. These data confirm the lack of relationship between the extent of QTc prolongation and the occurrence of torsade de pointes. In addition, the results also indicate that terfenadine is less proarrhythmic than clofilium and that clofilium is a useful drug to include as a positive control in future studies in this *in vivo* model of torsade de pointes.

Supported by the British Heart Foundation (PG/96100).

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15P NO EVIDENCE THAT SODIUM SALICYLATE INHIBITS LPS-INDUCED EXPRESSION OF COX-2 IN ANAESTHETISED RATS

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Although the anti-inflammatory properties of salicylates are well established their mechanism of action is still debated. In particular, sodium salicylate may inhibit prostaglandin production *in vivo* despite having only a weak inhibitory effect on cyclo-oxygenase (COX) *in vitro*. As an explanation for this discrepancy it has been proposed that salicylate exerts its anti-COX effect by decreasing transcription of mRNA for COX rather than by directly inhibiting COX activity (Xu *et al.*, 1999). Here we have tested this hypothesis using a model of LPS-induced COX-2 expression in the rat.

Male Wistar rats (220-250 g) were anaesthetised with Inactin® (120 mg kg^{-1}, i.p.), instrumented, and then injected (t = 0 h) with either saline (2 ml kg^{-1}, i.p.) or *E. coli* LPS (0127:B8; 6 mg kg^{-1}, i.p.). Rats in the LPS group were treated either at time t = -1 h or t = 4 h with vehicle (10% DMSO in saline) or sodium salicylate 20 or 120 mg kg^{-1}. For comparison, other LPS-treated rats were given diclofenac (3 mg kg^{-1}, i.p.) or the COX-2-selective inhibitor, DFP (10 mg kg^{-1}, i.p.) (Leblanc *et al.*, 1999), at t = 4 h. At t = 6 h all rats were injected with a bolus of arachidonic acid (AA, 3 mg kg^{-1} i.v.). Blood samples (300 µl) were taken via the carotid artery at t = 0, 2, 4 and 6 h, and 1 min after AA bolus (t = 6AA). The samples were centrifuged and the plasma separated and snap frozen. PGI_2 production was subsequently determined by the plasma concentrations of the stable breakdown product 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$).

At t = 6 h, 6-keto-$PGF_{1\alpha}$ levels were significantly higher in LPS-treated rats than controls (2± 0.3 vs. 0.9± 0.15 ng ml^{-1}, n=6-7). Injection of AA caused a 19.5-fold rise in circulating 6-keto-$PGF_{1\alpha}$ levels in LPS-treated animals, but of only 5.4-fold in vehicle treated animals. Administration of salicylate 20 or 120 mg kg^{-1} at t = -1 significantly inhibited the accumulation of 6-keto-$PGF_{1\alpha}$ up to t = 6 h but not the burst of PGI_2 production following injection of AA (Fig. 1). Sodium salicylate 20 or 120 mg kg^{-1} given at t = 4 h similarly failed to reduce the rise in 6-keto-$PGF_{1\alpha}$ plasma levels that followed AA injection. In contrast, diclofenac and DFP inhibited the rise in plasma 6-keto-$PGF_{1\alpha}$ following AA injection by 92.8± 2.6 % and 90.3± 5.5 %, respectively.

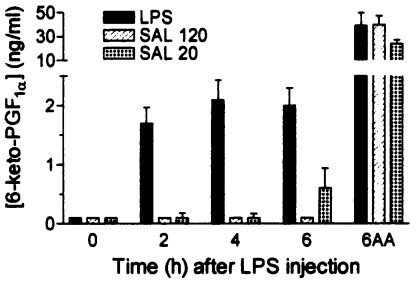

Figure 1. Effect of sodium salicylate 20 or 120 mg kg^{-1} on the accumulation (t = 0 to 6 h) and acute formation (t = 6AA) of 6-keto-$PGF_{1\alpha}$ in LPS treated rats.

In conclusion, our results show that sodium salicylate inhibited the rise in plasma 6-keto-$PGF_{1\alpha}$ following LPS treatment but not that following administration of exogenous AA. This observation supports the suggestion that salicylate causes only a weak inhibition of COX-2 that is readily overwhelmed by supply of substrate (AA) (Mitchell *et al.*, 1997) but not the suggestion that salicylate reduces the expression of COX-2. Thus, our findings do not support the hypothesis that a mechanism other than cyclo-oxygenase inhibition underlies the anti-COX properties of sodium salicylate.

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16P LIPOPOLYSACCHARIDE (LPS)-STIMULATED L-ARGININE UPTAKE IN RAT ALVEOLAR MACROPHAGES (AMΦ) IS DRIVEN BY iNOS-DEPENDENT L-ARGININE TURNOVER

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LPS-induced NO synthesis in rat AMΦ depends critically on the cellular uptake of L-arginine (L-Arg) (Hammermann *et al.*, 1998). Induction of iNOS is accompanied by an upregulation of L-Arg transport and an enhanced expression of the cationic amino acid transporter CAT-2B (Messeri Dreißig *et al.*, 2000).

Rat AMΦ (10^6 cells $well^{-1}$) were cultured for 20 h in DME-F12 medium containing 5% FCS in the absence or presence of LPS (Hey *et al.*, 1995). Thereafter, L-Arg uptake was studied by measuring the cellular radioactivity after 2 min of incubation with ^{3}H-L-Arg (37 kBq, 0.1 µM) (Racké *et al.*, 1998). In some experiments the incubation time was shortened to 1 min.

After culture in the absence or presence of LPS rat AMΦ incubated for 2 min with ^{3}H-L-Arg accumulated 6.8±0.8 and 16.5± ±1.7 pmol ^{3}H-L-Arg per mg protein, respectively (each n=25, means±s.e.mean of n experiments). When the iNOS inhibitor 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT up to 100 µM) had been present during the culture period together with LPS, ^{3}H-L-Arg uptake subsequently studied in the absence of AMT was reduced by maximally 77±2%. AMT alone present during the culture period caused a reduction of ^{3}H-L-Arg uptake maximally by 22±2%. iNOS inhibition did not affect LPS-induced increase in CAT-2B mRNA expression (determined by RT-PCR). AMT added to LPS-containing culture medium only for the last 30 min, caused a reduction of ^{3}H-L-Arg uptake (studied in the absence of AMT) maximally by 68±2% (EC_{50}: 0.6 µM). AMT present only during the acute uptake period only marginally affected ^{3}H-L-Arg uptake into AMΦ not stimulated by LPS. However, AMT present during the uptake period inhibited ^{3}H-L-Arg uptake into LPS-stimulated AMΦ, but its potency was significantly lower compared to the experiments in which AMΦ had been loaded with AMT by 30 min preincubation. When the incubation period with ^{3}H-L-Arg was shortened to 1 min the concentration inhibition curve of AMT was further shifted to the right and 100 µM AMT reduced ^{3}H-L-Arg uptake in LPS-stimulated AMΦ by only 33±9%. The NO releasing compound DETA-NONOate (0.1, 0.3 and 1 mM) present either 30 min prior and/or during the uptake period did not oppose the inhibitory effect of AMT.

In conclusion, the iNOS inhibitor AMT abrogates the LPS-induced activation of L-Arg transport, but affected only marginally basal L-Arg transport. The kinetics of this effect suggest an intracellular site of action of AMT. Therefore, inhibition of iNOS rather than interactions at the transporter site might be responsible for the reduction of LPS-stimulated L-Arg uptake. However, NO appears not to be an activator of L-Arg uptake. Therefore, L-Arg flow through the NOS pathway is considered as the driving force.

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17P REGULATION OF CYP2B1 EXPRESSION BY ENDOGENOUS NITRIC OXIDE

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Inflammatory stimuli cause down regulation of many forms of cytochrome P450 (P450) in whole animals or in cultured hepatocytes. Several studies have implicated NO, produced by the inducible form of NO synthase (NOS2), in the reduction of P450 mRNA and/or protein levels caused by inflammatory stimuli (Khatsenko & Kikkawa, 1997). On the other hand, studies in our laboratory have failed to detect any effects of inhibiting NO synthesis on down regulation of constitutively expressed P450s (Sewer *et al*, 1998). In the present study, we tested the hypothesis that NO is involved in the down regulation of phenobarbitone (PB)-inducible CYP2B1 in rat cultured hepatocytes.

Hepatocytes were prepared by *in situ* collagenase perfusion of the livers of male F344 rats (170-200 g), and cultured in 60-cm dishes coated with Matrigel. Cells were cultured in Waymouth's 752 medium for 48 h before the addition of 1 mM PB to the medium. After 48 h of PB treatment, the medium was replaced with medium containing PB, bacterial endotoxin (LPS), cytokines and/or drugs. Cells were harvested at various times thereafter (24 h unless otherwise specified). RNA was prepared by acid phenol extraction, and microsomes were prepared by differential centrifugation. The expression of CYP2B mRNA and proteins was assayed by Northern and Western blotting, respectively. RNA data were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA. All analyses were performed on groups of at least five independent samples. One-way analysis of variance and Newman-Keuls test were used to determine statistically significant differences between treatment groups.

CYP2B1 mRNA was maximally suppressed by 1 nM LPS after 24 hours of treatment, whereas 100 nM LPS was required to suppress CYP2B1 proteins or to induce NO production. CYP2B1 mRNA was down-regulated by 5 ng/ml interleukin-1 or tumour necrosis factor-α, but not by interleukin-6 or tumour growth factor-β (5 ng/ml). The NO synthase inhibitors aminoguanidine (9() μM) or N^w monomethyl arginine (NMA, 60-300 μM) failed to inhibit the down regulation of CYP2B1 mRNA by LPS, despite inhibiting NO production completely. In contrast, either NMA or aminoguanidine could prevent suppression of CYP2B protein measured 6 h or 24 h after LPS stimulation. The effect of NO synthase inhibition by NMA (60 μM) on CYP2B protein was reversed by the inclusion of 5-20 mM arginine in the incubation medium. The compound LY83583 (Calbiochem, 3 μM), which inhibits the induction of NOS2 by an unknown mechanism (Geng *et al*, 1998), also prevented the down regulation of CYP2B protein by either LPS or interleukin-1. Lastly, the NO donors Snitrosopenicillamine (0.5 mM) and S-nitrosoglutathione (50 μM) mimicked the down regulation of CYP2B1 protein by LPS.

We conclude that NO is not involved in the pretranslational down-regulation of CYP2B1 by LPS, but that it contributes to the decline in CYP2B proteins.

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18P LIPOPOLYSACCHARIDE-INDUCED AIRWAY HYPERRESPONSIVENESS (AHR) TO METHACHOLINE AND SUBSTANCE P IN GUINEA-PIGS: EFFECT OF ANTAGONISTS OF NK_1 AND NK_2 RECEPTORS

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Lipopolysaccharide (LPS) has been associated with AHR in both animals and man. In guinea pigs, capsaicin-sensitive nerves appear to be involved in the induction of AHR to histamine (Loeffler et al. 1997). We report here a pronounced AHR to methacholine and substance P induced by LPS in the guinea pig and an analysis, using selective antagonists, of the role of tachykinin receptors in this phenomenon.

Male Dunkin-Hartley guinea-pigs (550-650g) were anaesthetised (phenobarbital 100 $mgkg^{-1}$ i.p. plus pentobarbital 30 $mgkg^{-1}$ i.p., a regime sufficient to maintain full anaesthesia throughout the experiment), ventilated, immobilised (gallamine (10 $mgkg^{-1}$ i.m.) and cannulated for measurement of cardiovascular parameters (carotid artery) and drug administration (jugular vein). Airway resistance (R_L) was calculated from measurement of airflow and transpulmonary pressure by use of a digital electronic respiratory analyzer (LFR, Mumed Ltd., U.K.). Sensitivity of the airways to i.v. injections of methacholine (1, 3 and 10 μgkg^{-1}) and substance P (0.3, 1 and 3 μgkg^{-1}) were assessed before and 1 h after an i.v. injection of 1 $mgkg^{-1}$ LPS (from E.Coli 0111.B4; Sigma).

The results are summarised in Figure 1. LPS induced pronounced increases in the bronchoconstrictor responses to methacholine and substance P. No inhibition of the augmented response to methacholine, was observed when the animals were pretreated 30 min before with either the selective NK_1 receptor antagonist, NKP608 (Lewis et al. 2000), or the selective NK_2 receptor antagonist, SR 48968, (1 $mgkg^{-1}$ intraduodenally); however, when administered together, these agents induced complete inhibition of the AHR to methacholine. With respect to substance P, pretreatment with NKP608 reduced the augmented response following LPS to a level below that in the vehicle-treated controls. SR 48968 also significantly reduced the augmented response to substance P, although not to the same extent as NKP608.

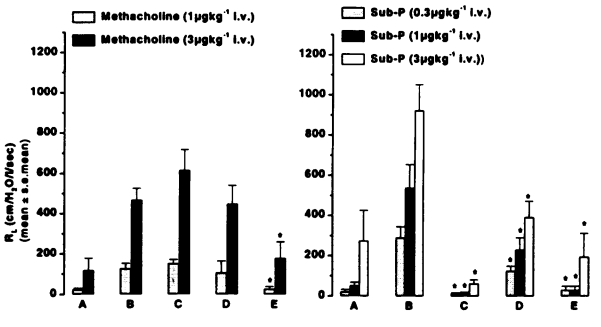

Figure 1 : The effects of tachykinin receptor blockade on LPS-induced AHR to methacholine and substance P in the guinea pig. Shown are the bronchoconstrictor responses to i.v. injections of methacholine (left graph) and substance P (right graph) 1 h after i.v. administration of saline (A) or LPS, 1 $mgkg^{-1}$ (B). NKP608 (C) SR 48968 (D) or a combination of the two (E) were administered intraduodenally at a dose of 1 $mgkg^{-1}$ 30 min prior to LPS. Groups A and B received the vehicle for the antagonists. Results are expressed as the mean ± s.e. mean of between 4 and 10 experiments. *$p<0.01$ that the value differs significantly from the equivalent value in group B.

Thus AHR to methacholine induced by LPS appears to involve endogenous tachykinins acting on both NK_1 and NK_2 receptors since antagonism at both sites is needed to inhibit the response. Potentiation of substance P may also reflect the consequence of endogenous tachykinin release since the augmented bronchoconstrictor response to this nominally selective NK_1 receptor agonist is blocked by SR 48968, a selective NK_2 receptor antagonist.

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19P INVOLVEMENT OF A_3 RECEPTORS IN ALLERGIC RESPONSES OF THE AIRWAYS IN CONSCIOUS, SENSITISED GUINEA-PIGS

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Adenosine promotes a wide variety of airways' responses relevant to asthma, high concentrations being detected after antigen challenge (Polosa and Holgate, 1997). Antigen challenge of sensitised animals releases mast cell-derived mediators resulting in early and late asthmatic responses, airway eosinophilia and hyperreactivity (Danahay & Broadley, 1997). We therefore investigated the effect of the A_1/A_2 adenosine receptor antagonists 8-phenyltheophylline (8-PT) or 8-sulphophenyltheophylline (8-SPT) or the A_3 receptor selective antagonist 9-chloro-2-(2-furanyl)-5-[9-(phenylacetyl)amino][1,2,4]-triazolo[1,5-c]quinazoline (MRS-1220) on the inflammatory events and hyperreactivity associated with asthmatic reactions.

Male Dunkin-Hartley guinea-pigs (250-300g) were sensitised to ovalbumin (OA, 10μg and 100mg $Al_2(OH)_3$ in 1ml normal saline i.p.) and experiments commenced 14 days later. Airway function was recorded in conscious guinea-pigs by whole body plethysmography and specific airway conductance (sGaw) (Danahay & Broadley, 1997). Guinea-pigs received 8-PT (10mg kg^{-1}), 8-SPT (60mg kg^{-1}), MRS-1220 (100μg kg^{-1}) or their corresponding vehicle 30min. before antigen (OA 100μg ml^{-1} in normal saline, 1h). sGaw was measured at intervals for 10 hours post-challenge. Airway hyperreactivity to inhaled histamine (nose-only, 1mM, 20s) was investigated, 24h before and after antigen challenge. Bronchoalveolar lavage was performed 24h after exposure to OA to determine total and differential cell counts (converted to Log_{10}). All values were represented as mean ±s.e.m. (n=6) and comparisons made by Student's paired or unpaired t-tests.

Exposure to OA typically produced an immediate early phase bronchoconstriction (45.7±13.5% reduction in sGaw) resolving to baseline by 6 hours and followed by a late phase bronchoconstriction (18.1±3.3% reduction in sGaw) between 6 and 10 hours. Neither 8-PT nor 8-SPT at doses which significantly inhibited the bronchoconstriction of 5'-adenosine monophosphate (5'-AMP, 3mM) attenuated the early (60.0±13.1%, 50.8±10.3%) or late phases (18.5±3.3%, 14.2±5.9% reduction in sGaw respectively). MRS-1220 significantly reduced the early phase response, the reduction in sGaw at 30 min (9.6±7.9%) being significantly less than vehicle-treated controls (35.3±5.7%) (P<0.05). The late phase was not inhibited by MRS-1220 (13.0±2.5%) compared to the controls (20.1±4.1%). Histamine challenge 24h before antigen exposure in the three vehicle-treated groups produced no significant bronchoconstriction, but 24h after antigen, significant bronchoconstrictions were seen (17.7±7.5%, 29.1±9.4% and 18.2±6.3% reduction in sGaw, respectively) indicating the development of hyperreactivity. MRS-1220 abolished hyper-reactivity to histamine, the reduction in sGaw before (3.0±3.9%) and after OA (1.5±5.7%) not differing significantly (P>0.05). However 8-PT and 8-SPT had no significant protective effect against hyperreactivity. Bronchoalveolar lavage revealed an increase in total cells ($6.2\pm0.4\times10^6$), macrophages ($3.6\pm0.2\times10^6$) and eosinophils ($2.5\pm0.3\times10^6 ml^{-1}$) in the antigen challenged vehicle-treated animals. MRS-1220 significantly reduced the total influx of inflammatory cells ($3.4\pm0.2\times10^6$), macrophages ($2.3\pm0.1\times10^6$) and eosinophils ($1.0\pm0.1\times10^6 ml^{-1}$) (P<0.05), whereas 8-PT and 8-SPT had no effect.

These results indicate A_3 receptor involvement in the early phase response, airway hyperreactivity and influx of inflammatory cells following antigen challenge. Thus adenosine released during the early anaphylactic response, contributes to the inflammatory reaction raising the possibility of A_3 receptor antagonists having a therapeutic application in allergic asthma.

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20P PAR2-MEDIATED INHIBITION OF ELECTROLYTE TRANSPORT IN HUMAN BRONCHIAL EPITHELIAL CELLS

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Protease activated receptor 2 (PAR2) has recently been identified in human bronchial epithelium with a proposed 'protective' bronchodilator role (Cocks *et al.*, 1999). This study was undertaken to determine whether PAR2 activation affected ion-transport processes in cultures of differentiated human bronchial epithelial cells (HBECs) as has been described in other epithelia (Nguyen *et al.*, 1999; Danahay *et al.*, 2000).

HBECs (Clonetics) were cultured on Snapwell permeable supports for 21 days, the final 14 days at an apical air interface. These conditions provided a differentiated transporting epithelial structure. Epithelia were placed in Ussing chambers bathed in Ringer solution containing (mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose (37°C, 5%CO_2:O_2) and voltage clamped at 0V. Changes in the short circuit current (I_{sc}) and transepithelial resistance (R_T) induced by activators of PARs were recorded in addition to responses to apically applied (AP) amiloride (10μM) and AP + basolaterally applied (BL) forskolin (FSK 2μM). Data are expressed as absolute changes (mean±s.e.mean) and significance assumed when P<0.05 (Student t-test).

HBECs displayed a basal I_{sc} of $10.1\pm0.4\mu Acm^{-2}$ and transepithelial resistance (R_T) of $1234\pm73\Omega.cm^2$ (n=12). The basal current was amiloride-sensitive (max 82.1±2.8% inhibition, IC_{50}=0.2±0.01μM). The addition of porcine trypsin (0.03-1.0μM,BL) induced a transient increase in I_{sc} reaching a maximum of $7.0\pm0.9\mu Acm^{-2}$ (P<0.001) at 1μM. There then followed a sustained reduction in basal I_{sc}, reaching a maximal decrease of $4.2\pm0.2\mu Acm^{-2}$ (P<0.001) at 1μM.

The sustained attenuation of I_{sc} was associated with a 63±6% increase in R_T (n=4). The residual amiloride-sensitive and FSK-induced currents were also significantly attenuated by 44.7±9.2% (P<0.05,n=4) and 71.2±3.4% (P<0.001,n=4) respectively. These effects were not observed after the AP addition of trypsin or BL thrombin ($5UmL^{-1}$) (data not shown). The addition of the PAR2 activating peptide, SLIGRL-NH_2 (30μM,BL,n=4) mimicked the effects of trypsin, inducing a small, transient increase in I_{sc} ($1.4\pm0.5\mu Acm^{-2}$, P<0.05) followed by a sustained attenuation of basal I_{sc} of $3.1\pm0.2\mu Acm^{-2}$ (P<0.001). The amiloride sensitive and forskolin-induced currents were also attenuated (56.2±5.1% and 51.8±2.0% inhibition respectively, P<0.01). The PAR1 activating peptide, TFRIFD-NH_2 (100μM,BL,n=4) was without effect (data not shown).

This study demonstrates that PAR2 activation of HBECs occurs only via the basolateral surface. The direct response was bi-phasic initially inducing a transient increase in I_{sc} followed by a sustained period of inhibition of I_{sc}. The subsequent amiloride-sensitive current (sodium reabsorption) was also attenuated together with the FSK-induced current. Inhibition of sodium absorption by the airway epithelium has been demonstrated to increase mucociliary clearance (Knowles *et al.*, 1996). Whether PAR2-mediated inhibition of these currents is physologically relevant to mucociliary clearance remains to be determined.

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21P EFFECT OF THE IMIDAZOLINE$_2$ (I_2) SITE-SELECTIVE LIGAND BU224 ON *IN VIVO* NORADRENALINE RELEASE AND PLASMA CORTICOSTERONE IN CONTROL AND CHRONICALLY STRESSED RATS

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Central noradrenaline (NA) regulates the hypothalamo-pituitary-adrenal (HPA) axis (Plotsky, 1987). Acute administration of the I_2 site selective ligand, BU224, increases extracellular NA in the rat brain (Hudson *et al.*, 1999) and elevates plasma corticosterone (CORT) in naive rats (Finn *et al.*, 1999). We have studied simultaneously, the effect of BU224 on NA release in the hypothalamic paraventricular nucleus (PVN) and on CORT in chronically stressed rats with adjuvant-induced arthritis (AA).

Male PVG rats (250-270g) were injected i.d. with 0.15 ml of *Mycobacterium butyricum* suspension in paraffin oil (10 mg ml^{-1}) into the tail base or vehicle alone. 13 days later rats were implanted with a microdialysis probe (1 mm membrane length; m.wt. cutoff 40,000) in the right PVN (AP: –2.6 lat: - 1.1, depth -8.4 mm relative to bregma and dura, probe at a 5° angle) under sodium pentobarbital anaesthesia (48 mg kg^{-1}, i.p.) and cannulated through the right jugular vein. An i.p. cannula was also implanted. 24 hrs later basal dialysate (30 µl) NA and blood (40 µl) CORT levels were established by HPLC-ECD and radioimmunoassay (Harbuz *et al.*, 1994) respectively. Rats were then given an i.p. injection of either saline vehicle (control) or BU224 (10 mg kg^{-1}). Sampling continued every 20 min for 180 min post injection.

Basal levels of CORT were significantly higher ($P < 0.05$, t-test) in AA rats (239 ± 51 ng ml^{-1}) compared to non-AA controls (100 ± 11 ng ml^{-1}). In both control and AA rats, BU224 administration resulted in a significant elevation in PVN NA release (Table 1) and CORT (Table 2) 20 and 40 min post injection (repeated measures anova followed by Dunnet's test to compare with pre-injection basal levels). Saline injections were without effect. There was a significant correlation between NA release in the PVN and CORT levels ($P < 0.001$, $r^2 = 0.8412$ for con + BU224 group). At 60 min, chronically stressed AA rats had a greater NA response to BU224 (change from basal = 21.6 ± 3.9 fmol) compared to non-AA controls (change from basal = 9.3 ± 3 fmol).

Table 1. NA release in the PVN (fmol/30 µl sample)

Time (min)	0 (Basal)	20	40	60
Con sal (6)	9.1 ± 1.8	8.2 ± 1.7	8.4 ± 1.7	8.6 ± 1.7
Con BU224 (4)	7.5 ± 1.4	31.6 ± 5.9**	35.1 ± 11**	16.8 ± 3.6
AA sal (5)	21.3 ± 6.8	18.5 ± 8.7	14.1 ± 5.1	19.9 ± 5.7
AA BU224 (5)	11.4 ± 1.9	42.7 ± 10+	50 ± 13++	33 ± 3.4$

Table 2. Plasma CORT (ng ml^{-1})

Time (min)	0 (Basal)	20	40	60
Con sal (6)	98 ± 14	131 ± 24	94 ± 16	76 ± 19
Con BU224 (6)	111 ± 27	859 ± 229**	538 ± 148*	373 ± 141
AA sal (8)	169 ± 67	255 ± 73	299 ± 114	331 ± 155
AA BU224 (6)	297 ± 88	811 ± 237+	974 ± 277++	542 ± 153

Tables 1 and 2. Values as mean ± s.e.mean (*n* numbers in brackets). $^{**}P < 0.01$ compared to basal Con BU224 levels; $^{+}P < 0.05$, $^{++}P < 0.01$ compared to basal AA BU224 levels; $^{\$}P < 0.05$ comparing AA BU224 Vs Con BU224 group at 60 min.

In addition, the BU224-induced elevation of NA in the AA group was more prolonged compared to the control group (20-180 min cumulative NA release = 161.1 ± 25 fmol for AA and 62.4 ± 12.8 fmol for controls, $P < 0.05$, t-test). These data are further evidence for I_2 binding sites affecting central noradrenergic and HPA function. In addition the NA response to BU224 in chronically stressed AA rats was enhanced relative to that of control animals.

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22P THE EFFECT OF LISINOPRIL ON THE HEALING OF EXCISIONAL SKIN WOUNDS OF THE RAT

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Attempts to modulate the formation of scar tissue have been the subject of much work (Sun *et al*, 1997). This communication studies lisinopril on its ability to alter wound contraction, myofibroblast activity and collagen deposition.

20 female Hooded Lister rats (160-198g) were randomly allocated to 1 of 4 groups (n = 5). All were dosed by gavage for 2 days prior to wounding using lisinopril suspended in 3% methyl cellulose at 3, 5 and 10 mgkg^{-1}day^{-1}. Controls received equivalent doses of methylcellulose. Dosing continued daily throughout the experiment. 14mm^2 full thickness squares of skin were excised from the left hindquarter using a template, under isolflurane anaesthesia. Wound areas were measured daily on conscious animals kept under minimal restraint. The resulting tracings (n = 10/wound/day) were quantified and the coefficient of the wound contraction rates calculated (Cross *et al.*, 1995).

The wounds were harvested on day 12 and the tissue processed and stained with Herovici's stain for collagen and α-smooth muscle actin to identify myofibroblasts. The size and frequency of blood vessels were assessed by randomly placing a 5 by 5 cm grid over the photographic montage of the wounds at 80x magnification. Statistical analysis was performed using the Mann-Whitney U-test.

Wound contraction slowed in a dose dependent manner, the 10mg dose being significantly lower than that of the controls (Table 1). A similar dose dependent reduction in both vessel cross sectional area and vessel number was also observed (Table 1). Observationally, collagen staining appeared more haphazard in the 5 and 10mg^{-1}kg^{-1} dosed animals; similarly there appeared to be an increase in the population density of myofibroblasts as indicated by the immunostaining technique.

	Wound coefficient of contraction rate		Cross sectional area (mm^2)	Vessel number
	Median	Range		
Control	-0.055	(-0.01- -0.070)	8.2	50
3mg	-0.071	(-0.01- -0.080)	4.5*	35*
5mg	-0.065	(-0.01- -0.080)	1.7*	24*
10mg	-0.074	(-0.07- -0.076)*	0.7*	17*

Table 1. The median and range of the wound contraction coefficients, cross sectional area of vessels (mm^2) and number of vessels in a 5 x 5 cm area of photographs between control and treated groups. *P<0.05 versus control.

The effects of ACE inhibition on cardiac healing are well established (Sun *et al.*, 1997). Angiotensin receptors have been identified on cardiac myofibroblasts and when blocked reduce collagen deposition (Sun *et al.*, 1997). They have also been identified on myofibroblasts and endothelial tissue in the skin pouch model (Zhang *et al.*, 1999). Receptor numbers increase in wounded skin, though their precise location is unknown (Akibo *et al.*, 1996).

Our results suggest that myofibroblast behaviour is altered by lisinopril, appearing to slow collagen deposition and wound contraction, complementing *in vitro* studies which suggest angiotensin II promotes contractility and collagen deposition (Campbell *et al.*, 1997). Lisinopril also appears to disrupt the formation of new blood vessels within the wound, a previously unreported effect. Modulation of angiogenesis in this way gives rise to the prospect of the modulation of angiogenesis dependent diseases.

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Zhang, J. *et al.* (1999) *J. Mol. Cell Cardiol.* 31, 1005-13.

23P EVIDENCE FOR HETEROGENEITY OF CGRP-LIKE RECEPTORS IN RAT VAS DEFERENS

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The existence of two CGRP receptor subtypes has been proposed on the basis of differential antagonistic affinity and agonistic potencies in peripheral preparations. The CGRP antagonist CGRP(8-37) is considered to possess higher affinity for CGRP1 receptors found in the atrium, whereas the agonist [Cys(Et]^{2,7}]hCGRPα is more potent for the CGRP2 receptor in vas deferens (Dumont *et al.*, 1997). In the present study we evaluated the interaction between the novel, potent and selective CGRP antagonist BIBN4096BS (Doods *et al.*, 2000) and the agonists h-αCGRP and [Cys(Et)^{2,7}]hCGRPα for CGRP1 and CGRP2 receptors in rat atrium and vas deferens respectively.

Methods:

Male Wistar rats were exsanguinated under sodium pentobarbitone anaesthesia. The hearts and vas deferens were dissected and immediately placed in oxygenated Krebs buffer. The left atria and vas deferens were mounted in 25ml organ baths containing Krebs solution of the following composition (mmol/L): NaCl 118; KCl 4.7; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; glucose 10; $CaCl_2$ 2.5. The Krebs solution was gassed with 95% O_2 + 5% CO_2 and maintained at 37°C. A resting tension of 1g was applied. Following a 60 min equilibration period, the atria were electrically driven at a frequency of 3 Hz (duration: 0.5 ms; voltage: initial value + 20%) and the vas deferens were stimulated at a frequency of 0.2 Hz (duration: 0.5 ms; voltage: 60v). Following a 30 min period of electrical stimulation, concentration-effect curves to CGRP agonists were obtained in a cumulative fashion. For experiments using the antagonists, the tissues were incubated for 15 min with antagonist prior to the construction of the concentration-effect curve for CGRP agonists. Only one concentration-effect curve was made in each preparation.

h-αCGRP and [Cys(Et)^{2,7}]hCGRPα produced concentration dependent positive inotropic effects in rat left atrium and inhibited the electrically-evoked twitch response in rat vas deferens. The pD_2 values are listed in table 1.

BIBN4096BS (1μM) induced a parallel rightward shift of the agonist dose-response curves. The antagonist was approximately 10-fold more potent in blocking h-αCGRP mediated responses in atrium compared to the vas deferens. BIBN4096 did not discriminate between the effects of [Cys(Et)^{2,7}]hCGRPα or h-αCGRP in rat atrium. However, interestingly, BIBN4096 was more potent in antagonizing [Cys(Et)^{2,7}]hCGRPα than h-αCGRP responses in rat vas deferens (table 1).

Table 1

	agonist	pD_2* agonist	pK_b* BIBN4096BS
Atrium	h-αCGRP	8.79 ± 0.07	8.5 ± 0.05
	[Cys(Et)^{2,7}]hCGRP	8.26 ± 0.07	8.8 ± 0.07
Vas deferens	h-αCGRP	8.66 ± 0.08	7.1 ± 0.04
	[Cys(Et)^{2,7}]hCGRP	8.20 ± 0.03	8.4 ± 0.053

* mean ± s.e.m; n=4

These findings suggest that rat vas deferens contains besides the CGRP2 receptor an additional receptor which is stimulated by [Cys(Et)^{2,7}]hCGRPα and for which the novel antagonist BIBN4096BS exhibits a high affinity.

Doods, H. *et al.* (2000) *Br. J. Pharmacol.* 129, 420-423.
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24P EFFECTS OF THE CGRP ANTAGONIST BIBN4096BS ON NEUROGENIC VASODILATION IN ANAESTHETISED RATS

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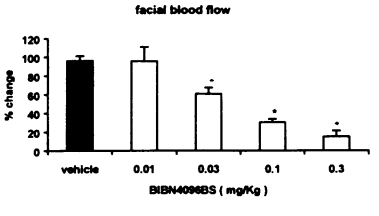
We recently reported that BIBN4096BS is a potent and selective CGRP antagonist (Doods *et al.*, 2000). This compound possesses high affinities for both the human and rat CGRP receptor. The affinity (ki) for the human CGRP receptor on SK-N-MC cells was 14.4 pM and for CGRP binding sites in rat spleen 3.4 nM. The present study investigated the effects of BIBN4096BS on neurogenic vasodilation in anesthetized rats.

Male Wistar rats (Chbb: Thom 280-320) were anaesthetized with sodium pentobarbitone. The trachea was cannulated and the animals were artificially respired. The left femoral artery and right femoral vein were cannulated for the continuous measurement of arterial blood pressure and intravenous administration of test agents, respectively. Heart rate was derived from the blood pressure signal. The rat was placed in a stereotaxic frame and a longitudinal incision was made in the scalp. One burr hole was drilled in the skull and a bipolar electrode was lowered, using a micromanipulator, into the trigeminal ganglion. The trigeminal ganglion was stimulated at 10 Hz, 1ms, 1mA, for 30 seconds. Microvascular blood flow changes in the facial skin were measured by Laser Doppler flowmeter. All the parameters were recorded on a 8 channel polygraph. The animals were subjected to three periods of electrical stimulation, separated by 30 min intervals. The first stimulation served as a control for the second and third stimulations. Test agents or vehicles were given intravenously 5 min prior to the second and third stimulations. Two different doses of the test compound were administered to each animal. Blood pressure and facial skin blood flow were measured continuously throughout the experiment. Radioimmunoassay was used for the measurement of CGRP levels in the external jugular vein blood samples. All values are the mean ± s.e.mean, n=6-8, $p<0.05$ are considered to be significant (ANOVA).

Electrical stimulation of the trigeminal ganglion caused a rise in facial skin blood flow ipsilateral to the side of stimulation, and a slight and more transient increase in blood flow of the contralateral facial skin. There was a significant increase in CGRP plasma levels in the external jugular vein blood samples following trigeminal ganglion stimulation, from 65 ± 7.93 to 151 ± 17.65 pg/ml ($p<0.05$).Administration of BIBN4096BS had no effect on blood pressure and resting facial blood flow values. BIBN4096BS dose-dependently inhibited the increase in facial blood flow provoked by stimulation of the trigeminal ganglion (figure). The ID50 value of the inhibitory effect was 0.052 mg/kg.



The present study confirms that CGRP release plays an important role in rat facial skin vasodilation elicited by electrical stimulation of the trigeminal ganglion. Migraine headache is believed to be associated with dilation of cranial vessels and activation of the trigemino-vascular system with consequently CGRP release (Goadsby *et al.*, 1991). Accordingly, CGRP-antagonists could be considered as a novel approach for the treatment of migraine.

Doods, H. *et al.* (2000) *Br. J. Pharmacol.* 129,420-423.
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25P RANOLAZINE REDUCES MYOCARDIAL INFARCT SIZE AND CARDIAC TROPONIN T RELEASE IN THE ANAESTHETISED RAT

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There is good evidence that ranolazine (Ran) exerts beneficial effects in animal models of experimental myocardial ischaemia and in patients with angina pectoris (Alley & Alps, 1990; Cocco *et al.*, 1992). Ranolazine modulates the metabolism of ischaemic myocytes by increasing activation of pyruvate dehydrogenase to promote glucose oxidation (Clarke *et al.*, 1993). Ranolazine is thought to switch substrate utilisation from fatty acids to glucose. This metabolic switch increases adenosine triphosphate production per mole of oxygen consumed, reduces the rise in lactic acid, and maintains myocardial function under conditions of reduced myocardial oxygen delivery (Wyatt et al., 1995). This mechanism of action of ranolazine may explain its anti-ischaemic (in the absence of any haemodynamic) effects in human and animal models. The aim of this study was to investigate whether ranolazine exerts beneficial effects in a rat model of regional myocardial ischaemia and reperfusion.

Male Wistar rats (240-350 g) were 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 was cannulated to measure mean arterial blood pressure (MAP) and the jugular vein was cannulated for the administration of drugs. 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). The animals were allowed to stabilise for 30 min and subsequently the LAD was occluded for 15 or 25 min and then reperfused for 2 h. At the end of the experiment, 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. Infarct size (IS) was determined by incubation of the slices of the heart with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1}). The plasma levels of cardiac Troponin T (cTnT) were determined by the short-turn-around-time assay (Boehringer Mannheim) using an Elecixs® System 2010 (Zacharowski *et al.*, 1999). The following 6 groups were studied (Table 1). Saline (bolus: 2.4 ml kg^{-1} & infusion: 2.4 ml kg^{-1} h^{-1}) or ranolazine (bolus: 10 mg kg^{-1} & infusion: 9.6 mg kg^{-1} h^{-1}) were infused 30 min prior to LAD-occlusion and throughout the experiment.

The mean AR were similar in all groups studied. When compared to vehicle (25 min study), ranolazine caused a significant reduction in IS and cTnT release (Table 1). Haemodynamic data: At baseline and throughout the experiment, heart rate was significantly reduced in the ranolazine group (25 min) when compared to control (data not shown).

Table 1: Area at risk, infarct size and cTnT data (* $P<0.05$ vs. saline, ANOVA followed by Bonferroni's test). Sham=no LAD-occlusion.

15 min LAD-occlusion				25 min LAD-occlusion			
Groups (n=)	**AR (%)**	**IS (%)**	**cTnT (ng ml^{-1})**	**Groups (n=)**	**AR (%)**	**IS (%)**	**cTnT (ng ml^{-1})**
sham (4)	52±3	<3*	<0.1*	**sham (4)**	47±3	<3*	<0.1*
saline (8)	49±3	45±7	18±5	**saline (12)**	48±2	61±2	65±14
ran (8)	48±1	39±5	15±7	**ran (12)**	48±1	41±5*	12±2*

This study demonstrates for the first time that ranolazine significantly reduces (1) infacrt size and (2) cTnT release in rats subjected to LAD occlusion and reperfusion.

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26P PHARMACOLOGICAL PRECONDITIONING OF THE RAT HEART WITH LIPOTEICHOIC ACID OR ENDOTOXIN IS NOT ABOLISHED BY A K_{ATP} CHANNEL INHIBITOR

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Pretreatment of rats with a low dose of endotoxin (lipopolysaccharide, LPS) or lipoteichoic acid (LTA) for 16 h protects the heart against ischaemia-reperfusion injury (Zacharowski *et al.*, 1999 & 2000). This phenomenon is referred to as a pharmacological-induced delayed preconditioning (Baxter *et al.*, 1996). There is evidence that the activation of K_{ATP} channels contributes to the cardioprotective effects of delayed preconditioning (Takashi *et al.*, 1999). Numerous pharmacological agents such as adenosine and monophosphoryl lipid A appear to protect the myocardium via a K_{ATP} channel-dependent signalling pathway (Baxter & Yellon, 1999). This study was designed to elucidate whether the cardioprotective effects of two bacterial wall fragments, namely LPS (1 mg kg^{-1} i.p.) or LTA (1 mg kg^{-1} i.p.) are abolished by the K_{ATP} channel inhibitor 5-hydroxydecanoate acid (5-HD, 5 mg kg^{-1} i.v.). LPS or LTA were administered 16 h prior to ischaemia, while 5-HD was injected 10 min prior to ischaemia.

Male Wistar rats (230-330 g) were 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 was cannulated to measure mean arterial blood pressure (MAP) and the jugular vein was cannulated for the administration of drugs. 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 30 min and subsequently the LAD was occluded for 25 min and then reperfused for 2 h. 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. Infarct size (IS) was determined by incubation of the slices of the heart with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1}).

The following groups (each n=6) were studied: (1) saline (control), (2) 5-HD, (3) LPS, (4) LTA, (5) LPS+5-HD or (6) LTA+5-HD.

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

Table 1: Area at risk (AR, %) and infarct size (IS, %) data.
* $P<0.05$ vs. control, ANOVA followed by Bonferroni's test.

	control	**5-HD**	**LPS**	**LTA**	**LPS+ 5-HD**	**LTA+ 5-HD**
IS	57±4	63±3	21±4*	24±6*	31±8*	29±6*
AR	48±3	46±3	52±2	53±4	50±1	48±4

Thus, pretreatment with LPS or LTA for 16 h induces delayed protection of the rat heart. Our results suggest that K_{ATP} channels are not involved in the cardioprotective effects of these agents.

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Zacharowski, K., Frank, S., Otto, M., *et al.*, (2000). *Arterioscler. Thromb. Vasc. Biol.*, (in press).

27P A CRUCIAL INFLUENCE OF PRECONTRACTION ON POTASSIUM-INDUCED RELAXATION IN THE RAT ISOLATED MESENTERIC ARTERY

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Cardiovascular Research Laboratories, Dept of Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD, UK.

Potassium ion (K^+) has been suggested to serve as an endothelium derived hyperpolarizing factor (EDHF) in rat arteries (Edwards *et al.*, 1998). Part of the evidence in support of this suggestion was the demonstration of clear and reproducible smooth muscle hyperpolarization and relaxation to K^+ ion in both the small mesenteric and hepatic arteries. However, recent work has failed to confirm the ability of K^+ ion to evoke relaxation in these arteries, and thus questions the involvement of K^+ as an EDHF (Lacy *et al.*, 2000; Andersson *et al.*, 2000). In the present study, we show that K^+'s ability to evoke hyperpolarization and relaxation depends critically on the extent of smooth muscle prestimulation.

Male Wistar rats (200-250 g) were stunned and killed by cervical dislocation. Segments of mesenteric arteries (2mm in length; 100-200 μm diameter, third order branch) were dissected and mounted in a Mulvany-Halpern myograph as described previously (Dora *et al.*, 2000). Arteries were incubated in Krebs buffer containing L-NAME and indomethacin and then precontracted with phenylephrine (PE, 0.2-3.0 μM). The integrity of the endothelium was assessed as relaxation (>95%) to acetylcholine. Next, the effect of increasing the extracellular K^+ concentration (from 4.8 up to 14 mM) on smooth muscle tension and membrane potential was assessed in the presence of variable precontraction (2 to 20 mN) and depolarization (-55 to -35 mV) with phenylephrine. Increasing K^+ to 10 mM completely reversed phenylephrine contractions of up to 5 mN, an effect associated with smooth muscle repolarization to resting levels (Figure 1, n=5). With higher levels of prestimulation both responses were reduced (n=3). In contrast, K^+ increases to 14 mM were still able completely to reverse precontractions up to levels approaching 20 mN, effects associated with marked repolarization/hyper-polarization (n=5). Hyperpolarization and relaxations to acetylcholine were not altered in a similar way by the extent of precontraction to phenylephrine.

These data show that the extent of prior smooth muscle depolarization and contraction can determine if small increases in extracellular K^+ can or cannot evoke repolarization and relaxation. They are consistent with our original suggestion that another mechanism, possibly myoendothelial transfer of hyperpolarization from the endothelial cells, forms a component of the 'EDHF' response in small mesenteric arteries (Edwards *et al.*, 1998). In addition, they highlight the importance of determining the appropriate experimental conditions in order to record relaxation to K^+ in vascular smooth muscle.

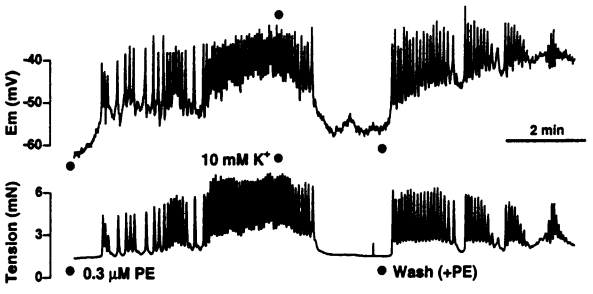

Figure 1. Effect of raising extracellular K^+ to 10 mM on membrane potential and tension in rat mesenteric artery.

Supported by the Wellcome Trust, UK.

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Edwards, G., Dora, K.A., *et al.*, (1998) *Nature* **396**, 269-272.
Lacy P.S., *et al.*, (2000) *Br.J.Pharmacol.*, **129**, 605-611.

28P INHIBITION OF EDHF-TYPE RELAXATION BY GLYCYRRHETIC ACID DERIVATIVES IN RABBIT SUPERIOR MESENTERIC ARTERY

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EDHF-type responses depend upon a signal passing from the endothelium to underlying smooth muscle via gap junctions (Chaytor et al., 1998). Lipophilic glycyrrhetinic acid derivatives are widely used as inhibitors of gap junctional communication and have been shown to inhibit EDHF-type responses. The effects of three such derivatives 18α-glycyrrhetinic acid (18α-GA), 18β-glycyrrhetinic acid (18β-GA) and carbenoxolone on EDHF-type relaxations and agonist-induced tone were compared in rings of superior mesenteric artery from male NZW rabbits (2-2.5Kg).

In the absence/presence of N^G-nitro-L-arginine methyl ester (L-NAME; 300μM) and indomethacin (10μM) rings were preconstricted by 10μM phenylephrine and cumulative concentration-response curves to acetylcholine (ACh) constructed following a one hour incubation with 18α-GA (10μM-100μM), 18β-GA (1μM-10μM) or carbenoxolone (30μM-300μM). Direct effects on vascular tone were examined by constructing concentration-response curves to each of these agents in endothelium-intact preparations in the absence/presence of L-NAME and in denuded rings. Maximal relaxations are expressed as mean ± s.e.m with EC_{50} values expressed as mean ± 95% confidence interval and comparisons made employing the Student's *t* test. Concentration-response curves were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. In the absence of L-NAME and indomethacin ACh evoked maximal relaxations of 92±2% which were reduced to 68±5%, 75±5% and 76±7% following incubation with 100μM 18α-GA, 10μM 18β-GA and 100μM carbenoxolone, respectively (n=4, P<0.05 in each case).

In the presence of L-NAME and indomethacin, 18α-GA at concentrations of 10μM and 30μM reduced maximal relaxation from 32±4% to 23±2% and 11±4%, respectively (n=7, P<0.05 for both). 100μM 18α-GA abolished EDHF-type responses (n=5). 18β-GA at concentrations of 1μM, 3μM and 10μM inhibited maximal relaxations from 34±4% to 33±9%, 23±8% and 12±6% (n=4, P<0.05 at 3μM and 10μM). Similarly, carbenoxolone at concentrations of 30μM and 100μM inhibited EDHF-type responses from 42±6% to 35±1% and 30±3%, respectively (n=4 and 5, P<0.05 at 100μM). 300μM carbenoxolone abolished EDHF-type relaxations (n=5). EC_{50} values for EDHF-type relaxations to ACh were unaffected by 18α-GA and 18β-GA. However, 100μM carbenoxolone caused a rightward shift from 210±160nM to 790±86nM (P<0.05). 18α-GA did not itself evoke relaxation, whereas 18β-GA and carbenoxolone caused concentration-dependent relaxations in both endothelium-intact and -denuded rings. 18β-GA evoked a maximal relaxation of 96±2% in endothelium-intact rings at ~1mM (n=9) and this was unaffected by L-NAME and indomethacin or by endothelial denudation (n=7 and 5, respectively). Carbenoxolone also evoked a maximal relaxation of 85±1% at ~10mM which was reduced to 67±6% in the presence of L-NAME and indomethacin and 74±3% by denudation (n=9 and 10, P<0.05 for both).

In conclusion, all three agents inhibit EDHF-type relaxations evoked by ACh, however, unlike 18α-GA, carbenoxolone and 18β-GA possess intrinsic vasorelaxant activity which in the case of carbenoxolone involves functional enhancement of NO activity in addition to direct effects on smooth muscle.

Chaytor, A.T., Evans, W.H. & Griffith, T.M. (1998) *J. Physiol.* 508, 561-573.

29P THE EFFECTS OF OUABAIN AND 18α GLYCYRRHETINIC ACID ON GAP JUNCTION INTERCELLULAR COMMUNICATION AND STABILITY

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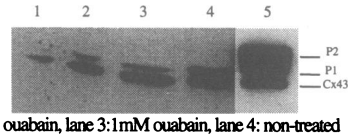
There is now evidence that EDHF-type relaxations involve intercellular communication between the endothelium and subjacent smooth muscle cells via gap junctions (Chaytor et al., 1998). These membrane structures are constructed from connexin (Cx) proteins and form channels which connect adjacent cells directly. In the present study we have examined the effects of the glycoside ouabain and 18α glycyrrhetinic acid (αGA) on the functionality and stability of gap junctions as both compounds are known to attenuate EDHF-type responses and possess similar steroidal structures. Two cell culture systems were employed: a) the rat smooth muscle cell line A7r5 which expresses Cx40 and Cx43 and is highly communication competent, and b) HeLa cells which are normally communication incompetent. The HeLa cells were transfected with cDNA encoding a chimeric Cx43 protein in which the carboxyl terminus on Cx43 was fused to the amino terminus of Green Fluorescent Protein (GFP). Cells expressing this protein allow connexin trafficking to be studied by fluorescent microscopic visualisation of the chimeric protein and analysis of the functional properties of gap junctions (Paemeleire et al., 2000).

The effects of ouabain and αGA on gap junction permeability were monitored by the ability of individual cells microinjected with the fluorescent dye Lucifer yellow to transfer the dye to neighbouring cells. The results show that preincubation with ouabain for 1 hour at 37°C reduces dye transfer in a concentration-dependent manner. Preincubation of Cx43GFP expressing HeLa cells with ouabain reduced dye transfer (quantified as % of cells transferring dye to more than 5 neighbouring cells ± s.e.m) by 90 ±12 % at 100μM, by 20-30% at concentrations in the range 0.3μM-10μM, but was without effect at at 0.1μM (n=3, >50 cells injected/experiment). Preincubation of A7r5 cells with ouabain inhibited dye transfer by ~70% at concentrations of 300μM and 1mM while 100μM had no effect. In both cases 25μM αGA resulted in > 95% inhibition of dye transfer within 30 minutes (n=3, >50 cells injected/experiment). Western Blot analysis of A7r5 cells treated with 300μM and 1mM ouabain demonstrated a reduction in the level of Cx43 phosphorylation and in cells treated with 25μM αGA phosphorylation was totally abolished (Figure1).

Figure 1: Western Blot analysis of A7r5 cells treated with αGA and ouabain. Cx43 was detected with an antiCx43 monoclonal antibody (Chemicon). Blots were developed by ECL.



Lane 1: 25μM αGA, lane 2: 300μM ouabain, lane 3: 1mM ouabain, lane 4: non-treated cells, lane 5: purified gap junctions. P1 and P2 denote Cx43 phosphorylation isoforms.

Incubation of Cx43GFP expressing HeLa cells with 25μM αGA caused a decrease in the number of plaques in the cell membrane. This was also evident, to a lesser degree, in cells treated with 1mM and 300μM ouabain. Confocal timelapse microscopy of A7r5 cells transfected with Cx43GFP cDNA showed the disassembly and internalisation of gap junction plaques 30-45 minutes post αGA treatment.

The results show that ouabain and αGA reduce gap junction communication in a manner that may be related to the phosphorylation status of Cx43. Chimeric CxGFP proteins can be used to study the effect of drugs on gap junction integrity in living cells.

Chaytor A. T., Evans W. H. & Griffith T. M. (1998). J. Physiol. 508, 561-573.
Paemeleire K. et al., (2000). Mol. Biol. Cell. (In Press).

30P CONSTRICTOR RESPONSES OF THE NOVEL PEPTIDE HUMAN UROTENSIN II (U-II) AND ENDOTHELIN-1 (ET-1) COMPARED IN ENDOTHELIUM-DENUDED HUMAN ARTERIES AND VEINS *IN VITRO*

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Human urotensin II (U-II) is reportedly ten-fold more potent than endothelin-1 (ET-1) in rat and monkey vasculature, making it the most potent constrictor yet described (Ames *et al.*, 1999). We have compared human U-II to ET-1 in human coronary artery (CA), internal mammary artery (MA), saphenous vein (SV), umbilical vein (UV) and, as control, rat thoracic aorta which is known to respond to U-II.

Rings (4mm) of endothelium-denuded arteries or veins were set up for isometric tension recordings in 5ml organ baths containing warm (37°C), oxygenated Krebs solution. Cumulative concentration-response curves were constructed to ET-1 (10^{-10}-$3x10^{-7}$M) or U-II (10^{-13}-10^{-7}M). Experiments were terminated by addition of 50mM KCl to determine the maximum contractile response and agonist responses were expressed as a percentage of this. Data were analysed by the iterative curve-fitting programme Fig P (Biosoft, Cambridge, U.K.) and values of pD_2 and maximum response (E_{max}) were expressed as mean±s.e.mean. n Values were the number of rats or patients from which blood vessels were obtained.

In rat aorta, U-II and ET-1 were equipotent with comparable maximum responses. UII was 5 times more potent in human veins and 50 times more potent in human arteries than ET-1. However, these differences only reached significance for CA. In all human vessels, unlike rat aorta, the maximum contractile response to U-II (~20% KCl) was always significantly less than that for ET-1 (~80% KCl). The U-II response was also more variable with 3/8 CA and 2/6 MA not responding to the peptide (Table 1). These data contrast with that reported by Ames and colleagues who found U-II constriction of monkey vasculature to be limited to arterial vessels.

The physiological/pathophysiological roles of U-II in the mammalian cardiovascular system are not known (Davenport & Maguire, 2000). Our data suggest that U-II, if present endogenously in humans, may act as a potent vasoconstrictor of both arteries and veins.

Supported by grants from the British Heart Foundation.

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	Endothelin-1			Urotensin II		
	pD_2	E_{max}	n	pD_2	E_{max}	n
Rat Aorta	9.43±0.3	79.2±21	3	9.38±0.3	64.0±18	3
Human Coronary Artery	8.28±0.1	84.3±7	8	9.99±0.6†	19.4±8‡	5/8*
Human Mammary Artery	8.25±0.1	81.3±8	5	10.0±1.1	18.7±11‡	4/6*
Human Saphenous Vein	8.67±0.6	93.9±6	2	9.4±0.7	27.1±10‡	4
Human Umbilical Vein	8.41±0.4	82.6±8	3	9.29±0.4	16.6±10‡	4

Table 1. Significantly different from corresponding ET-1 value: † $p<0.01$ ‡ $p<0.005$ Student's two-tailed *t*-test. * Data from responders only.

31P THE ENDOTHELIN$_A$ (ET_A) RECEPTOR ANTAGONIST, BSF 302146, IS A POTENT INHIBITOR OF PORCINE VEIN GRAFT THICKENING, *IN VIVO*

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Vein graft thickening is the main cause of late failure following coronary artery bypass graft surgery using autologous saphenous vein (Jeremy *et al.*, 1997). This process is mediated by: 1) medial thickening and neointima formation both of which involve the proliferation and migration of vascular smooth muscle cells (VSMC) and 2) deposition of matrix proteins, such as collagen (Mehta *et al.*, 1998). No pharmacological intervention has hitherto proved succesful in preventing late vein graft failure in man (Jeremy *et al.*, 1997). However, we have recently demonstrated that the neointimal and medial region of porcine vein grafts contain high levels of immunoreactive endothelin-1 (ET-1) and ET_A receptor subtypes (Dashwood *et al.*, 1997). Since ET-1 promotes the proliferation of VSMCs, we investigated the effect of administration of the ET_A receptor antagonist, BSF 302146 [(+)-(S)-2-(4,6-Dimethyl-pyrimidin-2-yloxy)-3,3-diphenyl-butanoic acid] on porcine vein graft thickening *in vivo*.

Saphenous vein-carotid artery interposition grafting was performed in two groups of Large White pigs (22 - 36 kg, n = 10 for each group) using established techniques (Mehta *et al.*, 1998). The ET_A receptor antagonist, BSF 302146 was administered orally (30 mg / kg / day) for four weeks to one group of pigs and placebo to the other (controls). Pigs were anaesthetised and the grafts were removed and pressure fixed (100 mm Hg) with 4 % paraformaldehyde. Histological sections were prepared at 6 equi-distant points along each graft, stained and graft morphology measured using computer-aided planimetry (Mehta *et al.*, 1998).

There was a marked reduction in medial thickness (MT), a significant reduction in intimal thickening (NT) and a marked increase in lumenal area (LA) in vein grafts from animals administered with BSF 302146 compared to untreated controls (table1).

Table 1. Effect of BSF 302146 administration on lumenal area (LA), neointimal thickness (NT) and medial thickness (MT) in porcine vein grafts (mean ± S.E.M., n = 10). *p < 0.001 treated compared to controls (paired t test).

	LA (mm^2)	NT (mm)	MT (mm)
Control	37.6 ± 2	0.15 ± 0.01	0.32 ± 0.01
BSF 302146	95.3 ± 8*	0.13 ± 0.01*	0.18 ± 0.01*

The potent inhibitory effect of BSF 302146 on porcine vein graft thickening consolidates that ET-1 and ET_A receptor subtypes are involved in medial thickening and neointima formation. It is concluded that the effects of BSF 302146 on vein graft morphology are mediated through the inhibition of VSMC proliferation and migration. The large lumenal areas in grafts from treated pigs also indicates that the BSF 302146 inhibits matrix protein synthesis and deposition. The administration of an ET_A antagonist may prove benefical in treating late vein graft failure in man.

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32P INHIBITION BY ENDOGENOUS NITRIC OXIDE OF ACETYLCHOLINE RELEASE IN THE MOUSE ISOLATED ILEUM

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Modulation of acetylcholine (ACh) release by NO has been studied in ileum preparations of guinea-pig and dog (Hebeiss & Kilbinger, 1998; Hryhorenko *et al.*, 1994). Mice are becoming increasingly important as subjects for neuroscience research because of the availability of mutant or genetically manipulated mice for studies of transmitter release. We have therefore investigated the effects of NO on ACh release and cholinergic contractions in the mouse ileum.

Longitudinal muscle myenteric plexus preparations were isolated from male C3H/He mice (30-45g). After incubation with [^{3}H]choline (2.5μCi/ml) in a 2ml organ bath and subsequent superfusion with a physiological salt solution (37°C), the muscle strips were stimulated twice 42 min apart (S1, S2; 1Hz, 180 pulses). The electrically evoked [^{3}H]outflow as measured in the presence of neostigmine (10 μM) consisted of 95 ± 6% (n=5) [^{3}H]acetylcholine. The evoked outflow was abolished by tetrodotoxin or calcium-free superfusion solution indicating its neurogenic origin. Statistics were calculated by ANOVA followed by Dunnett multiple comparisons test.

In order to block the endogenous NO-synthase pathway, L-nitroarginine (L-NOARG) was added 27 min before S2. L-NOARG (10, 100, 300μM) concentration-dependently enhanced the electrically evoked [^{3}H]ACh release (118 ± 5%, p>0.05, n=6; 130 ± 6%, p<0.01, n=5; 132 ± 6% of control, p<0.05, n=3).

For interaction experiments either L- or D-arginine was added to the superfusate 55 min before S1. The facilitatory effect of 100 μM L-NOARG was stereospecifically prevented by 1mM L-arginine (97 ± 3%, n=5) but not by D-arginine (126 ± 6%, n=4).When ODQ (1μM), an inhibitor of the NO-sensitive soluble guanylyl cyclase, was added to the preparations instead of L-NOARG, there was a similiar increase of the stimulated [^{3}H]ACh release (134 ± 8% of control, n=4, p<0.01). A concentration of 0.1 μM ODQ did not significantly facilitate the evoked release (102 ± 4%, n=4, P>0.05). The resting outflow of [^{3}H]ACh was not affected by L-NOARG or ODQ. The electrically evoked isometric contractions of the smooth muscle were similarly increased by L-NOARG (10, 100, 300μM) to 109 ± 5% (p>0.05), 147 ± 4% (p<0.01), 151 ± 9% (p<0.01) of control (n=7 each). The NO donor S-nitroso-N-acetylpenicillamine (SNAP, 100μM), added 27 min before S2 caused a small but significant inhibition of the electrically evoked [^{3}H]ACh release (84 ± 4% of control, n=5, p<0.05). This effect was abolished by ODQ (0.1 μM).

The results suggest that endogeneous NO inhibits the release of acetylcholine and thus cholinergic contractions in the mouse ileum. The experiments with ODQ indicate that the NO-induced inhibition of transmitter release is mediated by activation of NO-sensitive soluble guanylyl cyclase.

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33P M3-MUSCARINIC RECEPTOR-MEDIATED CONTRACTILE RESPONSES IN PORCINE DETRUSOR MUSCLE *IN VITRO*

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The bladder receives a dense parasympathetic innervation, with acetylcholine acting on postjunctional muscarinic receptors to induce contraction and mediate bladder emptying. However which muscarinic receptor subtypes are present in the bladder is debatable. Both the M2 and M3 subtypes have been found to be present in detrusor muscle of several species including man (Wang et al., 1995) with the M2-receptor predominating in all these species. In spite of this, it appears to be the minor population of M3-receptors which mediates contractile responses *in vitro* (Longhurst et al., 1995). The pig is an important model for the study of bladder physiology and pathophysiology since it is thought to closely resemble the human. However, a recent study failed to identify any M3 receptors in the pig bladder using radioligand binding assays (Goepel et al., 1998). Since in all species so far examined the M3-receptor subtype mediates contractile responses *in vitro*, we have investigated the role of muscarinic receptor subtypes in mediating the contraction of pig detrusor muscle strips.

Strips of detrusor muscle were taken from the dome of female pig bladders and the urothelium and serosa removed. The strips were then suspended in gassed Krebs at 37°C under a resting tension of 1.0g. Contractile responses to carbachol were obtained in the absence and presence of various muscarinic antagonists (4-DAMP, methoctramine, darifenacin, oxybutynin, tolterodine and pirenzapine) (30min equilibration period).

The M3-selective antagonists, 4-DAMP and darifenacin had high affinities (pK_B 9.37±0.07 (n=12) and 8.61±0.10 (n=18) respectively, whilst the M2-selective agent methoctramine had a relatively low affinity (pK_B 6.1±0.1 (n=18)). Schild plots for 4-DAMP and methoctramine were close to unity (0.90±0.1 and 0.89±0.15) and maximum responses were not affected by these antagonists, confirming a competitive mode of action. However, darifenacin produced unsurmountable antagonism, with maximum responses being significantly reduced ($P<0.01$) to 3.93±1.18g at a darifenacin concentration of 30nM (compared to the control response of 10.55±2.62g). Oxybutynin, tolterodine and pirenzapine had affinities of 8.2 (n=13), 8.1 (n=11) and 6.8 (n=13) respectively and appeared to act competitively with Schild plots close to unity (0.97±0.05, 0.86±0.20, and 1.09±0.23 respectively) and maximum responses being unaffected by these antagonists. The mean affinity values obtained for the antagonists at pig detrusor were correlated with those published for these antagonists at the five cloned muscarinic receptor subtypes. The pig detrusor receptor correlated best with the m3 and m5 subtypes with correlation coefficients of 0.92. However, only the correlation plot for the m3 receptor had a slope close to unity (1.06±0.17), with the plot for the m5-receptor being significantly flatter (slope=0.75±0.11). Poor correlations were found between the detrusor receptor and the cloned m1, m2 and m4 receptor subtypes (correlation coefficients of 0.68, 0.19 and 0.66 respectively).

These data suggest that a small population of M3-muscarinic receptors must mediate direct contractile responses of the pig detrusor muscle to muscarinic receptor stimulation *in vitro*.

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34P RELATIVE INTRINSIC EFFICACY OF ADENOSINE A1 RECEPTOR AGONISTS MEASURED USING FUNCTIONAL AND RADIOLIGAND BINDING ASSAYS

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For a G-protein-coupled receptor (GPCR), agonist intrinsic efficacy may represent the degree to which the agonist can convert the receptor into a conformation that binds and activates G-proteins. Relative efficacy measurements should be independent of the stage of the transduction pathway at which they are measured, unless saturation of a physiological process occurs. We describe here some selective A1 agonists including novel low-efficacy agonists which were tested in a range of assays and which maintained their relative intrinsic efficacies in accordance with this prediction.

Experiments were performed using Chinese hamster ovary cells stably expressing both the human adenosine A1 receptor (a GPCR), and a reporter gene for secreted placental alkaline phosphatase (SPAP) which responded to changes in cyclic AMP. Functional assays were as follows: inhibition of forskolin-stimulated SPAP secretion as in McDonnell et al., 1998; [^{35}S]GTPγS binding as in Cohen et al., 1996; and elevation of intracellular Ca^{2+} measured using a Molecular Devices 'FLIPR' reader. Radioligand binding studies used cell membranes in 50mM HEPES, pH 7.4. 'GTP-shift' experiments measured the reduction in compound pK_I induced by 0.1mM GTP with [^{3}H]-8-cyclopentyldipropylxanthine (DPCPX) as radioligand. This was also used as antagonist radioligand and [^{3}H]-2-chlorocyclopentyladenosine as agonist radioligand in experiments measuring affinity for G-protein-uncoupled and –coupled receptor states. N^6-ethylcarboxamidoadenosine (NECA) was reference full agonist in every experiment. Test compounds were GR79236 (Gurden et al., 1993), GR190178 (Brown et al., 1999), GR161144 (9-[3R,4S-dihydroxy-5-[4-methyl]-1,2,4-oxadiazol-2-yl]-tetrahydro-furan-2(R)-yl]-6-[[tetrahydroyran-4-yl]amino]-9H-purine) and GR162900 ((2R,3R,4S,3R)-2-[6-[(1-methylethyl)amino]-9H-purin-9-yl]-5-[4H-5-methyl-1,2,4-triazol-2-yl]tetrahydrofuran-3,4-diol).

GR79236 behaved as a full agonist in each system except elevation of intracellular Ca^{2+}, where it was slightly but significantly less efficacious. GR190178, GR161144 and GR162900 were of progressively lower relative intrinsic efficacy (Table 1).

	Relative intrinsic efficacy (% relative to NECA; mean ± SEM or 95% conf. limits, $n \geq 3$)			
	GR79236	GR190178	GR161144	GR162900
cAMP-linked reporter gene	100 ± 0	99 ± 1	100 ± 0	83 ± 20
GTPγS binding	105 ± 5	84 ± 5	55 ± 7	14 ± 10
Ca^{2+} (FLIPR)	81 ± 4	45 ± 17	28 ± 6	11 ± 4
Binding (GTP shift)	87 ± 36	54 ± 12	29 ± 69	9 ± 10
Binding (ag / antag ligands)	86 (60-153)	68 (38-123)	11 (2-60)	5 (3-10)

The relative intrinsic efficacy of these compounds was thus maintained across every experimental preparation, even though the assay readouts varied from being either a receptor affinity state change (agonist/antagonist binding and GTP shift), GTP turnover on the G-protein (GTPγS binding), a Giα-mediated response (SPAP) or a Giβγ-mediated response (Ca^{2+}).

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35P BIPHASIC [^{35}S]GTPγS FUNCTIONAL RESPONSES OF HUMAN ADENOSINE A_1 RECEPTORS EXPRESSED IN CHINESE HAMSTER OVARY (CHO) CELL MEMBRANES

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The adenosine A_1 receptor is a member of the seven transmembrane G-protein coupled superfamily and couples primarily to the activation of $G\alpha_{i/o}$ G-proteins. Activation of these receptors results in the dissociation of bound GDP from the G-protein, association of GTP and the initiation of the G-protein activation cycle. [^{35}S]GTPγS binding can be used to monitor the extent of G-protein activation as it is resistant to hydrolysis once bound to the G-protein. We have used this assay to investigate the consequence of receptor activation in CHO cells stably expressing different levels of the adenosine A_1 receptor.

Membranes (20 μg/tube) from CHO cells stably expressing high (9 pmol.mg^{-1} protein) and low (0.4 pmol.mg^{-1} protein) levels of the A_1 receptor were pre-incubated for 30 min at 30°C in a buffer of 20mM HEPES, 100mM NaCl, 10mM $MgCl_2$, 0.3U/ml adenosine deaminase and 10μM GDP, pH 7.4, with agonist (0.1nM - 100μM) either alone or with the antagonist 8-cyclopentyl-1,3-dimethylxanthine (DPCPX, 10-100nM) or the allosteric enhancer PD 81,723 (3μM; Bruns and Fergus, 1990). [^{35}S]GTPγS was added (0.1nM final concentration) and incubated for a further 30 min. Bound radioactivity was separated by rapid filtration and counted using liquid scintillation spectrometry.

In the low expressing cell line, the agonist N^6-cyclohexyladenosine (CHA) produced a concentration dependent increase in [^{35}S]GTPγS binding with a pEC_{50} of 8.15±0.03 and maximum response (expressed as fold-stimulation/basal) of 2.9± 0.1 (n=13). In the high expressing cell line basal [^{35}S]GTPγS was 2.4±0.3 fold higher than in the low expressing cell line. CHA (0.1-10nM) produced a concentration dependent increase in [^{35}S]GTPγS binding with a pEC_{50} of 9.12±0.04 and maximum response of 3.2±0.2 fold. At higher concentrations (30nM-10μM), CHA inhibited 59±5% of the maximum stimulated [^{35}S]GTPγS response in a concentration-dependent manner with a pIC_{50} of 7.09±0.05 (n=13). This biphasic response was mimicked by the agonists R(-)N^6-(2-phenylisopropyl)adenosine and 5'-N-ethylcarboxamidoadenosine. The selective A_1 antagonist DPCPX inhibited CHA responses in low expressing membranes and inhibited both phases of response in high expressing membranes with similar pA_2 values of 8.53±0.19, 8.52±0.15 and 8.42±0.14, respectively (n=3). In addition, in high, but not low, expressing membranes DPCPX produced a concentration-dependent inhibition of basal [^{35}S]GTPγS binding with a pIC_{50} of 8.32±0.21 and a maximum inhibition of 36±4%. The allosteric enhancer PD 81,723 potentiated by 3-fold the response to CHA in low expressing membranes and both phases of response in high expressing membranes (n=4). In addition, PD 81,723 enhanced basal activity by 35±1% in high, but not low expressing membranes. In kinetic studies, the magnitude of the inhibitory response to 10μM CHA in high expressing membranes increased with the time of exposure to [^{35}S]GTPγS, reaching 71% inhibition of the response to 10nM CHA at 120 min. However, it was independent of the duration of exposure to CHA (30-120 min).

The novel inhibitory response to agonists is seen in high but not low expressing membranes, suggesting that it is dependent on the density of A_1 receptors. Antagonism by DPCPX and potentiation by PD 81,723 of both the stimulatory and inhibitory responses to CHA provide evidence that both effects are mediated by the A_1 receptor. Kinetic studies suggest that the inhibitory response to CHA is dependent on the duration and extent of G-protein activation. The data also suggest that constitutive receptor activation is dependent on the density of the A_1 receptor.

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36P CHARACTERISATION OF BIPHASIC [^{35}S]GTPγS RESPONSES OF HUMAN ADENOSINE A_1 RECEPTORS USING PARTIAL AGONISTS AND THE ALLOSTERIC ENHANCER PD 81,723

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A biphasic response to high efficacy agonists in Chinese hamster ovary (CHO) cells expressing a high level of the human adenosine A_1 receptor has been described (Browning *et al.*, this meeting). Using [^{35}S]GTPγS binding, we describe the further characterisation of this phenomenon using agonists of varying efficacies and the allosteric enhancer PD 81,723 (Bruns and Fergus, 1990).

[^{35}S]GTPγS binding in membranes from CHO cells expressing high (9 pmol.mg^{-1} protein) and low (0.4 pmol.mg^{-1} protein) levels of the A_1 receptor was conducted as previously described (Browning *et al.*, this meeting) using CHA or the lower efficacy adenosine A_1 agonists GR190178, GR161144 and GR 162900 (0.1nM - 10μM; Sheehan *et al.*, this meeting) either alone or in the presence of the allosteric enhancer PD 81,723 (3μM). The affinities of these ligands for the G protein-coupled (K_H) and uncoupled (K_L) receptor were determined by competition binding using the antagonist radioligand [^{3}H]DPCPX as previously described (Browning *et al*, 2000). The ratio K_H/K_L was used as an index of agonist efficacy (Birdsall *et al.*, 1978).

In low and high expressing CHO A_1 membranes, CHA, GR190178, GR161144 and GR169200 produced concentration-dependent increases in [^{35}S]GTPγS binding. In the low expressing cell line GR190178 was a full agonist (compared with CHA) whilst GR161144 and GR162900 were partial agonists with intrinsic activity (i.a.) values of 0.75±0.04 and 0.43±0.05 respectively (n=4). The intrinsic activity values were increased by PD 81,723 (Table 1). This order of intrinsic activities was compatible with the K_H/K_L ratios of 600, 80, 10 and 1, respectively. In high expressing membranes, all four compounds produced a similar maximal level of [^{35}S]GTPγS stimulation. GR161144 and GR162900 yielded monophasic concentration-effect curves whilst high concentrations of CHA and GR190178 also inhibited 63±4 and 48±3% of the maximum [^{35}S]GTPγS response with pIC_{50} values of 7.1±0.1 and 6.7±0.1 respectively, (n=4). PD 81,723 augmented the inhibitory response to both CHA and GR190178 to 87±1 and 73±3% inhibition, respectively, of the maximum [^{35}S]GTPγS response whilst GR161144 and GR162900 now yielded biphasic concentration-effect curves with the expected rank order of inhibitory intrinsic activities (Table 1). In both cell lines PD 81,723 increased the potencies of all agonists examined by 2-5 fold.

Table 1. A_1 agonist responses and the effects of PD 81,723.

	Low expression		High expression					
	pEC_{50}	i.a.	pEC_{50}	i.a.	pIC_{50}	i.a.	K_H	K_L
control								
CHA	8.1	1	9.2	1	7.1	1	8.4	5.6
GR190178	7.3	1	8.3	1.1	6.6	0.8	7.9	6.0
GR161144	7.3	0.8	8.3	0.9	-	-	8.0	7.0
GR162900	7.4	0.4	8.0	0.9	-	-	7.4	7.4
+ PD81,723								
CHA	8.6	1	9.5	1	7.8	1		
GR190178	7.7	1	8.6	1.0	7.2	0.9		
GR161144	7.7	0.9	8.5	1.0	7.2	0.6		
GR162900	7.6	0.7	8.5	0.8	7.2	0.4		

All data are mean (n=4, control; n = 3, PD 81,723). s.e.m < 0.1.

These data suggest that the inhibitory responses to agonists seen in the high expressing cell line are dependent on the efficacy of the agonist. This is also the first report that the allosteric enhancer PD 81,723 can increase the efficacy of partial adenosine A_1 agonists.

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37P ROLE OF CALCIUM IN THE M_3 MUSCARINIC RECEPTOR-MEDIATED POTENTIATION OF A_{2B} ADENOSINE RECEPTOR-INDUCED CYCLIC AMP ACCUMULATION IN HEK 293 CELLS

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HEK (Human Embryonic Kidney) 293 cells endogenously express A_{2B} adenosine receptors positively coupled to cyclic AMP (cAMP) accumulation (Cooper *et al.*, 1997). We have previously shown that co-stimulation of these cells with histamine (HA, 100 μM) produces a greater than additive cAMP generation in an HEK 293 cell clone stably over-expressing recombinant human H_1 histamine receptor DNA (568 ± 69 fmol/mg protein) (Walker *et al.*,1998). HA alone (100 μM) did not elicit a significant cAMP generation (Walker *et al.*,1998). In this study, we have investigated this potentiation of A_{2B} adenosine receptor-mediated cAMP generation by $G_{q/11}$ coupled M_3 muscarinic receptors, which are endogenously expressed in these cells (Jackson *et al.*, 1999).

HEK 293 cells stably transfected with recombinant human H_1 histamine receptor DNA (Presland & Hill, 1998) were used for all experimental work and were grown as described previously (Cooper *et al.*, 1997). Competition radioligand binding studies were performed on total cell particulate preparations using [^{3}H]-QNB as radioligand. Non-specific binding was defined using 1 μM atropine. Cyclic AMP and total inositol phosphates (IP) accumulations, and changes in intracellular Ca^{2+} were measured as described previously (Neil *et al.*, 1997, Walker *et al.*, 1998, Iredale *et al.*, 1995).

M_3 muscarinic receptors were expressed at a level of 82 ± 12 fmol/mg protein (n = 4, data are mean ± s.e.m. in this and in all subsequent cases). Carbachol (CCh, 300 μM) did not elicit any cAMP generation (-0.5 ± 1.7% of 10 μM NECA cAMP response, n = 8). Co-stimulation with NECA (10 μM) and CCh (300 μM) produced a significantly greater cAMP response ($P<0.001$, n = 6, paired t-test) than stimulation with NECA alone(218 ± 13 % of 10 μM NECA response). Incubation in Ca^{2+} free medium (containing 0.1 mM EGTA) significantly ($P<0.05$, one-way ANOVA, Neuman Keuls *post-hoc* test) reduced (by 37 ± 11%, n = 3) the cAMP generation following co-stimulation with NECA and CCh. Chelation of intracellular Ca^{2+} with BAPTA/AM (50 μM) under the Ca^{2+} free conditions described above significantly ($P<0.001$, one-way ANOVA, Neuman Keuls *post-hoc* test) reduced (by 80 ± 3%, n = 3) CCh induced potentiation of cAMP accumulation following co-stimulation with NECA and CCh. Maximal IP accumulations elicited by carbachol (300 μM, 27.1 ± 1.5 fold over basal) and histamine (100 μM, 26.3 ± 0.4 fold over basal) were not significantly different (n = 4, paired t-test).

In summary, potentiation of A_{2B} receptor-mediated cAMP generation occurs with activation of either endogenously expressed or stably overexpressed $G_{q/11}$ coupled receptors in HEK 293 cells, dependant predominantly on intracellular calcium elevations. Given the similarities in maximal IP accumulations and levels of cAMP potentiation by H_1 and M_3 receptors, and the differences in expression levels of these receptors, coupling efficiency is also important in this cross-talk phenomenon.

AMJ holds an MRC studentship.

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38P *IN VIVO* INACTIVATION OF IMIDAZOLINE$_2$ BINDING SITES BY A NOVEL IRREVERSIBLE LIGAND

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Our group has recently reported the synthesis of a selective irreversible ligand for the imidazoline$_2$ binding site (I_2-BS) BU99006, Coates *et al.*, (2000). Previously a radio-iodinated photoaffinity ligand ([^{125}I]AZIPI) has been prepared and shown to label at least three subtypes of I_2 sites that differ in their ligand recognition properties, apparent molecular weight and tissue distribution (Lanier *et al.*, 1993). However, since a photo-affinity label requires photoactivation it is not possible to use such a ligand *in vivo*. Here we present evidence of irreversible inactivation of I_2-BS *in vivo*.

Rats (Male, Wistar, 240g) were anaesthetised using sodium pentobarbital (60mg kg^{-1} i.p.) and treated with either vehicle (saline) or BU99006, 15mg kg^{-1}, i.v. 30 minutes post injection the rats were perfused with ice-cold phosphate buffered saline approximately 1ml g^{-1} to wash off any unreacted BU99006. The brains were removed and membranes prepared based on the methods of Lione *et al.*, (1998). Sodium orthophosphate (50mM) was used instead of TRIS HCl (50mM), to prevent the isothiocyanate of BU99006 reacting with the amino group of TRIS. The effect of the BU99006 treatment on the I_2-BS was investigated using [^{3}H]-2BFI binding (Lione *et al.*, 1998). The treated rat brain membranes (~400μg) were incubated in triplicate with increasing concentrations of [^{3}H]-2BFI (0.001 - 5nM) in buffer (50mM sodium orthophosphate, 1mM $MgCl_2$, pH 7.4), in the absence (total binding) or presence (non-specific binding) of 10μM BU224 (Lione *et al.*, 1998) in a final volume of 500μl. The data were analysed using the nonlinear regression analysis supplied with GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.

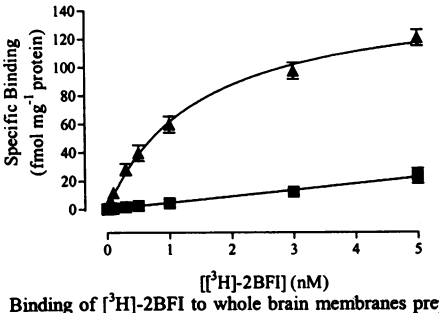

Figure 1. Binding of [^{3}H]-2BFI to whole brain membranes prepared from treated rats. Vehicle saline controls (▲) and BU99006 15mg kg^{-1} (■). Each curve represents the means of three animals (assay performed in triplicate), vertical bars represent the s.e.mean.

Figure 1 shows the saturation binding of [^{3}H]-2BFI to the rat brain membranes prepared from the treated animals. The K_D and B_{MAX} for the vehicle control, 1.6 ± 0.3 nM and 153 ± 8 fmol mg^{-1} protein respectively, agree with previously published data (Lione *et al.*, 1998). However, it was not possible to obtain a true value for the BU99006 treated animals for either K_D or B_{MAX}, also the curve obtained for these animals was the same as the non-specific binding (data not shown) indicating that all the I_2-BS were no longer available for binding. These data show that BU99006 inactivates I_2-BS *in vivo* and will be a very useful tool in the further understanding of their function in brain.

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