81P COMPLEX RELATIONSHIP BETWEEN INS(1,4,5)P_3 AND $[Ca^{2+}]_i$ SIGNALLING IN SH-SY5Y CELLS REVEALED BY DIFFERENTIATION WITH RETINOIC ACID

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The human neuroblastoma cell line, SH-SY5Y, is used extensively as a model to study many aspects of neuronal function including intracellular signalling. However, following differentiation these cells display a more neuronal phenotype (Perez-Polo *et al.*, 1979) and we have, therefore, studied the effects of differentiation on Ins(1,4,5)P_3 and Ca^{2+} signalling following activation of muscarinic (predominantly M3) and bradykinin (B2) receptors.

Cells were differentiated using retinoic acid (RA) (10μM) with 1% heat-inactivated serum for 6 days. Ins(1,4,5)P_3 was measured by radioreceptor assay (Challiss *et al.*, 1990) and intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) determined in adherent fura 2-loaded cell populations (Willars & Nahorski, 1995). B_{max} and K_D of muscarinic receptors on membrane preparations were determined by antagonist binding with 1-[*N-methyl*-^{3}H]scopolamine methyl chloride ([^{3}H]NMS). Data are mean±SEM ($n \geq 3$). EC_{50}'s are $-\log_{10}$ M values. Statistical analysis was by Student's t-test.

In undifferentiated cells, methacholine evoked a rapid peak followed by a lower sustained phase of both Ins(1,4,5)P_3 (104±8, 77±10 pmol/mg protein) and $[Ca^{2+}]_i$ (727±30, 198±9 nM) from resting levels of 18±2 pmol/mg protein and 98±4 nM respectively. RA treatment produced phenotypic changes consistent with differentiation (cessation of cell proliferation, neurite formation). Time-courses of responses were unaffected. However, RA significantly ($p<0.01$) increased the magnitude of the peak Ins(1,4,5)P_3 responses following maximal methacholine stimulation (1.5-fold) and decreased the EC_{50} value (5.4±0.07 versus 4.9±0.03). In contrast, the magnitude and potency of peak $[Ca2+]_i$ elevations in response to methacholine (EC_{50} 5.59±0.19) were unaffected by RA treatment. Differentiation increased muscarinic receptor number as assessed by binding (529±22 versus 272±11 fmol/mg protein, $p<0.01$) and Western blots using an M3 receptor-specific antibody, but did not alter the K_D (0.49±0.1nM).

Ins(1,4,5)P_3 accumulation in response to a maximal bradykinin dose (10μM) peaked at 5s with rapid decline to basal in both differentiated and undifferentiated cells. Differentiation significantly ($p<0.01$) increased the peak response (70±6 versus 21±2 pmol/mg protein). In contrast, peak $[Ca^{2+}]_i$ elevations to bradykinin were dose-related under both conditions with identical EC_{50} (7.65±0.2) and maximal (535±41 nM) values.

Thus, whilst differentiation of SH-SY5Y cells enhanced Ins(1,4,5)P_3 signalling in a manner consistent with increased receptor number, $[Ca^{2+}]_i$ signalling was unaffected. Furthermore, for equivalent peak accumulations of Ins(1,4,5)P_3 in response to methacholine or bradykinin, methacholine-evoked $[Ca^{2+}]_i$ elevations were greater. Together, these data imply a complex relationship between Ins(1,4,5)P_3 accumulation and $[Ca^{2+}]_i$ elevation and show the relationship differs for different agonists.

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82P RELAXATION OF RABBIT AORTA BY IODINATED RADIOGRAPHIC CONTRAST MEDIA (IRCM) MAY BE PARTIALLY MEDIATED BY Ca^{2+} SEQUESTRATION INTO THE SARCOPLASMIC RETICULUM

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Vasorelaxation of rabbit isolated aorta by IRCM is an endothelium-independent mechanism and hence mediated by a direct action vascular smooth muscle (Pitman *et al* 1996). In the present study we have investigated if uptake of free $[Ca^{2+}]_i$ into the sarcoplasmic reticulum (SR) contributes to the relaxant properties of two such agents, iohexol and iodixanol whose effects were compared in agonist-(phenylephrine PhE) and depolarisation-(high K^+) contracted rings of endothelium-denuded rabbit isolated thoracic aorta.

In rings precontracted with PhE (0.3 μM), the relaxation to 100 mg Iodine/ml iohexol (58.1 ± 6.2 %; n=12) and iodixanol (46.1 ± 2.9 %; n=10) was significantly greater than the corresponding relaxation (22.9 ± 3.2 %; n=7 and 24.8 ± 1.9 %; n=13 respectively), obtained in tissues constricted by 120 mM $[K^+]_o$. Incubation of the tissue with 30 μM cyclopiazonic acid (CPA) for 40 min prior to contrast application attenuated the vasodilatory action of iohexol (40.8 ± 4.6 %; n=7) and iodixanol (33.4 ± 3.6 %; n=9; $P < 0.01$) by ~ 30 % in PhE-contracted arteries. In preparations contracted by high K^+, however, incubation with CPA had no effect on the relaxation to iohexol (20.5 ± 4.5; n=5) or iodixanol (19.9 ± 3.5 %; n=5). Similarly, in K^+- contracted tissue ryanodine (10 μM) had no inhibitory influence on the relaxation to iohexol (15.3 ± 1.6 %; n=5) or iodixanol (23.7 ± 4.6; n=5), whereas in PhE-contracted rings there was a significant reduction in relaxation to both iohexol (36.2 ± 2.1 %; n=9) and iodixanol (21.1 ± 2.4 %; n=4) by 30 and 50 % respectively. Mannitol (0.3-180 mM), an "inert" sugar, was used to increase osmolality to similar levels produced by administration of IRCM and gave only a small < 15% relaxation of PhE-induced tone which was unaltered by prior incubation with either CPA or ryanodine. Moreover, mannitol caused no significant relaxation of K^+-evoked contraction and CPA and ryanodine did not modify the observed response.

In conclusion, IRCM induced vasodilatation is partially mediated (by ~ 50 %) through enhanced Ca^{2+} sequestration within the sarcoplasmic reticulum, in PhE-, but not K^+-constricted rabbit aorta. This phenomenon cannot be attributed directly to the effects of osmolality on vascular smooth muscle. The difference between the responses to IRCM under the two different methods of constriction may be explained by the observations that K^+-evoked contraction promotes SR loading (Sturek *et al* 1992) whereas PhE stimulation depletes the Ca^{2+} store (Low *et al* 1991). As the buffering ability of the SR will be reduced when the SR is maximally loaded and increased under conditions when it is depleted, IRCM-induced sequestration of Ca^{2+} within stores will thus depend on the mode of contraction. Although CPA, which inhibits the SR Ca^{2+} ATPase and prevents Ca^{2+} uptake, and ryanodine, which locks Ca^{2+} release channels open, have differents sites of action, the net effect of both agents will be functional inactivation of the SR. Further work is necessary to elucidate the mechanism of the residual vasodilatation observed in the presence of CPA or ryanodine in PhE- and K^+-contracted aorta.

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83P AN ACTIVATOR OF c-Src TYROSINE KINASE (pY)EEI PEPTIDE INCREASES VOLTAGE-OPERATED CALCIUM CHANNEL CURRENTS IN VASCULAR SMOOTH MUSCLE CELLS ISOLATED FROM RABBIT EAR ARTERY

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Receptor and non-receptor tyrosine kinases are important in regulating voltage-operated calcium channels (VOC) in vascular smooth muscle cells (Wijetunge & Hughes 1995a & 1995b). Intracellular application of human c-Src, a non-receptor tyrosine kinase increased VOC currents in rabbit ear artery cells (Wijetunge & Hughes 1995b). Since c-Src is found in large amounts in smooth muscle (Di Salvo *et al.* 1989) it is possible that this endogenous tyrosine kinase may have a role in regulating VOC. (pY)EEI peptide is a synthetic peptide which is derived from the Hamster polyoma virus middle-T antigen and is known to activate endogenous c-Src *in vitro* (Liu *et al.* 1993). We have used this peptide to examine the effect of activation of endogenous c-Src on VOC in rabbit ear artery cells.

Single smooth muscle cells were freshly dispersed from rabbit ear arteries by an enzymatic method as previously described (Benham & Bolton 1986). Calcium channel currents were measured by the whole cell configuration of the patch clamp technique. Currents were evoked by a depolarizing step to +10mV for 20ms from a holding potential of -60mV. The experiments were carried out in a "high barium" solution ($BaCl_2$ 110mM, HEPES 10mM and buffered to pH 7.2 with TEA-OH) to increase the size of the inward current and to minimize calcium-dependent inactivation of currents. Peptides were included in the intracellular pipette solution which contained (mM): NaCl 126, $MgCl_2$ 1.2, EGTA 2, MgATP 2, TEA 10, and HEPES 11 buffered to pH 7.2 with NaOH. Data are presented as means ± s.e. means of (n) observations.

(pY)EEI (100μM) peptide increased VOC currents from 58 ± 8pA to 90 ± 14pA (n=6) which corresponds to a 55 ± 14% increase in current. The inactive non-phosphorylated analogue (YEEI peptide) (100μM) had no effects on the currents (control = 44 ± 6pA, YEEI= 42 ± 6pA, n=5). Peptide-A (Sato *et al*, 1990) a synthetic peptide known to inhibit c-Src, reduced VOC currents from 50 ± 7pA to 30 ± 4pA (41 ± 5% inhibition of current, n=8) and also abolished the effect of (pY)EEI peptide on VOC currents (control = 57 ± 19pA, (pY)EEI + peptide-A = 45 ± 15pA, n = 5).

These results indicate that activation of intrinsic c-Src by (pY)EEI peptide increases VOC currents and support a role for endogenous c-Src or a related tyrosine kinase in the regulation of VOC in arterial smooth muscle cells.

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84P EFFLUX OF INOSITOL PHOSPHATES FROM NEURONAL TISSUES *IN VITRO*

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In vivo microdialysis studies have indicated that extracellular levels of inositol- 1,4,5 trisphosphate (IP_3) are increased after stimulation of muscarinic receptors (Minisclou *et al.*, 1994; Roberts *et al.*, 1996), presumably through an increase in its export out of cells. Previous studies, however, have failed to demonstrate an increase in external IP_3 *in vitro* despite increases in internal levels (Roberts *et al.*, 1996), possibly owing to binding of IP_3 to the tissue. In this communication we report the efflux of inositol phosphates from brain slices and SH-SY5Y cells *in vitro*.

Hippocampal slices from male wistar rats (180-250g) prelabelled with [^{3}H]-myo-inositol (20μCi/ml for 45 min) were perfused (0.25ml/min) with Kreb's buffer containing 5mM LiCl in a Brandel suprafusion system. Perfusate was collected directly onto Dowex chloride columns over 10 min for up to 50 min. There was a significant increase in the amount of inositol phosphates ([^{3}H]-IPs) in the perfusate 10 min after 1mM carbachol was added to the perfusion buffer (122% ± 7% of basal levels (mean ± S. E. mean); $p<0.05$, n=4) which reached a maximum 20 min after addition of the agonist (223% ± 56%). A combination of 1mM carbachol and 30mM KCl in the perfusion buffer caused a greater efflux of [^{3}H]-IPs (429% ± 21% of basal levels at 30 min; $p=0.0002$; n=3).

In a further set of experiments, in the absence of LiCl, perfusate was collected over 30 min in vials containing 1ml 7.5% PCA. To prevent the external breakdown of IP_3, the experiment was performed in the absence of Mg^{2+}. [^{3}H]-IPs were separated out on Dowex-1 columns in formate form. Stimulation of hippocampal slices with 1mM carbachol and 30mM KCl produced significant increases over basal levels in IP (394% ± 69%), IP_2 (444% ± 63%), IP_3 (452% ± 64%), and IP_4 (379% ± 140%); (all $p<0.05$, n=4).

The increase in external [^{3}H]-IPs accumulated after stimulation with carbachol and KCl could be inhibited by 5mM probenecid. Mean values in these particular experiments were: control; 998% ± 142%, compared to 609% ± 107% with 5mM probenecid at 30 min ($p<0.05$; 2-tailed paired t-test, n=5). However, preliminary experiments suggest that this decrease may be due to inhibition of internal [^{3}H]-IP production rather than an inhibition of export. In rat brain cortical slices, 5mM probenecid reduced the internal levels of [^{3}H]-IPs stimulated by 1mM carbachol and 15mM KCl in the presence of 5mM LiCl from 413% ± 40% to 192% ± 27% of controls ($p<0.05$; 2-tailed unpaired t-test, n=3) after 30 min.

In another set of experiments, SH-SY5Y cells prelabelled with [^{3}H]-myo-inositol (1μCi/well for 48 h) were stimulated with either 1mM carbachol, 30mM KCl, or a combination of both in a total volume of 1ml of Mg^{2+}-free Hanks basal salt solution containing 20mM HEPES and 20mM LiCl. After a 30 min incubation time there was an increase in total [^{3}H]-IPs in the external buffer after stimulation with 1mM carbachol (158% ± 13%, n=8) which was potentiated in the presence of 30mM KCl to 270% ± 43% despite there being no potentiation in the internal [^{3}H]-IP levels (1532% ± 179% of basal levels with 1mM carbachol compared to 1363% ± 129% with 1mM carbachol and 30mM KCl). This suggests that there may be an involvement of K^+-activated channels in IP export, although the physiological role of IP efflux remains to be elucidated.

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85P PHOSPHOINOSITIDE C-LINKED MUSCARINIC RECEPTORS, BUT NOT GnRH RECEPTORS, UNDERGO RAPID DESENSITIZATION IN αT3-1 CELLS: IMPLICATIONS FOR MECHANISMS OF DESENSITIZATION

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Many G-protein coupled, phosphoinositidase C- (PIC) linked receptors desensitize within seconds of agonist binding. Mechanisms likely to underlie this include receptor phosphorylation and/or substrate ($PtdIns(4,5)P_2$) depletion (Fisher, 1994). The gonadotropin releasing hormone (GnRH) receptor of the mouse pituitary cell line, αT3-1, does not, however, rapidly desensitize (Davidson *et al.*, 1994). We have examined if recombinant human muscarinic m3 receptors, when expressed in αT3-1 cells, display the rapid partial desensitization which occurs in other cells in order to assess cell and receptor specificity and to determine the role of $PtdIns(4,5)P_2$ availability in shaping phosphoinositide responses.

The cDNA for the muscarinic m3 receptor was transfected into αT3-1 cells. We report results from a clone with a B_{max} for antagonist binding of 376±21 (3) fmol/mg protein (untransfected cells 42±9 (3)) (all data are mean±sem (n)). Methods are given in detail elsewhere (Jenkinson *et al.*, 1994; Tobin & Nahorski, 1993).

$Ins(1,4,5)P_3$ accumulation in response to a maximal concentration of methacholine (MC, 1mM), consisted of a rapid peak (20s, 148±6 (3) pmol/mg protein, basal 6±3 (3)) followed by a lower sustained phase (85±5 (3) at 5min). In contrast, a maximal concentration of GnRH (1μM) evoked a sustained increase which was maximal by 60s (277±5 (3)). The GnRH response remained monophasic when the concentration was reduced (100nM) to evoke an $Ins(1,4,5)P_3$ accumulation equivalent to the peak MC response. In cells labelled with [^{3}H]inositol (48h) and in which Li^+ (10mM) was used to trap the products of phosphoinositide hydrolysis, MC caused a biphasic accumulation of [^{3}H]$InsP_x$. This consisted of an initial phase followed (>20s) by a lower sustained phase (4.2±0.4 fold of basal at 5min) indicating rapid partial desensitization of PIC. The response to 100nM GnRH was approximately linear (5.2±0.6 fold at 5min) suggesting no desensitization. Despite similar initial (0-10s) rates, the sustained response to GnRH (10453±1936 dpm/min) was greater ($p=0.03$, paired *t*-test) than that to MC (5529±1015). GnRH (100nM) also caused a rapid (20-60s) and sustained fall of [^{3}H]$PtdIns(4,5)P_2$ (no Li^+ present) (65±15% of basal at 5min). In contrast, MC caused a rapid (20s) fall (88±18% of basal) which recovered by 60s.

Thus, in αT3-1 cells, phosphoinositide responses to activation of muscarinic- but not GnRH-receptors undergo rapid, partial desensitization. Further, sustained GnRH-mediated depletion of $PtdIns(4,5)P_2$ contrasts to a transient depletion by MC suggesting that lack of substrate may not underlie desensitization in αT3-1 cells. We also demonstrate agonist-dependent phosphorylation of muscarinic m3 receptors. Although the lack of a suitable antibody precludes similar assessment of GnRH receptors, we suggest a lack of phosphorylation sites may underlie the lack of desensitization.

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86P INCREASED BASAL CYCLIC GMP LEVELS IN CORONARY ARTERIES FOLLOWING HEART FAILURE

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In heart failure, large epicardial coronary arteries exhibit increased endothelium-dependent relaxations to acetylcholine (Ach;Larosa *et al.*, 1994), but no change in the relaxation response to nitroglycerin (GTN;Forster *et al.*, 1990). A possible mechanism for this exaggerated response is an increased activation of nitric oxide synthase resulting in enhanced production of cyclic GMP. Accordingly, we measured basal, Ach- and GTN-stimulated cyclic GMP levels in canine circumflex coronary arteries before and at heart failure.

Twenty male mongrel dogs were divided into 2 groups (non-paced; N=9 and paced [right ventricle at 250 bpm for 4 weeks] to end-stage heart failure; N=11: Larosa & Forster, 1996). The dogs were killed and the circumflex arteries were dissected and cyclic GMP levels were determined in endothelium-intact and denuded segments in response to Ach (10^{-5}M) and GTN (10^{-5}M). Briefly, arterial rings [mean ± s.e.m. weight: non-paced (intact), 30.5 ± 1.6mg; non-paced (denuded), 34.5 ± 2.9mg; heart failure (intact), 36.8 ± 1.5mg; and heart failure (denuded), 38.2 ± 2.5mg] were pulverised under liquid nitrogen. Trichloroacetic acid (6%) was added, incubated (on ice) for 1 hour and then centrifuged for 15 min at 4,000 x g. The supernatants were extracted with water-saturated ether. Cyclic GMP was measured using radioimmunoassay, and expressed as $pmol.ml^{-1}$ (mean ± s.e.m.). Table 1 shows the cyclic GMP levels in intact and denuded coronary arteries from non-paced and paced dogs where * is $P<0.05$ vs intact, ^ $P<0.05$ vs basal and # vs non-paced.

These data show: (1) basal and Ach-stimulated cyclic GMP are lower in endothelium-denuded arteries; (2) Ach increases cyclic GMP in both non-paced and paced endothelium-intact arteries; (3) basal cyclic GMP is increased in paced coronary arteries; (4) basal and Ach-stimulated cyclic GMP is decreased in heart failure denuded arteries; and (5) GTN increases cyclic GMP regardless of endothelial and heart failure status. This suggests that the mechanism for enhanced endothelium-dependent relaxations in coronary arteries in heart failure is, in part, due to a composite of increased basal and Ach-stimulated nitric oxide synthase relative to a decreased activation of nitric oxide synthase in endothelium-denuded coronary arteries.

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Table I. Cyclic GMP levels in canine coronary arteries.

	NON-PACED		PACED	
	intact	denuded	intact	denuded
basal	6.3 ± 2.9	1.8 ± 0.9	13.2 ± 4.4#	0.4 ± 0.1*#
Ach	30.1 ± 4.4^	3.1 ± 0.7*	27.9 ± 4.9^	0.7 ± 0.4*#
GTN	30.0 ± 10.0^	54.2 ± 11.5^	35.1 ± 6.4^	33.4 ± 15.5^

87P INHIBITION OF THE ACTIVITY OF POLY(ADP-RIBOSE) SYNTHETASE REDUCES INFARCT SIZE IN A RABBIT MODEL OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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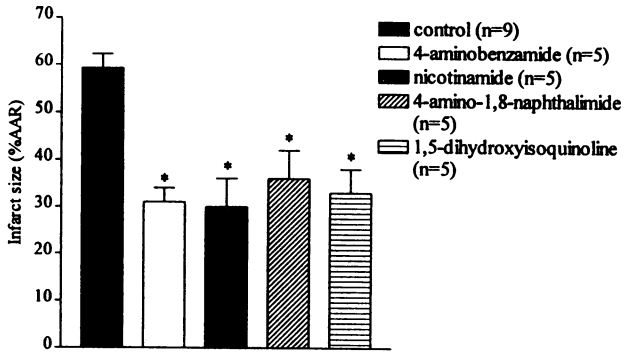
Reperfusion of the ischaemic myocardium results in the generation of oxygen-derived free radicals (Bolli *et al.*, 1988). In cultured cells, radicals cause strand breaks in DNA, which activate the nuclear enzyme, poly (ADP-ribose) synthetase (PARS). This results in a depletion of intracellular NAD and ATP, and ultimately cell death (Schraufstatter *et al.*, 1986). At this meeting we demonstrate that 3-aminobenzamide, an inhibitor of PARS activity, reduces infarct size, when given before reperfusion, of the previously ischaemic rabbit myocardium (Bowes & Thiemermann). Here we investigate the effects of several, chemically distinct, inhibitors of PARS activity on infarct size resulting from regional myocardial ischaemia and reperfusion in the anaesthetised rabbit.

Male New Zealand white rabbits (2.5-3.5 kg) were premedicated with Hypnorm (0.1 ml·kg^{-1}, i.m.). General anaesthesia was induced (20 mg·kg^{-1}, i.v.) and maintained with sodium pentobarbitone. The animals were ventilated with room air. A left intercostal thoracotomy was performed, and a ligature was placed around the first antero-lateral branch of the left coronary artery (LAL). Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded. The LAL was occluded for 45 min and reperfused for 2 h. 4-aminobenzamide (10 mg·kg^{-1}), nicotinamide (20 mg·kg^{-1}), 4-amino-1,8-naphthalimide (1 mg·kg^{-1}), 1,5-dihydroxyisoquinoline (1 mg·kg^{-1}) or vehicle (saline or 10% DMSO) were injected into the left ventricle 1 min prior to reperfusion. At the end of the experiment the LAL was reoccluded and Evans blue dye (2%w/v) was injected into the left ventricle for determination of area at risk (AAR). Infarct size was determined by incubation of the AAR with nitro-blue tetrazolium (0.5 mg·ml^{-1} for 20 min.). Values are expressed as mean ± s.e.mean (*p< 0.05 compared to control by ANOVA followed by a Bonferroni's test for multiple comparisons).



AAR (range 37±2% to 36±3% of left ventricle), and haemodynamic parameters were not significantly different between groups (p>0.05). Injection of the PARS inhibitors prior to reperfusion resulted in a 40-50% reduction in infarct size (expressed as a % of AAR, see figure).

Thus, four chemically distinct, inhibitors of PARS activity cause reductions in infarct size and prevent myocardial reperfusion injury in the anaesthetised rabbit. Thus, we propose that activation of PARS by radicals during reperfusion results in a fall in ATP levels and extension of infarct size.

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88P D-MYOINOSITOL 1,2,6 TRISPHOSPHATE (pp56) CAN DISCRIMINATE BETWEEN PHOSPHOLIPASE C (PLC)-DEPENDENT AND PLC-INDEPENDENT MECHANISMS OF CONTRACTION IN CARDIAC MYOCYTES

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The use of D-myoinositol 1,2,6 trisphosphate (pp56) as a selective inhibitor of phospholipase C (PLC)-dependent cellular contraction in rat ventricular cardiomyocytes has been assessed, by investigating the effects of the compound on the contractile responses elicited by :(1) the α-adrenoceptor agonist, noradrenaline (in the presence of propanolol), employed as a positive control, as it acts by a mechanism dependent on the stimulation of PLC; (2) forskolin and isoprenaline, used as negative controls, as they act by PLC-independent processes; (3) neuropeptide Y (NPY), since it has been proposed that pp56 is a specific inhibitor of NPY responses (Edvinsson *et al.*, 1990); (4) endothelin-1 (ET-1), calcitonin gene-related peptide (CGRP), secretin, vasoactive intestinal polypeptide (VIP), for which the contractile coupling mechanisms have not yet been firmly established.

Ventricular cardiomyocytes, isolated from the hearts of adult rats, were stimulated to contract at 0.5Hz in the presence of calcium ion (2 mM). Maximum diastolic shortening (DL) was calculated as a percentage of prestimulus length and data were expressed as differences (ΔDL, mean ± standard error) from basal values, with or without pp56, as appropriate. Statistical analysis was performed using a one-factor analysis of variance followed by a multiple range test (Scheffe).

In the absence of agonist, contractile amplitudes after 20 min preincubation with pp56 were not different from that observed in the absence of pp56. pp56 inhibited the positive contractile response to noradrenaline (NOR, 5μM), elicited in the presence of propanolol (PROP, 500nM), such that the response was almost completely attenuated at the highest concentration of the inhibitor. pp56 did not inhibit the positive contractile responses to forskolin (40μM) or isoprenaline (ISO, 100nM). NPY alone does not influence the basal level of contraction of cardiomyocytes, but can attenuate isoprenaline-stimulated contraction and can increase contractile amplitude from basal when the transient outward current is blocked with 4-aminopyridine (4-AP) (Millar *et al.*, 1991). In the presence of ISO (100 nM), the negative response to NPY (100 nM) was attenuated by pp56. With 4-AP, the positive contractile response to NPY (200 nM) was decreased by pp56, although this was not statistically significant. pp56 inhibited the positive contractile responses to CGRP (1 nM) and ET-1 (20 nM) completely, but did not affect the responses to secretin (20 nM) or VIP (20 nM).

Table 1. Effects of pp56 (1-100μM) on contractile responses (ΔDL) to inotropic agents in rat ventricular cardiac myocytes; n=5 experiments; *p<0.05 with respect to the absence of pp56.

		1μM	10μM	100μM
Basal		-0.4±0.01	-0.5±0.01	-0.5±0.01
NOR(+PROP)	3.2±0.29	1.4±0.77*	0.9±0.56*	0.5±0.34*
Forskolin	7.1±1.10	6.2±0.74	5.8±1.06	7.6±0.52
ISO	2.6±0.24	3.1±0.25	3.3±0.31	3.1±0.56
ISO+NPY	-0.3±0.55	1.1±0.62*	3.0±0.33*	2.3±0.70*
4-AP	1.4±0.26	1.6±0.40	1.3±0.28	1.5±0.34
4-AP+NPY	3.2±0.34	2.5±0.50	1.8±0.25	2.2±0.20
CGRP	2.1±0.63	-0.5±0.40*	0.0±0.33*	-0.3±0.66*
ET-1	1.6±0.29	-0.2±0.21*	-0.4±0.43*	-0.5±0.72*
Secretin	3.2±0.38	4.0±0.42	3.5±0.49	3.6±0.70
VIP	1.7±0.36	2.7±0.28	2.0±0.43	2.2±0.29

In summary, pp56 is a useful pharmacological agent with which to distinguish between PLC-dependent and PLC-independent contractile coupling mechanisms in mammalian ventricular cardiomyocytes. On this basis, it is concluded that the positive contractile responses to NPY, CGRP and ET-1 and the negative contractile response to NPY in these cells are each attributable to the activation of PLC-dependent pathways, while the positive contractile responses to secretin and VIP are mediated by PLC-independent pathways.

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89P FUNCTIONAL STUDIES ON THE MECHANISM OF ACTION OF NICORANDIL IN THE RAT MESENTERIC ARTERY AND ITS POTENTIATION DURING IMPAIRED METABOLISM

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Nicorandil produces vasodilatation of arteries via two mechanisms; an increase in intracellular cGMP and opening of ATP-sensitive potassium (K_{ATP}) channels (Kukovetz *et al*, 1991). Our experiments investigated the actions of nicorandil in rat mesenteric arteries using glibenclamide, a K_{ATP} channel blocker and LY 83583, a guanylyl cyclase inhibitor. The effect of impaired oxidative phosphorylation and glycolysis on nicorandil-induced vasodilatation was studied using cyanide m-chlorophenyl hydrazone (CCCP) and low external glucose.

Adult Wistar rats were killed by cervical dislocation. Second and third order mesenteric arteries were removed and rings were mounted in a small vessel wire myograph for isometric force recordings. The arterial rings were maintained at 36°C in oxygenated 5mMK^+ buffer. All the buffer solutions contained 20μM L-nitro arginine methyl ester. The vessels were contracted using 20mMK^+ solution and 200nM BAYK 8644. Once a stable tone was achieved cumulative dose response curves to nicorandil were obtained. All nicorandil responses were expressed as a percentage of the maximal contraction. Log EC_{50} values were obtained from individual curves and are given as means±s.e.mean (antilog mean, number of measurements). ANOVA and t-tests were used for statistical analysis.

Nicorandil (1 to 300μM) produced a concentration dependent relaxation, with a log EC_{50} of -4.77±0.07 (17μM, 21). Glibenclamide (10μM) caused a significant ($P<0.01$) parallel rightward shift of the nicorandil curve; log EC_{50} -4.32±0.05 (48μM, 6). LY 83583, at 10nM, 0.1μM, 1μM and 10μM produced dose-related rightward shifts, with log EC_{50}s of -4.59±0.09 (26μM, 6), -4.32±0.26 (48μM, 4), -4.16±0.15 (69μM, 5) and -3.48±0.11 (331μM, 4) at each concentration respectively. Cumulative effects were seen when 1μM LY 83583 and 10μM glibenclamide were applied together. 30nM CCCP shifted the concentration effect curve for nicorandil to the left ($P<0.05$), from a control log EC_{50} value of -4.68±0.09 (21μM, 9) to -4.88±0.04 (13μM, 8), while reducing glucose to 0.5mM had a similar effect. These effects were inhibited by glibenclamide, suggesting that the increased potency of nicorandil under conditions of metabolic inhibition resulted from its effects on K_{ATP} channels. In 30nM CCCP+10μM glibenclamide the log EC_{50} was -4.42±0.03 (38μM, 5).

We conclude that nicorandil relaxes rat mesenteric arterial branches by activating both guanylyl cyclase and K_{ATP} channels. The potency of nicorandil is enhanced when metabolism is impaired, an observation that may have significance for its clinical use.

Supported by the MRC. We thank Chugai Pharmaceuticals and Rhône-Poulenc Rorer for the gift of nicorandil.

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90P NITRIC OXIDE (NO) STORE AS DINITROSYL-IRON COMPLEXES IN LIPOPOLYSACCHARIDE-TREATED RAT ISOLATED AORTA: LOCALIZATION AND MECHANISM OF FORMATION

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Recently, we have demonstrated an association between N-acetylcysteine-induced relaxation and the appearance of protein-bound dinitrosyl iron complexes (DNIC) in lipopolysaccharide (LPS)-treated vascular tissue (Muller *et al.*, 1996). The aim of the present study was to investigate the localization of DNIC and the mechanism of their formation.

Whole thoracic aortae or aortic rings from male Wistar rats (10-12 weeks-old) were incubated for 18h at 37°C in Minimum Essential Medium in the absence or in the presence of LPS (10μg/ml), with or without L-arginine (L-arg, 1mM) or N^{ω}-nitro-L-arginine methyl ester (L-NAME, 3mM). In some preparations, the endothelium was removed and/or the adventitial layer was carefully separated from the media with forceps. NO spin trapping studies were performed using diethyldithiocarbamate (DETC)-Fe technique (Vanin *et al.*, 1993). Tissues were incubated for 1h at 37°C with DETC (5mM) and $FeSO_4$ (50μM) in the presence of L-arg (1mM) and then frozen in liquid nitrogen. Electron paramagnetic resonance (EPR) spectra were recorded on an x-band spectrometer at 77K. For quantification of EPR signals, a stable paramagnetic $[Fe^{2+}(S_2O_3^{2-})(NO)_2]$ complex of known concentration was used. Results are expressed as the mean ± s.e.mean of 5-8 experiments. Unpaired Student *t* test was used for statistical comparisons.

Incubation of whole thoracic aorta in the presence of LPS and L-arg resulted in the appearance of a distinct axial EPR signal with $g_{\perp} = 2.04$ and $g_{\parallel} = 2.015$ attributed to DNIC with sulfur groups of protein(s). The concentration of DNIC in the aortae was estimated to be 5.1±0.6μM. No obvious EPR signals for DNIC were detected when the 18h incubation period was performed in the presence of L-NAME, in the absence of L-arg or in the absence of LPS. Removal of the endothelium in aortae after incubation with LPS and L-arg did not change the amount of DNIC (4.9±0.7μM). The media layer contained about 4 times more DNIC than the adventitia (3.1±0.5 versus 0.8±0.1μM, $P<0.01$). However, incubation of the media alone in the presence of LPS and L-arg for 18h did not result in DNIC formation, suggesting the crucial role of the adventitial layer in DNIC generation. This was confirmed by evaluating NO synthase activity (using NO spin trapping) in media and adventitia separated from LPS-treated aortic rings. It was found out that adventitia generated about 6 times more NO than media (13.3±1.7 versus 2.2±0.2 pmol/μgDNA/h, $P<0.01$).

In conclusion, this study demonstrates: (1) the formation of DNIC in isolated rat aorta via LPS-induced, L-arg dependent NO synthase activity; (2) the predominant localization of DNIC in the media layer; (3) the major contribution of adventitial-derived NO in the mechanism of DNIC formation.

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91P NITRIC OXIDE (NO) STORE AS DINITROSYL-IRON COMPLEXES IN LIPOPOLYSACCHARIDE-TREATED RAT ISOLATED AORTA: CYCLIC GMP-INDEPENDENT RELAXING EFFECT OF NO RELEASED BY N-ACETYLCYSTEINE

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We reported that within vascular tissue, nitric oxide (NO) generated by lipopolysaccharide (LPS)-induced NO-synthase activity can be stored as protein-bound dinitrosyl-iron complex (DNIC) (Kleschyov *et al.*, 1996). In addition, we provided evidence that upon reaction with thiols such as N-acetylcysteine (NAC), low molecular weight DNIC can be released from these storage sites and induce vascular relaxation (Muller *et al.*, 1996). The aim of the present study was to investigate the mechanisms of the NAC-induced relaxation in LPS-treated vascular tissue.

Thoracic aortic rings from male Wistar rats (10-12 weeks-old) were incubated for 18h at 37°C in Minimum Essential Medium containing L-arginine (L-arg, 1mM) and LPS (10μg/ml). The rings were then either mounted in organ bath filled with Krebs solution (kept at 37°C and gassed with a mixture of 95% O_2, 5% CO_2) for isometric tension recording, or incubated in similar conditions with the non specific phosphodiesterase inhibitor isobutylmethylxanthine (100μM) for cGMP accumulation studies (by radioimmunoassay). The relaxing effect of NAC is expressed as percentage of precontraction induced by noradrenaline (NA, 3μM) or KCl (25 or 50 mM) plus N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 3mM, in order to block NO generation by NO-synthases), in the presence or in the absence of different inhibitors. Results are expressed as the mean ± s.e.mean of at least 5 experiments. Analysis of variance was used for statistical comparisons.

NAC (0.1 to 10mM, added in a cumulative manner) evoked a concentration-dependent relaxation which was not affected by the selective inhibitor of protein-kinase G, Rp-8-bromoguanosine 3',5'-cyclic monophosphorothioate (Rp-8-BrcGMPS, 60μM, added 30 min before NAC) (Figure 1a). Cyclic nucleotide determination performed on LPS-treated aortic rings showed that L-arg (100μM) evoked a four-fold increase in cGMP level (from 198±26 to 789±83fmol/μgDNA, p<0.001), whereas NAC (10mM in the presence of L-NAME 3mM) did not increase the aortic content of this nucleotide (133±30 fmol/μgDNA) over basal values (122±28 fmol/μgDNA). The relaxing effect of NAC was also studied in the presence of either tetrabutylammonium (TBA, 3mM, as a non-selective blocker of K^+ channels) or glibenclamide (Glib, 10μM, a selective inhibitor of ATP-sensitive K^+ channels). TBA or Glib was added 15 min before NAC. In these conditions TBA, but not Glib, significantly inhibited the relaxation induced by NAC (Figure 1a). The relaxing effect of NAC was also significantly reduced in rings precontracted with depolarizing concentrations of KCl (25 or 50mM) and L-NAME (Figure 1b).

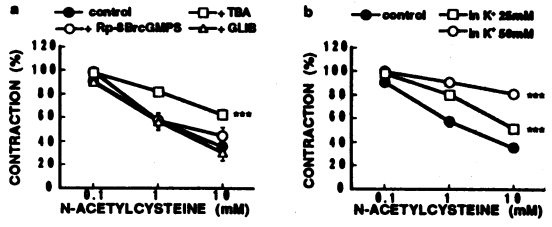

Figure 1: Relaxing effect of NAC obtained on rat aortic rings incubated for 18h in the presence of LPS and L-arg. The effect of NAC was studied on NA (or KCl) plus L-NAME precontracted rings (a) in the presence or in the absence of Rp-8BrcGMPS (60μM), TBA (3mM) or Glib (10μM); (b) in rings precontracted with KCl (25 or 50mM). P< 0.001, in comparison to control . Vertical lines indicate ± s.e.mean; they are not shown when the size of the symbol exceeds the value of the s.e.mean.

Taken together, these results suggest that in LPS-treated vascular tissue, NO released by NAC as low molecular weight DNIC from NO stores (protein-bound DNIC) induces relaxation by cGMP-independent mechanisms, which involve the activation of TBA-sensitive (but glibenclamide-insensitive) K^+ channels.

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92P THE NATRIURETIC PEPTIDES BNP AND CNP ARE POTENT INHIBITORS OF CYTOKINE-STIMULATED NITRIC OXIDE PRODUCTION IN PRIMARY CULTURES OF HUMAN PROXIMAL TUBULAR CELLS

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The natriuretic peptides BNP and CNP possess both renal and vasoactive effects mediated via the NPR-A and B (Davidson & Struthers, 1994, Amin *et al*, 1996). Recent evidence also suggests that both peptides may interact with the NPR-C (Savoie *et al*, 1995) and may also possess immunological properties (Vollmar & Schulz, 1995). We have previously demonstrated that human proximal tubular cell (PTC) nitric oxide (NO) synthase can be stimulated by an immune challenge (Chatterjee *et al*, 1994) and that this response can be inhibited by both ANF and the NPR-C specific ligand $C_{(4-23)}$ANF (Chatterjee *et al*, 1995).

Renal PTC possess immunological functions which may involve NO and natriuretic peptides. In view of the evidence that BNP and CNP possess immunological functions, the aim of this study was to investigate whether these natriuretic peptides could influence cytokine-stimulated NO production by human PTC.

Primary cultures of human PTC were prepared using collagenase digestion, sieving and Percoll density centrifugation of cortex obtained from nephrectomy specimens (Rodilla and Hawksworth, 1996). PTC were cultured on 24 well plates ($1x10^5$ cells/well) in DMEM/Ham's F12 medium containing 10% fetal calf serum and grown to confluence at 37°C. Cultures were either incubated with the combination of cytokines IL-1β (10 u/ml), TNF-α (10 ng/ml) and IFN-γ (100 u/ml) for 24 hours at 37°C or were preincubated with ANF, BNP, CNP or $C_{(4-23)}$ANF (10^{-10}, 10^{-8}, 10^{-6} M) for 24 hours at 37°C prior to incubation with cytokines. NO was measured spectrophotometrically as nitrite after the Griess reaction whilst protein content was determined using the Lowry assay.

Incubation with ANF, BNP, CNP or $C_{(4-23)}$ANF for 24 hours prior to cytokine stimulation produced a significant reduction in NO production in confluent primary cultures of human proximal tubular cells.

Effect of ANF, BNP, CNP and $C_{(4-23)}$ upon cytokine-stimulated NO production by primary cultures of human PTC

	NO production (μM/mg protein/24 hours)		
Peptide concentration	10^{-10} M	10^{-8} M	10^{-6} M
Control	1.66±0.57	1.61±0.55	2.19±0.29
Cytokines only	50.1±7.67$^+$	49.1±7.19$^+$	48.4±5.83$^+$
ANF preincubation	13.0±1.93*	9.55±2.96*	7.67±2.99*
BNP preincubation	11.1±3.73*	9.08±1.87*	8.39±2.07*
CNP preincubation	6.29±2.10*	7.63±0.28*	7.76±1.53*
$C_{(4-23)}$ANF preincubation	7.87±1.43*	8.19±1.21*	7.91±0.60*

$^+$P<0.001 vs. control cells, *P<0.002 vs. cytokine-stimulated cells, n=4

These results demonstrate that like ANF, BNP and CNP are both potent inhibitors of cytokine-stimulated NO production in primary cultures of human PTC. However, at low concentrations, CNP appears to possess twice the inhibitory activity of ANF. The similar response observed for the C receptor specific ligand $C_{(4-23)}$ANF suggests that the inhibition of NO production may be mediated via the NPR-C.

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93P NATRIURETIC PEPTIDES STIMULATED NATRIURETIC PEPTIDE AND RECEPTOR EXPRESSION IN RAT PROXIMAL TUBULAR CELLS

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The natriuretic peptides ANF, BNP and CNP, are known to influence the control of salt and water modulation, cell growth (Appel, 1994) and immune response (McLay, *et al*, 1995) in the kidney, via specific natriuretic peptide receptors (NPR-A, B and C). Previously we described natriuretic peptide receptor subtype switching during *in vitro* culture of rat proximal tubular (RPT) cells (Mistry, *et al*, 1996), which also suggested that the natriuretic peptides may have a regulatory role for NPR expression. Natriuretic peptide regulation of NPR's in the kidney may be important in the regulation of electrolyte transport and cell growth.

The objectives of the present study were to use RT-PCR to identify natriuretic peptide and NPR expression in freshly isolated primary cultures of RPT cells and (ii) to determine how incubation of freshly isolated RPT cells with ANF, BNP and CNP might affect NPR and peptide expression.

Renal proximal tubular cells were isolated from male Sprague Dawley rat (250-300g) kidney cortex by collagenase digestion, sieving and Percoll density centrifugation. Cells were rested on ice in DMEM/Hams F12 for 1 hour, and then incubated with concentrations of 10^{-6}M ANF, BNP and CNP for 1 and 3 hours. Total RNA was prepared from freshly isolated RPT cells and from cells incubated with the natriuretic peptides. Natriuretic peptide and NPR expression were determined using RT-PCR and specific pairs of primers for ANF, BNP, CNP, NPR-A, NPR-B and NPR-C. All PCR products for the natriuretic peptides and NPR were sequenced and compared with those deposited in the GENBANK database.

RT-PCR analysis of freshly isolated RPT cells revealed expression of mRNA for NPR-C, however no mRNA for NPR-A or B could be detected. Similarly no mRNA for the natriuretic peptides could be detected in freshly isolated RPT cells. After 1 and 3 hours incubation of RPT cells with ANF, BNP and CNP, mRNA transcripts became apparent for the natriuretic peptides and the NPR (Table 1 & 2).

Table 1: mRNA transcript expression obtained after 1 hour incubation with the natriuretic peptides ANF, BNP and CNP (n=3)

NPR & Natriuretic Peptides	DMEM	ANF	BNP	CNP
NPR-A	-	-	-	+
NPR-B	-	+	+	+
NPR-C	+	+	+	+
ANF	-	+	+	+
BNP	-	+	+	+
CNP	-	+	+	+

Table 2: mRNA transcript expression obtained after 3 hours incubation with the natriuretic peptides ANF, BNP and CNP (n=3)

NPR & Natriuretic Peptides	DMEM	ANF	BNP	CNP
NPR-A	-	-	-	+
NPR-B	-	+	+	+
NPR-C	+	-	+	+
ANF	+	+	+	+
BNP	+	+	+	+
CNP	-	+	+	+

These results suggest that incubation with the natriuretic peptides accelerates mRNA expression for both the natriuretic peptides and NPR in rat proximal tubular cells.

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94P EFFECT OF NITRIC OXIDE SYNTHASE (NOS) INHIBITION WITH AMINOETHYL-ISOTHIOUREA ON RENAL FUNCTION AND HAEMODYNAMICS IN ENDOTOXAEMIA IN THE RAT

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In septic shock, inhibition of NOS improves hypotension and restores vascular responsiveness to vasopressor agents, but regional perfusion may be impaired. We have investigated the effect of aminoethyl-isothiourea (AE-ITU), an inhibitor of NOS activity with preferential activity against the inducible isoform (Southan *et al.*, 1995), administered as pre- and post-treatment in a model of endotoxaemia assessing systemic and intrarenal haemodynamics and simultaneous renal function in the rat.

Male Wistar rats (245-364g) were anaesthetised with sodium thiopentone (100 mg·kg^{-1} ip). An ultrasonic flow probe was placed around the left renal artery to measure renal blood flow (RBF). A surface Laser Doppler probe was placed on the renal cortex, and a needle probe was implanted into the renal medulla. Clearance of ^{3}H-inulin (C_{in}) (in 0.9% NaCl at 3 ml·h^{-1}) was used as an index of renal function. Endotoxaemia was induced by infusion of lipopolysaccharide from *Escherichia coli* (LPS, 1 mg·kg^{-1} over 30 min). AE-ITU (1 mg·kg^{-1}·h^{-1}) was administered as a constant infusion commencing either 60 min before or 120 min after LPS infusion. Mean arterial pressure (MAP), RBF, cortical (C flux) and medullary (M flux) Laser Doppler flow were measured constantly, and C_{in} was measured hourly.

Infusion of LPS caused a small, delayed fall in MAP from 106±5 mmHg (3 h) to 89±4 mmHg (6 h; P<0.05). This hypotension was not affected by either pre- or post-treatment with AE-ITU (P>0.05). C_{in} fell immediately after LPS to 44±4% of the baseline value and remained reduced for 6 h. Pretreatment with AE-ITU had no effect on C_{in}, whereas C_{in} improved over time to ~80% when AE-ITU was given as post-treatment (Table 1). At 6 h after LPS, there was no significant fall in RBF in any of the groups. However, injection of LPS caused (within 2 h) a 20% reduction in C flux, which remained reduced for the remainder of the experiment. This fall in C flux was not affected by the post-treatment with AE-ITU, but significantly worsened by the pretreatment with AE-ITU (P<0.05). Endotoxaemia caused a transient rise in M flux, followed at 2 h by a 20% fall in M flux. Neither pre- nor post-treatment with AE-ITU had any effect on the alterations in M flux caused by LPS.

Conclusions: (1) The renal dysfunction caused by LPS in this model is not due to changes in perfusion pressure of the renal vascular bed, but (at least in part) due to an enhanced formation of nitric oxide. (2) AE-ITU improves renal function when administered post-endotoxaemia.

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Table 1. Inulin clearance and cortical laser doppler flux following endotoxaemia. * P<0.05 versus control (ANOVA)

		Baseline	Post LPS 1-2 h	Post LPS 3-4 h	Post LPS 5-6 h
C_{in} (% baseline)	Control (n=6)	100	44±4%	47±3%	44±2%
	AE-ITU pre (n=6)	100	38±%	48±4%	44±5%
	AE-ITU post (n=7)	100	36±8%	69±5% *	83±6% *
C flux (% change)	Control	0	-19.7±2.7%	-21.0±4%	-31.1±4.2%
	AE-ITU pre	0	-17.7±2.7%	-37.5±7.9% *	-38.0±8.4%
	AE-ITU post	0	-23.7±9.1%	-23.3±3.9%	-27.3±4.1%

95P EFFECTS OF ENDOTOXIN ON ENDOTHELIUM-DEPENDENT AND INDEPENDENT RESPONSES IN THE RAT ISOLATED PERFUSED HEART

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Septic shock is a hyperdynamic state characterised by low systemic vascular resistance, high cardiac output, hypotension and inadequate tissue perfusion. The cardiovascular features of septic shock result from a complex cascade of endogenous mediators whose release is triggered by endotoxin (Bogle & Vallance, 1996). Endotoxin induces expression of inducible nitric oxide synthase which leads to vasorelaxation and hyporesponsiveness to vasoconstrictors. The aim of this study was to determine whether endotoxin also alters responses to dilator agents and to explore the specificity to any hyporesponsiveness observed.

Male Wistar rats (220-300g) were stunned, exanguinated and the thorax opened. The heart was excised and perfused retrogradely with Krebs solution through the aorta at a constant flow of 10 ml min^{-1} at 37°C. Changes in coronary perfusion pressure (CPP, mmHg) were recorded. To assess the effects of vasodilators CPP was increased by perfusion with Krebs solution containing 3.2mM K^+ (Criddle *et al.*, 1991). Hearts were also obtained from animals treated with endotoxin (*E. Coli.*, 055:B5, 2.5mg kg^{-1}, i.p.) for 2, 6 or 24h. Nitrate levels in plasma samples were measured by capillary electrophoresis. Endothelium-dependent and independent responses were assessed by injection (10µl) of either bradykinin (BK) or sodium nitroprusside (SNP) into the coronary perfusate. Results are mean±SEM with $P<0.05$ considered statistically significant (unpaired t-test).

Endotoxin resulted in an increase ($P<0.05$) in plasma nitrate levels from 4±1µM in control animals to 30±6µM at 2h, 426±121µM at 6h and 47±3µM at 24h (n=4). In control animals basal CPP was 53±2mmHg which was increased ($P<0.05$) to 108±3mmHg following perfusion with 3.2mM K^+ for 10min. Hearts obtained from animals treated with endotoxin for either 2h or 6h had significantly higher basal CPP (99±7 and 83±9mmHg respectively; $P<0.05$, n=5). CPP was not further increased by perfusion with 3.2mM K^+. Injection of BK (10^{-14}-10^{-9}mol) or SNP (10^{-13}-10^{-8}mol) resulted in a dose-dependent reduction in CPP. The response to BK or SNP was reduced 6h after endotoxin injection. Maximum dilation to BK was reduced from 55±2 to 25±2mmHg (n=5, $P<0.05$) and to SNP from 57±5 to 17±4mmHg (n=5, $P<0.05$). In contrast, 24h after endotoxin treatment CPP responses evoked by BK or SNP (54±2mmHg and 56±1mmHg respectively) were not significantly different from control values (n=4). Concentration-dependent relaxation to the endothelium independent, non-nitric oxide releasing vasodilator nicardipine (10^{-10}-10^{-5} mol) were not significantly different in animals treated with endotoxin for 6h (n=5).

These results suggest that endotoxin results in coronary vascular dysfunction involving endothelium-dependent and -independent pathways. The dysfunction may lie at the level of guanylate cyclase since responses to endogenous and exogenous nitric oxide are impaired.

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96P ROLE OF INTRACELLULAR CALCIUM STORES IN FLOW- AND AGONIST-EVOKED NO RELEASE FROM ENDOTHELIUM IN RABBIT ISOLATED ABDOMINAL AORTA

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It has been reported that thapsigargin (TSG), an inhibitor of the endothelial endoplasmic reticulum (ER) Ca^{2+}-ATPase, abolishes release of NO in response to agonists but not to low levels of shear stress in intact vessels (Macarthur *et al.*, 1993). It has also been demonstrated that ryanodine receptors are present in vascular endothelium (Lesh *et al.*, 1993) but their involvement in agonist- or flow-induced NO release remains to be established. We have attempted to clarify the role of intracellular Ca^{2+} stores in flow- evoked NO release over a range of physiological shear stress levels and investigated the potential involvement of the ryanodine receptor in flow- and agonist-evoked NO release.

Relaxations to increases in shear stress and acetylcholine were studied, at 37°C, in intact segments of abdominal aorta, from male N.Z. white rabbits (2-2.5 kg), in cascade bioassay (see Hutcheson and Griffith, 1996) and as isolated rings in organ bath. Indomethacin (10 µM) was present in the perfusate to eliminate the effects of prostanoids. In cascade bioassay shear stress was manipulated with dextran (1-4% w/v, 80000 MW) to increase perfusate viscosity. Experiments were performed prior to and following a 20 minute incubation of donor aorta and recipient ring with TSG (1 µM) and ryanodine (30 µM) or in the continuous presence of the ER Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA, 10 µM). L-NAME (100 µM) was used to confirm the specificity of the responses and sodium nitroprusside was used to assess smooth muscle function.

In cascade bioassay all three agents induced relaxations of the recipient ring (CPA 24.4±3.8%; TSG 63.6±10.5%; ryanodine 23.6±1.6%, expressed as % of phenylephrine tone) which were abolished by 100 µM L-NAME. Co-incubation with superoxide dismutase (200 U/ml) abolished relaxations to ryanodine and significantly reduced TSG-induced relaxations to 23.2±3.8% (n=4, $P<0.01$). In isolated rings only CPA and thapsigargin induced L-NAME-sensitive relaxations (CPA 52.7±6.5%; TSG 61.3±7%). Viscosity-related NO release was significantly attenuated at the highest viscosity (1.93 mPa.s) by CPA (19.2±2.4% to 8.8±1.6%, $P<0.001$; n=6) and TSG (21.8±3% to 15.2±3.7%, $P<0.05$; n=7) but not by ryanodine (19.8±1.7% to 20.9±1.6%, n=6). Acetylcholine-induced relaxations in both preparations were abolished by pretreatment with CPA and TSG but were again unaffected by ryanodine. Ryanodine and CPA caused a small but significant increase in the EC_{50} to SNP by 0.24±.08 µM to 0.61±0.1 µM, n=7, $P<0.05$ and 0.26±0.11 µM to 0.63±0.1 µM, n=6, $P<0.05$.

We conclude that release of Ca^{2+} from endothelial CPA- and TSG-sensitive intracellular stores is necessary for stimulation of NO release in response to both acute changes in shear stress and acetylcholine in rabbit abdominal aorta. However, ryanodine has no effect on acetylcholine- or flow-induced NO release indicating that Ca^{2+}-induced Ca^{2+} release via the ryanodine-sensitive Ca^{2+} release channel plays no significant role in these responses.

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97P NITRIC OXIDE (NO) RELEASE BY THE ISOLATED PERFUSED RAT HEART: IS QUANTIFICATION WITH AN ON-LINE AMPEROMETRIC SYSTEM POSSIBLE?

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Conflicting data have been reported with regard to the release of NO by the isolated perfused heart. According to Kelm and Schrader (1990), who measured NO on the basis of its capacity to convert oxyhemoglobin into methemoglobin, the guinea pig heart releases 160 pmol/min, whereas Engelman *et al.* (1995), using an on-line amperometric sensor (ISO-NO, World Precision Instruments, Inc., Sarasota, FL, USA), measured right atrial NO concentrations of 260 nM in the working rat heart preparation. We used the above mentioned on-line amperometric sensor in order to quantify NO release by the rat heart under basal and stimulated conditions. Hearts were obtained from Wistar rats (300-400 g) and perfused according to Langendorff at 37 °C. The lowest detectable NO concentration was ≈ 1 nM. NO was measured both in the pulmonary artery (PA) and the right atrium (RA). After preparation and a 30-min stabilisation period, hearts were perfused with L-arginine (L-Arg) (0.3-30 mM, final conc. in aorta) or bradykinin (BK) (0.1-100 nM), followed by a perfusion with NO (1-100 nM). After the NO perfusion a second perfusion with either L-Arg, BK or NO was given. Each consecutive perfusion was followed by a washout period in order to let coronary flow (CF) return to baseline.

CF (baseline 9±2 ml/min; mean±SD, n=11) increased to 16±4 ml/min with L-Arg (n=8), to 20±4 ml/min with BK (n=3) and to 21±3 ml/min with NO (n=11). EC_{50} values were 17±10 mM (L-Arg), 15±7 nM (BK) and 8±5 nM (NO). A second perfusion with L-Arg following NO perfusion did not result in an increase in CF, whereas a second NO or BK perfusion elicited the same flow response as the first perfusion.

Baseline NO release was below the limit of detection, both in the PA and the RA. Moreover, even at doses of L-Arg, BK and NO that elicited maximal flow responses, coronary venous NO remained below the detection limit of the amperometric sensor. Only at NO doses approximately 25-50 fold above its EC_{50} value, NO could be detected in the RA or PA. Based upon data obtained during infusion of even higher doses of NO, it appeared that more than 95% of the arterially delivered NO was metabolised during coronary passage.

Thus, in view of the fact that NO is extracted almost completely in the coronary vascular bed, whereas its maximal effects on CF are reached at arterial concentrations below 50 nM, we conclude that the baseline NO levels in the RA and PA of the isolated perfused rat Langendorff heart are at least 100 fold below the detection limit of the amperometric sensor. Due to its rapid degradation, coronary venous NO remains undetectable in the presence of concentrations of L-Arg, BK and NO that elicit maximal responses on CF. The decreased sensitivity of the heart to L-Arg following NO perfusion is in agreement with the contention that NO inhibits NO synthase (Buga *et al.*, 1993).

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98P THE ROLE OF POTASSIUM CHANNELS IN RELAXATION OF THE RABBIT ISOLATED CAROTID ARTERY TO THE NO-DONOR SIN-1

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Acetylcholine (ACh)-evoked relaxation and repolarization of the rabbit carotid artery are both mediated by the release of nitric oxide (NO; Plane *et al.*, 1996). NO can activate large conductance calcium-dependent potassium channels in vascular smooth muscle cells either directly (Bolotina *et al.*, 1994), or via cyclic GMP (Robertson *et al.*, 1993). However, the contribution which these pathways make to NO-evoked relaxation is unclear. In this study, the role of potassium channels in relaxation to the NO donor 3-morpholino-sydnonimine (SIN-1) was investigated.

New Zealand White rabbits of either sex were anaesthetised (sodium pentobarbitone, 60 mg kg^{-1}) and killed by rapid exsanguination. Segments of the carotid artery (2 mm in length) were mounted in a myograph under a normalised tension and maintained at 37°C in oxygenated Krebs buffer containing indomethacin (1 μM). All data are expressed as mean ± s.e. mean. Differences between mean values were calculated using the Student's t-test.

The resting membrane potential of the smooth muscle cells was -55.2 ± 4.6 mV (n=20 cells from 12 tissues). Phenylephrine (PE; 1 μM) evoked depolarization and contraction of the arterial segments which was concentration-dependently reversed by SIN-1 (0.1-10 μM). The maximum reversal of depolarization and tension by SIN-1 (10 μM) was 92.5 ± 4.5 % and 97.5 ± 3.4 % (n=4), respectively. In the presence of raised extracellular potassium (25 mM), SIN-1-evoked repolarization and relaxation was significantly attenuated (maximal responses reduced to 26.5 ± 7.6 % (n=3) and 67.9 ± 2.5 %; n=7; P<0.01, respectively). Neither charybdotoxin (CTX; 50 nM), iberiotoxin (100 nM) nor apamin (500 nM) significantly altered responses to SIN-1 (n=4; P>0.05), if the concentration of PE used in the presence of the potassium channel inhibitors was reduced (0.3-0.5 μM) to ensure that the level of depolarization and tone was comparable to controls. However, if 1 μM PE was used to stimulate the tissues in the presence of CTX, significant inhibition of both SIN-1-evoked relaxation and repolarization was observed (n=4; P<0.01), although raising the level of depolarization and tone with PE alone did not significantly alter SIN-1-evoked responses (n=4; P>0.01). Pre-incubation with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10μM; 10 mins), an inhibitor of soluble guanylyl cyclase, almost completely inhibited repolarization and relaxation to SIN-1 reducing the maximum responses to 5.0 ± 4.5 % (n=3) and 8.8 ± 2.3 % (n=5: P<0.01), respectively, and abolished SIN-1-evoked increases in cyclic GMP (n=6 P<0.01). In contrast, ODQ had much less effect on relaxation and repolarization to ACh (0.03-10 μM) and around 59 % of both components of the response persisted in the presence of the inhibitor (n=4; P<0.01).

These data demonstrate that, in the rabbit carotid artery, SIN-1-evoked relaxation is accompanied by marked repolarization which does make a significant contribution to the change in tone. However, the identity of the potassium channel mediating SIN-1-evoked repolarization is unclear, as inhibition of the response in the presence of CTX was only observed at higher levels of pre-stimulation. Both relaxation and repolarization to SIN-1 were almost abolished by ODQ indicating that activation of soluble guanylyl cyclase may underlie both components of the response. This is in contrast to endothelium-derived NO which appears to evoke smooth muscle repolarization and relaxation in the absence of increases in cyclic GMP. In conclusion, this study demonstrates that differences appear to exist between the actions of the NO-donor SIN-1 and endothelium-derived NO in the carotid artery.

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99P LEFT VENTRICULAR HYPERTROPHY AND CORONARY MICROVASCULAR ENDOTHELIUM IN THE GUINEA-PIG: PHENOTYPIC CHANGES IN NITRIC OXIDE SYNTHASE AND ANGIOTENSIN CONVERTING ENZYME ACTIVITY

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Left ventricular hypertrophy (LVH) is associated with endothelial cell dysfunction (Shah, 1996). Though the mechanism of this dysfunction is unknown, evidence suggests it involves changes in the enzymes nitric oxide synthase (NOS) (Shah, 1996) and angiotensin converting enzyme (ACE) (Schunkert *et al.*, 1990). In the present study we investigated changes in the activity of these enzymes in coronary microvascular endothelial cells (CMVE) freshly isolated (Piper *et al.*, 1990) from a guinea pig, supra-renal aortic-banded (band diameter 0.5mm), pressure overload model of LVH (method as used by Linz & Scholkens (1992) for rats), with optimum LVH developing 6 weeks post-banding. NOS activity was measured indirectly via cGMP production (Lang *et al.*, 1988) and ACE activity by the method of Holmquist *et al.*, (1979). Data are expressed as mean±s.e.mean (n≥6). Freshly isolated CMVE from both control and LVH animals responded to exposure to sodium nitroprusside (SNP, 1μM for 2min) with similar increases in cGMP (4.52±0.54 [n=6] & 4.37±0.48 fmol/μg protein respectively). In the CMVE from control animals, exposure to bradykinin (BK) and the calcium ionophore A23187 (both 1μM for 90s) induced significant ($P<0.01$) increases in cGMP from 0.82±0.07 to 1.63±0.13 & 2.57±0.25 fmol/μg protein respectively. In the CMVE from animals with LVH, the basal cGMP levels were significantly ($P<0.05$) lower (0.41±0.05 fmol/μg protein) compared to control animals, as were the cGMP responses to BK and A23187 (both 1μM for 90s) (0.95±0.15 & 1.06±0.14 fmol/μg protein [$P<0.01$ cf. LVH basal levels] respectively). The latter increases in cGMP were completely inhibited by pre-incubation with the NOS inhibitor L-nitro arginine benzyl ester (1μM for 20min) in cells from both groups of animals. Freshly isolated CMVE also express ACE activity (0.36±0.04 U/mg protein), which was significantly ($P<0.01$) higher in the LVH animals (1.06±0.11 U/mg protein). Thus in this model of LVH, CMVE exhibit impaired NO release and increased ACE activity. It is likely that these changes contribute to the development of LVH following aortic banding in the guinea pig.

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100P ACETYLCHOLINE INDUCES NO-DEPENDENT VASODILATATION IN THE HEPATIC ARTERIAL VASCULATURE OF THE ISOLATED DUAL-PERFUSED RAT LIVER PREPARATION

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We have shown that nitric oxide (NO) plays a prominent role in ACh and ATP-induced vasodilatation in the hepatic arterial (HA) vascular bed of the perfused rabbit liver (Browse *et al* 1994). However ACh induces an NO - independent hyperpolarisation in isolated ring segments of (extrahepatic) rat HA (Zygmunt *et al* 1994). This study was conducted to determine whether ACh and ATP also induce NO-independent vasodilatation in the HA vascular bed of the perfused rat liver.

6 male Wistar rats (250-300g) were anaesthetised with sodium pentobarbitone (3mg $100g^{-1}$ i.p.) and the common bile duct, common HA and portal vein (PV) were cannulated under an operating microscope. The livers were excised and perfused with Krebs-Bülbring buffer (pH 7.4, 37^0C) saturated with 95% O_2/5% CO_2 via the HA and PV cannulae at constant flow rates of 0.53±0.03 (mean±SE) and 1.47±0.09 ml min^{-1} g $liver^{-1}$ respectively, according to our previous protocols (Yang *et al* 1995). After a 10-15 min equilibration period, the vascular tone was raised by the addition of 3μM methoxamine. The basal perfusion pressures of the HA and PV were 44.2±0.8 and 12.8±3.5 mmHg respectively following equilibration. The HA perfusion pressure increased to 130.8±7.2 mmHg and remained stable after addition of methoxamine. There was no measurable difference in the PV perfusion pressure after addition of methoxamine. Dose-related HA vasodilatation, measured as transient decreases in perfusion pressure, was observed following HA bolus injections (50μl) of ACh (10^{-8} - 10^{-5}moles) and sodium nitroprusside (SNP) (10^{-6}-$5x10^{-4}$moles). ATP (10^{-8} -10^{-5}moles) produced dose-related vasoconstriction and was measured as transient increases in perfusion pressure. 3 dose-response curves were completed and the NO synthase inhibitor L-N-arginine methyl ester (L-NAME) was then added to the perfusate at a concentration of 300μM and the curves repeated.

SNP produced HA, endothelium-independent (direct smooth muscle) vasodilatation in a dose-related manner (- log ED_{50} =5.02±0.20, G_{max}=57.5±3.5mmHg),which was not significantly attenuated by L-NAME (-logED_{50} = 4.85±0.26, G_{max}= 56.6±4.3mmHg). ACh produced dose-related HA vasodilatation (-logED_{50} = 6.40 ± 0.08, G_{max} = 53.3 ± 2.84mmHg) which was significantly inhibited by L-NAME (-logED_{50}= 6.57±0.13, G_{max} =32.5 ± 2.82 mmHg***). ATP produced HA dose-related vasoconstriction which was significantly enhanced by L-NAME at -5.3 log moles ATP (94.0 ± 9.24 vs 127.0 ± 7.9mmHg***, before and after L-NAME respectively). There were no measurable pressure changes in the PV to HA injections of ACh, ATP or SNP. In summary, ACh induces NO-dependent vasodilatation and ATP predominantly induces vasoconstriction. Our observations indicate that the vascular reactivity of the (intrahepatic) HA vascular bed differs from the previously reported data in the extrahepatic rat HA. $^{**}P<0.01$, $^{***}P<0.001$, Student's paired t-test. Results are mean ± sem.

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101P CONTROL OF VASCULAR TONE BY ENDOTHELIN-1 IN HUMAN PIAL ARTERIES

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We previously demonstrated that activation of α_2-adrenergic receptors (α_2-AR) decreases basal and serotonin-induced production of endothelium-derived endothelin-1 from isolated segments of rabbit cerebral artery (Thorin *et al.*, 1996). The aim of this study was to further characterize the involvement of endothelial α_2-AR and endothelin-1 in the regulation of tone of isolated human pial artery.

Segments of human pial arteries were isolated from redundant cortex tissues obtained during brain surgery. Rings (459±36µm outer diameter, 2mm long, n=32 from 8 donors) were mounted on a microvessel myograph within 1 h of surgery. Changes in isometric tension were recorded. Contractions are expressed as % of the maximal response (E_{max}) to 127mM KCl-physiological salt solution. Relaxations are expressed as % inhibition of the preconstricting tone. In some experiments, endothelium was removed by gentle scraping of the lumen with a hair. All experiments were performed in the presence of indomethacin (10µM). Results are given as s.e.mean. n=number of rings (at least 3 different donors were used per protocol).

In control arterial segments, serotonin (10µM) induced tone (21±6% E_{max}) that was potentiated by removal of the endothelium (42±8% E_{max}, n=4; P<0.05 *versus* control). Oxymetazoline (α_2-AR agonist; 0.01-30µM) induced a concentration-dependent relaxation of preconstricted rings. After endothelial denudation, the relaxation was significantly decreased: 10µM oxymetazoline induced 106±7% relaxation in control compared to 20±11% relaxation in denuded arteries (n=4; P<0.05).

In intact vessels, inhibition of nitric oxide synthase with N^{ω}-nitro-*l*-arginine (*l*-NA; 100µM) increased basal tone (7±1% E_{max}, n=10). Serotonin-induced contraction was potentiated (49±5% E_{max} *versus* 21±6% E_{max} in control, P<0.05), whereas oxymetazoline-induced relaxation was attenuated; for example, 10µM oxymetazoline induced 62±7% relaxation compared to 106±7% relaxation in control (n=10; P<0.05). Pretreatment with BQ123 (1µM; ET_A receptor antagonist), in the presence of *l*-NA, decreased serotonin-mediated contraction (5±1% E_{max}, P<0.05 *versus l*-NA). Furthermore, the preconstricting tone was unsustained. In contrast, addition of a subthreshold concentration of endothelin-1 (1nM; n=3) in the presence of *l*-NA potentiated serotonin-induced contraction (74±1% E_{max} compared to 49±5% E_{max} in the presence of *l*-NA; P<0.05). In these conditions, oxymetazoline-induced relaxation was abolished, but not the relaxation mediated by substance P (0.1µM; 88±1% compared to 75±6% relaxation in the presence of *l*-NA).

In summary, serotonin-induced contraction was strongly potentiated by endothelial denudation, nitric oxide synthase inhibition and a subthreshold concentration of endothelin-1. By opposition, inhibition of endothelin-1 ET_A receptors antagonized serotonin-induced tone. Endothelium-dependent relaxation mediated by oxymetazoline was sensitive to nitric oxide synthase inhibition and fully antagonized by exogenous endothelin-1. The results suggest that 1) serotonin induces contraction of intact human pial arteries by stimulating endothelin-1 release; 2) α_2-AR agonists relax serotonin-preconstricted human pial arteries by inhibiting endothelium-derived endothelin-1 release.

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102P THE ATYPICAL CARDIOSTIMULANT β-ADRENOCEPTOR IS DISTINCT FROM β_3-ADRENOCEPTORS AND COUPLED TO A CYCLIC AMP-DEPENDENT PATHWAY IN HUMAN AND RAT MYOCARDIUM

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β_3-Adrenoceptors (β_3AR) mediate cardiodepression in human ventricle through a pertussis toxin-sensitive G (Gi?) protein (Gauthier et al., 1996). In rat atria an atypical β-adrenoceptor, distinct from β_3AR, mediates cardiostimulant effects (Kaumann & Molenaar, 1996). The atypical βAR has also been described in human atrium (Kaumann, 1996) and we now provide more evidence that it is distinct from the β_3AR and likely to be coupled to a cyclic AMP-dependent pathway. Atrial strips paced at 1 Hz at 37°C were incubated with (-)-propranolol (200 nM). Positive inotropic effects of (-)-CGP 12177 (pEC_{50} 7.2 ± 0.1, n = 15) were enhanced (pEC_{50} = 7.7 ± 0.1, n = 15) by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 60 µM) but unaffected by 6-60 µM of the β_3AR agonists SR 58611A, BRL 37344, ZD 2079, CL 316243 or the β_3AR antagonist SR 59230A (6 µM). The β_3AR agonists were devoid of cardiostimulant or cardiodepressant effects. (±)-Cyanopindolol (CYP) caused inconsistent positive inotropic effects (2 out of 6 patients) but consistent effects (pEC_{50} = 6.9 ± 0.1, n=8) in the presence of IBMX. (-)-CGP 12177 (1 µM) and CYP (1 µM) in the presence of (-)-propranolol 200 nM and IBMX 60 µM increased contractile force of human ventricular trabeculae by 23.2 ± 4.3 % (n = 4) and 9.9 ± 3.4 % (n = 3) respectively of the effect of (-)-isoprenaline (600 µM). (-)-CGP 12177 and CYP hastened relaxation of both atrial and ventricular tissues.

Experiments in rat atria are also consistent with a cyclic AMP pathway. In rat left atria (paced at 2 Hz in the presence of 200 nM (-)-propranolol) IBMX (10 µM) increased the pEC_{50} of (-)-CGP 12177 from 7.67 ± 0.03 (n = 6) to 8.23 ± 0.10 (n = 6). In paced rat left atria (200 nM (-)-propranolol) (-)-CGP 12177 (10 µM) and (-)-isoprenaline (200 µM) enhanced cyclic AMP levels (measured by enzyme-immunoassay) from a basal of 13.7± 1.0 (n = 10) to 21.6 ± 2.0 (n = 7) and 33.1 ± 3.1 (n = 5) $pmol.mg^{-1}$ protein respectively. All data are expressed as mean ± s.e.mean.

We suggest that the cardiac atypical βAR is distinct from the β_3AR and coupled to a Gs protein/adenylyl cyclase system.

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103P ALLOSTERIC MODULATION BY ALCURONIUM, GALLAMINE AND W84 IS NOT A COMMON FEATURE OF G-PROTEIN COUPLED RECEPTORS BUT SPECIFIC FOR CARDIAC MUSCARINIC RECEPTORS

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The dissociation of radioligands from the muscarinic M_2-receptor can be retarded by compounds which allosterically interact with the binding site. These allosteric modulators posses a heterogenous stucture (Lee & El-Fakahany, 1991) and display different potencies of stabilizing the antagonist-binding to the receptor (Tränkle *et al.*, 1994). Muscarinic M_2-receptors belong to the superfamily of G-protein coupled receptors. The aim of the present study was to investigate whether allosteric modulators of muscarinic M_2-receptors show any effect on the dissociation characteristics of other G-protein coupled receptors, namely cardiac left ventricular α_1-adrenoceptors (G_q), ß-adrenoceptors (G_s), and cerebral A_1 adenosine receptors (G_i).

The effects of three representative allosteric modulators, alcuronium, W84 (N,N,N',N'-tetramethyl-N,N'-bis-(3-phthalimido-propyl)-N,N'-hexane-1,6,-diyl-bis-ammonium dibromide), and gallamine on the equilibrium binding and the dissociation of the adenosine A_1-receptor ligand [^{3}H]-cyclopentyl-dipropylxanthin (CPX), the α_1-adrenoceptor ligand [^{3}H]-prazosin (PRAZ) and the ß-adrenoceptor ligand (-)-[^{125}I]-iodocyanopindolol (ICYP) have been investigated. The membrane fraction of homogenized rat cardiac left ventricle (ICYP, PRAZ) or rat whole forebrain (CPX) was incubated for 45 min with PRAZ or for one hour with ICYP or CPX, respectively. At the end of the incubation period the specific binding or, after the addition of an excess concentration of unlabelled cyclopentyl-dimethylxanthin (A_1, 10 µM), phentolamine (α, 10 µM) or (±)-CGP 12,177 (ß, 10µM) the dissociation was determined in the presence or the absence of the allosteric modulators. All values are given as mean ± SEM for 4-6 observations. CPX showed a monophasic dissociation pattern with a half-life of 6.0 ± 0.3 min in the absence and of 6.0 ± 0.2, 6.5 ± 0.1 and 6.0 ± 0.1 min. in the presence of 30 µM alcuronium, 30 µM W84 and 1000 µM gallamine, respectively. No influence on the CPX-equilibrium binding was detectable. The same monophasic dissociation was observed for PRAZ with the half-lives of 16 ± 1 min in the absence and of 17 ± 1, 8 ± 1 and 13 ± 1 min, respectively. In the presence of the allosteric modulators, the PRAZ equilibrium binding amounted to 36 ± 2% (alcuronium), 93 ± 2% (W84) and 42 ± 2% (gallamine) of control. Apparently, alcuronium and gallamine interfere with the association of PRAZ. Since the dissociation of ICYP was slow and after 3 hours more than 40% of the radioligand were still bound, it was impossible to calculate a reliable half-life. However, at no time point the binding of ICPY in the absence and the presence of 30 µM W84 showed differences. In the presence of gallamine and alcuronium, the dissociation curve of ICYP revealed an initial offset from the starting level followed by the major phase of dissociation being parallel to the control curve. The equilibrium binding of ICYP amounted to 92 ± 6% (alcuronium), 83 ± 8% (W84) and 91 ± 5% (gallamine) of control values, respectively.

In conclusion, the influence on the radioligand dissociation by alcuronium, W84, and gallamine appears to be specific for the muscarinic M_2-receptor and hence it is not a common feature of G-protein coupled receptors.

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104P α_{2C}-ADRENOCEPTORS MEDIATE CONTRACTIONS OF HUMAN SAPHENOUS VEIN

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Subtypes of postjunctional α_2-adrenoceptors of vascular smooth muscle have not been widely studied, due to the limited number of tissues in which they can be demonstrated. We have previously reported that pressor responses to α_2-adrenoceptor agonist in the pithed rat are mediated predominantly by α_{2D}-adrenoceptors, whereas contractile responses in human saphenous vein may be mediated predominantly by α_{2C}-adrenoceptors (Gavin & Docherty, 1996a). We have investigated in more detail the subtype of α_2-adrenoceptor which mediated contractions in human saphenous vein.

Human saphenous veins and male wistar rat aorta were set up (Connaughton & Docherty, 1990) and contracted with noradrenaline (NA) or KCl. Potency of antagonists was assessed as the ability to shift potency of NA. The following antagonist drugs were used in single concentrations: ARC 239, BDF 8933, BRL 44408, chlorpromazine, HV 723, prazosin, SKF 104078, WB 4101, yohimbine (see Connaughton & Docherty, 1990; Smith et al., 1995).

Noradrenaline produced contractions with a pD_2 of 6.18±0.06 (mean ± s.e. mean) and a maximum contraction of 1.27±0.17g (n=45). All antagonists except chlorpromazine produced parallel shifts in noradrenaline concentration-response curves: chlorpromazine (1 µM) produced a non-parallel shift and chlorpromazine (10 µM) reduced the maximum contraction. Chlorpromazine (1 µM), but not prazosin (3 µM), significantly reduced the maximum contraction to KCl in rat aorta. Hence, chlorpromazine may have actions in addition to α_2-adrenoceptor blockade.

The pK_B values obtained for prazosin, ARC 239, yohimbine, BRL 44408 and chlorpromazine in human saphenous vein were 6.62±0.15 (n=15), 7.19±0.15 (n=7), 7.23±0.09 (n=18), 5.72±0.21 (n=5) and 6.98±0.24 (n=4). However, chlorpromazine showed much higher potency in human saphenous vein than would be expected from its affinity for any of the ligand binding sites, a potency which may not reflect its true action at α_2-adrenoceptors.

When chlorpromazine results were omitted, the correlation with the postjunctional α_2-adrenoceptor mediating contraction of the human saphenous vein was best for the human recombinant α_{2C}-adrenoceptor ligand binding site (see Gavin & Docherty, 1996b) (r=0.92, n=8, P<0.001), as compared to correlations with the α_{2B}-ligand binding site of rat kidney (r=0.62, n=8, n.s.) and with the α_{2A}-ligand binding site of human platelet (r=0.23, n=8, n.s.). It is concluded that the contractile responses to NA in the human saphenous vein are mediated by α_{2C}-adrenoceptors.

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105P HYPERTHYROID- AND HYPOTHYROID-INDUCED MODULATION OF α_1-ADRENOCEPTORS AND THEIR SUBTYPES IN RAT LEFT VENTRICLE

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Previously we have shown that the influence of chronic hyperthyroidism on ß-adrenoceptor density in the left ventricle is time-dependent (Zwaveling, et al. 1996). Therefore, we also investigated the influence of L-thyroxine (T4) and propylthiouracil (PTU) treatment on α_1-adrenoceptor density in the left ventricle after 1,4 and 8 weeks of hyper- and hypothyroidism, respectively.
α_{1A}-/α_{1B}-Adrenoceptor distribution was determined after 4 weeks of treatment. Furthermore, in these animals α_1-adrenoceptor mediated responses were investigated in paced (5Hz) Langendorff hearts using methoxamine.
Hyperthyroidism was induced in male Wistar rats by feeding the animals with 5 mg/kg T4-containing chow during 1, 4 and 8 weeks. Hypothyroid rats were obtained by adding 0.05% PTU to the drinking water. α_1-Adrenoceptor density was assessed using [^{3}H]-prazosin as a ligand and α-adrenoceptor subtype distribution was determined by the inhibition of [^{3}H]-prazosin binding using the selective α_{1A}-adrenoceptor antagonist 5-methyl-urapidil.

T4 treatment caused significant increases in serum T3 levels compared to control animals whereas T3 serum levels were decreased after PTU treatment (table 1). The basal left ventricular pressure in hearts from hyperthyroid and hypothyroid was the same as in hearts from control rats. Potency of the inotropic response (pD_2) to methoxamine was increased in hearts from hypothyroid rats (table 1), whereas the maximal response (E_{max}) was the same in all groups. Binding characteristics are presented in table 1, showing that in hearts from hyperthyroid and hypothyroid rats the maximal number of α-adrenoceptor binding sites were decreased, independent of the treatment schedule. Furthermore, the proportion of high affinity binding sites (α_{1A}) is increased in hearts taken from hypothyroid rats. Affinity constants of high- and low affinity sites of the three groups of rats were the same.
From these data we conclude that the numbers of α_1-adrenoceptors after T4 and PTU treatment are not influenced in the opposite way and that the observed down-regulation is not transient. The discrepancy between functional effects and receptor density may be explained by either alterations in post receptor mechanisms or by the subtype specific modulation of α_1-adrenoceptors.

Table 1 serum T3 levels (nmol/l), pD_2-values for methoxamine, α-adrenoceptor density (B_{max}, fmol/mg protein) and % high affinity sites for 5-methyl-urapidil. Data presented as means ± s.e.mean; n=4-9. *P<0.05 vs. control group.

	T3	pD_2	B_{max}	% α_{1A}
controls	1.2±0.1	5.22±0.07	159±9	15.4±1.9
T4-treated				
- 1 week	3.5±0.3*	n.d.	115±9*	n.d.
- 4 weeks	4.1±0.3*	5.23 ±0.11	106±4*	13.6±1.6
- 8 weeks	4.1±0.5*	n.d.	106±9*	n.d.
PTU-treated				
- 1 week	0.55±0.03*	n.d.	83±6*	n.d.
- 4 weeks	0.62±0.06*	5.92±0.16*	88±6*	26.2±2.5*
- 8 weeks	0.64±0.04*	n.d.	69±8*	n.d.

Zwaveling et al. (1996) *Br. J. Pharmacol.* 117, 253P.

106P PHARMACOLOGY OF Ro 70-0004 (RS-100975), A NOVEL α_{1A}-ADRENOCEPTOR (AR)-SELECTIVE ANTAGONIST

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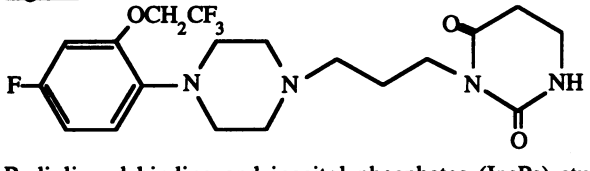
α_1-ARs have been subdivided into α_{1A}-, α_{1B}- and α_{1D}-subtypes. The α_{1A}-AR subtype has received considerable interest, as this subtype predominates in smooth muscle from the lower urinary tract (LUT) of man, and is reported to mediate NA-induced contractions in these tissues (Marshall *et al.*, 1995; Ford *et al.*, 1996a). The present studies have compared the *in vitro* and *in vivo* pharmacology and α_1-AR subtype selectivity of the novel α_1-AR antagonist Ro 70-0004 (Figure 1) with those of prazosin and tamsulosin.

Figure1 Chemical structure of Ro 70-0004



Radioligand binding and inositol phosphates (InsPs) studies (results shown in Table 1) used Chinese hamster ovary (CHO-K1) cells expressing the human cloned α_{1A}-, α_{1B}- or α_{1D}-AR. Binding affinities (pK_i) were estimated from competition studies using [^{3}H]prazosin (whole cells, Ham's buffer, 40min incubation at 37°C). Antagonist affinities (pK_B) to inhibit NA-stimulated InsPs accumulation were obtained as described previously (Ford *et al.*, 1996a).

Antagonist affinities (pA_2) to inhibit NA-stimulated contractions of human isolated LUT tissues or human isolated renal artery were determined (see Ford *et al.*, 1996b). Values obtained were: prazosin 8.7±0.1, 8.8±0.1; tamsulosin 10.4±0.1, 10.2±0.1 and Ro 70-0004 8.8±0.1, 6.8±0.3 (pA_2 for human LUT / artery, mean±s.e.mean, n≥3).

Table 1. Affinity estimates at cloned α_1-AR (CHO-K1 cells).

		Prazosin	Tamsulosin	Ro 70-0004
α_{1A}-AR	pK_i	9.0±.1	10.0±.1	8.9±.1
	pK_B	8.7±.1	10.5±.1	8.6±.1
α_{1B}-AR	pK_i	9.9±.1	9.7±.1	7.1±.1
	pK_B	9.6±.1	9.4±.2	6.7±.1
α_{1D}-AR	pK_i	9.5±.1	9.8±.1	7.2±.1
	pK_B	9.6±.3	9.8±.3	7.1±.2

Values shown are means ± s.e. mean, n≥3.

Studies in anaesthetised dogs comparing the ability of each antagonist (escalating i.v. doses) to inhibit hypogastric nerve stimulation-induced rises in intraurethral pressure (HGNS-IUP) or phenylephrine-induced rises in diastolic blood pressure (PE-DBP) were performed as described in Blue *et al.* (1996). Selectivity ratios (ID_{50} PE-DBP/ID_{50} HGNS-IUP) for prazosin and tamsulosin were 0.6 (95% c.l. 0.3-1.1) and 0.6 (95% c.l. 0.4-0.8). In contrast, Ro 70-0004 was found to inhibit HGNS-IUP at doses markedly below those affecting PE-DBP: ID_{50} doses were 4.7 and 254.5µg/kg respectively, yielding a selectivity ratio of 76.1 (95% c.l. 39.7-145.9).

Thus, binding and functional studies using human recombinant α_1-AR subtypes and isolated tissues have shown that, in contrast to prazosin and tamsulosin, Ro 70-0004 has marked (approximately 50-fold) selectivity for the α_{1A}-AR over the α_{1B}- and α_{1D}-AR. These data, supported by those from studies in anaesthetised dogs, suggest that Ro 70-0004 may have therapeutic advantage over existing treatments for bladder outlet obstruction resulting from benign prostatic hyperplasia.

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107P THE CONSCIOUS "REFLEX-COMPROMISED" RAT: A MODEL FOR EVALUATING THE HYPOTENSIVE POTENCIES OF THE α_1-ADRENOCEPTOR ANTAGONISTS PRAZOSIN, TAMSULOSIN AND Ro 70-0004

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The role of α_1-adrenoceptor (AR) subtypes in the maintenance of blood pressure (BP) is not well understood, although a role for α_{1B}- and α_{1D}-ARs has been suggested (Bylund et al., 1995). In the present study, we developed a conscious rat model in which BP is critically dependent upon α_1-AR tone and then evaluated the hypotensive potency of prazosin (PRA), tamsulosin (TAM) and the novel $\alpha_{1A/1L}$-AR antagonist Ro 70-0004 (3-(3-{4-[fluoro-2- (2,2,2- trifluoro- ethoxy)- phenyl]-piperazin- 1- yl}-propyl)-5-methyl-1H-pyrimidine 2,4-dione).

The femoral artery and vein of male Sprague-Dawley rats (250-400g) were cannulated under ether anaesthesia. Once the incision site was closed, rats were placed in Bollman cages and allowed to recover for 60min. In initial studies, the hypotensive potency of PRA was assessed by constructing cumulative dose-response curves (0.1-3000μgkg^{-1}, iv) in rats (n≥4) pretreated with either vehicle, a β_1-AR antagonist (CGP 20712A; Dooley et al., 1986; $1mgkg^{-1}$, iv), an AT_1-receptor antagonist (L-158,809; Siegl et al., 1992; $1mgkg^{-1}$, iv) or a non-selective α_2-AR antagonist (RS-42206; [8aR-(8aa,12aa, 13aa)]-N-[3-[(5,8a,9,10,11,12a,13,13a-octahydro-3- methoxy -6H-iso-quinol[2,1-g][1,3]naphthyridin - 12(8H) - yl)sulfonyl] propyl] -methanesulfonamide; $0.3mgkg^{-1}$, iv; pK_i at α_2-AR subtypes ≈ 9.3, unpublished data). BP and heart rate were measured 5min after administration of each dose of antagonist.

PRA produced significant (P≤0.05; relative to vehicle) decreases in BP at all doses except 0.1, 1 and 3000μgkg^{-1} (max effect: -16mmHg at 30μgkg^{-1}). Significant tachycardia was observed. The hypotensive effect of PRA was unaffected by pretreatment with CGP 20712A, even though the tachycardia was attenuated. Following pretreatment with L-158,809, PRA (doses ≥ 10μgkg^{-1}) produced significantly greater decreases in BP than PRA alone (max effect: -54mmHg at $3mgkg^{-1}$). The dose-response curve to PRA, however, was biphasic following pretreatment with L-158,809. The curve was rendered monophasic by pretreatment with RS-42206, suggesting that the plateau and second phase resulted from reflexogenic activation of α_2-ARs followed by antagonism of α_2-AR-mediated tone by PRA, respectively. Pretreatment with RS-42206 alone did not alter the hypotensive effect of PRA. Thus, reflexogenic activation of both the renin-angiotensin system and vascular α_2-ARs appear to be major compensatory mechanisms countering α_1-AR-mediated hypotension in conscious rats.

The hypotensive potencies of intravenously-administered PRA, TAM and Ro 70-0004 were subsequently evaluated in conscious "reflex-compromised" rats (rats pretreated with CGP 20712A, L-158,809 and RS-42206). The ID_{50} (μgkg^{-1}; mean: 95% confidence limits) and maximum hypotensive response (mmHg; mean ± s.e.mean) were as follows (n≥4): PRA (6.7: 5.5-8.1; -47 ± 1.4), TAM (2.4: 1.6-3.5; -34 ± 2.7) and Ro 70-0004 (1637.7: 1238.5-2165.6; -38 at $3mgkg^{-1}$; maximum response not calculated as curve did not reach plateau). BP increased significantly at the two highest doses (i.e. 1 and $3mgkg^{-1}$) of tamsulosin. The reason for this increase in BP was not investigated.

Studies with Ro 70-0004 demonstrate clearly that selective antagonism of $\alpha_{1A/1L}$-ARs does not lower BP in reflex-compromised rats. These results indicate the usefulness and sensitivity of the reflex-compromised rat model in assessing the hypotensive potency of subtype-selective α_1-AR antagonists.

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108P THE ROLE OF α_{1D}-ADRENOCEPTORS IN PROSTATIC CONTRACTION EXAMINED USING PROTECTION STUDIES

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Molecular biological, functional and binding studies have previously demonstrated that in human prostate the α_1-adrenoceptor population is predominantly composed of α_{1A} receptors[1]. However, about 30% of the α_1-adrenoceptor mRNA encodes for the α_{1D} receptor[2] but its functional presence in human prostate has yet to be established. In this study, protection experiments using the irreversible antagonist, phenoxybenzamine (PBZ) and the selective competitive α_{1D} antagonist, BMY 7378 (BMY) (pK_i: α_{1A}=6.1-6.6; α_{1D}=8.2-9.4)[3], were performed to determine the functional contribution, if any, of the α_{1D} receptor to α_1-adrenoceptor-induced prostatic responses. For comparison, rat aorta, a tissue known to possess predominantly the α_{1D} receptor subtype, was also used.

Human prostatic strips and denuded rat aortic strips (male wistar rats - 250g) were suspended in gassed Krebs solution (37°C) under a resting tension of 1g. An equilibration period of 90mins was allowed before and after conducting the first agonist dose-response curve with noradrenaline (NA). Tissues were then incubated with PBZ (30 mins) in the presence or absence of BMY. Following a 30min washout, a second curve to NA was obtained. All dose-response curves were conducted in the presence of corticosterone (10μM), cocaine (10μM) and propanolol (1μM).

PBZ reduced the maximum NA-induced response in rat aorta and human prostate in a dose-dependent manner with only 4.8% (aorta) and 30.5% (prostate) of the total response remaining in tissues incubated with 10nM and 10μM PBZ respectively (table 1). PBZ also reduced the potency of NA, decreasing the pEC_{50} value from 6.86 ± 0.15 to 5.68 ± 0.04 for aorta (p<0.001) and from 5.59 ± 0.04 to 5.29 ± 0.06 for prostate (p<0.001).

	Rat Aorta		Human Prostate	
	% max	(n)	% max	(n)
PBZ*	4.8 ± 2	9	30.5 ± 3.6	25
PBZ+BMY 7378				
- 1nM	3.8	2	—	
- 10nM	**89.3 ± 1.7	5	33.3 ± 7.5	10
- 100nM	**98.8 ± 2.6	4	25.5 ± 4.2	15
- 300nM	**102.7 ± 4.3	4	22.8 ± 5.4	10
Control (no PBZ)	98.0 ± 4.2	12	108.9 ± 3.3	27

Table 1 Effect of PBZ and BMY on contractile responses in human prostate and rat aorta. Results expressed as a mean % (±SE) of the pre-antagonist maximum response.
* [PBZ] = 10nM (aorta); 10μM (prostate)
** p<0.001 (student 't' test) compared to PBZ.

In rat aorta, incubation with 100 & 300nM BMY 7378 fully protected α_{1D} receptors with the tissue responses matching those of controls. In contrast, responses in the prostate were not protected by any of the BMY 7378 doses used but instead remained close to those observed with PBZ alone. These results suggest that in human prostate functional α_{1D} receptors do not contribute to the contractile response induced by noradrenaline.

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109P AGONIST AND ANTAGONIST AFFINITY ESTIMATES FOR THE HUMAN CLONED α_{2A} RECEPTOR EXPRESSED IN CHL CELLS USING THE CYTOSENSOR MICROPHYSIOMETER

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The Cytosensor Microphysiometer measures the extracellular pH of living cells superfused with physiological medium. pH changes therefore reflect changes in cellular metabolic activity. In studies to characterise receptors the instrument has been used thus far to estimate antagonist affinities. We have used the cytosensor to study human cloned α_{2A} adrenoceptors expressed in Chinese Hamster Lung cells to determine affinity estimates for both agonists and antagonists.

Cells were grown in DMEM with 5% FBS and 250 μgml^{-1} of G-418 and harvested using Trypsin/EDTA (0.05%/0.53mM). $3x10^5$ cells were plated in each Cytosensor capsule cup and incubated at 37°C in 5% CO_2 for 24 hrs. In the Cytosensor the cells were perfused with DMEM (-bicarbonate, +100μM ascorbic acid, pH 7.35) at 37°C using a 90 s pump cycle of which the pump is off for the final 35 s allowing the accumulation of acid metabolites. The rate of acidification is measured between 60 and 88 s of each pump cycle.

In experiments to estimate antagonist affinities, concentration-effect (E/[A]) curves to the agonist were constructed by non-cumulative addition (6 min exposure) at 40 min intervals. Such E/[A] curves are generated in ~ 6 hours. The maximum response (increase in acidification rate over basal) to noradrenaline (NA) was 86±12% and pEC_{50} was 6.91±0.10 (n=3). NA effects were competitively antagonised by rauwolscine and spiroxatrine (Table 1) with Schild plot slopes of 0.95 (mean, 95% confidence limits 0.91-1.00) and 1.36 (0.95-1.77) respectively. Agonist affinity estimates were determined using irreversible receptor alkylation with phenoxybenzamine (Pbz; Furchgott, 1966). However, in preliminary experiments Pbz (10μM) abolished responses to NA (10μM) which gradually recovered to a maximum of ~70% cf controls at 6 hours. This recovery of responses ruled out construction of E/[A] curves by non-cumulative addition. Instead E/[A] curves were constructed by cumulative addition of the agonist and E/[A] curves were generated in 70 mins. Receptor alkylation was achieved using Pbz (0.32μM) for 15 min. Agonist affinity estimates were made using direct operational modelling techniques (Leff *et al.*, 1990). Affinities for the human α_{2A} receptor are compared to those obtained using conventional ligand binding techniques (Jasper *et al*, 1996; Table 1).

Table 1 Agonist and antagonist affinities for the human α_{2A} receptor. Values are the mean±s.e.mean of 3-5 estimates.

	Cytosensor	*Binding*
Agonists	pK_A	pK_i
Noradrenaline	5.33±0.04	5.12±0.10
A-54741	7.83±0.06	7.38±0.03
Oxymetazoline	7.52±0.03	8.19±0.03
Dexmedetomidine	7.77±0.03	7.99±0.04
Antagonists	pK_B	pK_i
Rauwolscine	8.43±0.02	9.02±0.20
Spiroxatrine	7.08±0.08	7.43±0.06
Prazosin	<5.0	6.12±0.06

The mechanism(s) underlying the recovery of responses following Pbz treatment is being investigated. Whether this problem is associated with other receptor systems is not known but may limit the experimental design adopted for agonist affinity estimation. The similarity of ligand affinity estimates obtained using the cytosensor to those from binding studies demonstrate the utility of this instrument in making both agonist and antagonist affinity estimates for the human cloned α_{2A} receptor.

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110P EFFECTS OF DOPAMINE ON RAT JEJUNAL ELECTROLYTE TRANSPORT THROUGH α_2-ADRENOCEPTORS

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Endogenous dopamine (DA) reduces jejunal sodium transport in young rats submitted to a high salt diet (Finkel et al., 1994). This effect is most probably related to a decrease in Na^+-K^+ ATPase activity (Vieira-Coelho et al., 1997). By contrast, exogenous DA has been described to stimulate active Na^+ and Cl^- absorption in the rabbit ileum; both DA receptors and α_2-adrenoceptors appear to be involved (Donowitz et al., 1982). DA was also found to stimulate ileal water and electrolyte absorption in the rat ileum (Donowitz et al., 1983). The present work was aimed to study the effect of DA on rat jejunal electrolyte transport and to evaluate the type of receptors and the intracellular signaling mechanisms involved in this response. Jejunal epithelial sheets from male Wistar rats (60 days old) were mounted in Ussing chambers (window 0.28 cm^2), maintained at 37°C and bathed on either side with 10 ml of Krebs-Hensleit solution gassed with 95% O2 and 5% CO2; the chambers were connected to an automatic voltage current clamp (WPI DVC 1000) via KCl/agar salt-bridges. Epithelial sheets were voltage clamped and changes in the short circuit current (I_{sc}), an index of electrogenic ion transport, were measured. Drugs were added to the serosal side unless stated otherwise. The results showed that DA (0.1-100 μM) produced a concentration dependent decrease in I_{sc} with a EC_{50} of 1.4±0.1 μM (n=5); the effect of DA was not changed by propanolol (1 μM), prazosin (1 μM and 10 μM) and (±)-sulpiride (1 μM) and completely abolished by phentolamine (1 μM). The addition of phentolamine (0.3 μM) or yohimbine (0.3 μM) produced a rightward shift of the DA concentration-dependent curve with an increase in EC_{50} values to 30.0 ±0.2 μM (n=5) and 11.7 ±0.1 μM (n=5), respectively. The removal of sodium from the medium bathing both chambers resulted in a complete suppression of the effects of DA on I_{sc}. The α_2-adrenoceptor selective agonist, UK14,304, also produced a concentration-dependent decrease in I_{sc} with a EC_{50} of 0.4 ±0.1 μM (n=5); the addition of yohimbine (0.3 μM) increased the EC_{50} value of UK14,304 to 6.8 ±0.1 μM (n=5). 5-(N-ethyl-N-isopropyl)-amiloride (10 μM), a selective Na^+/H^+ exchanger inhibitor added to the apical side, was found not to change the effect of DA, whereas ouabain (1mM) antagonized only the effect produced by the highest concentrations of DA (50 and 100 μM). Forskolin (10 μM) and N^6,2'-O-dibutyryl cAMP (1mM) added to both the apical and serosal sides completely abolished the effect of DA on I_{sc}. In conclusion, DA increases jejunal electrolyte transport (probably sodium) in rat, the effect of which appears to be mediated through an α_2-adrenoceptor. This effect of DA is sensitive to increases in intracellular cAMP and is partially dependent on the activation of Na^+K^+ ATPase.

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111P WHICH RECEPTOR SUBTYPE MEDIATES RENAL NEUROPEPTIDE Y EFFECTS?

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Neuropeptide Y (NPY) reduces renal blood flow (RBF) and in parallel enhances diuresis and natriuresis in anaesthetized rats (Bischoff et al. 1996). Using the subtype-selective agonists NPY, peptide YY (PYY), [Leu^{31},Pro^{34}]NPY and NPY_{13-36} we have previously shown that all three effects are mediated by a Y_1-like NPY receptor subtype (Bischoff et al. 1995). Recently an additional NPY receptor subtype has been cloned and designated Y_5, which is discriminated from Y_1 receptors by its high affinity for the long C-terminal fragment PYY_{3-36} and the low affinity for the Y_1-selective antagonist, BIBP 3226 (Gerald et al. 1996). Since our previous data cannot distinguish Y_1 and Y_5 receptor-mediated effects, we have now compared the agonistic effects of NPY and PYY_{3-36}, and have investigated NPY antagonism by BIBP 3226.

Experiments were performed as previously described (Bischoff et al. 1996). Briefly, in pentobarbital-anaesthetized male Wistar rats BIBP 3226 (10 µg/kg/min) was infused via the femoral vein starting 90 min after completion of the surgery. Thirty min thereafter NPY or PYY_{3-36} (2 µg/kg/min) or saline was infused for another 120 min. RBF was determined by an electromagnetic flow sensor placed on the renal artery. Urine samples were collected via a ureter catheter in 15 min intervals. Urinary sodium concentrations were determined by flame photometry. Date are mean ± s.e. mean of 6-8 animals. Significances of differences for basal values were analyzed by two-tailed t-tests, while treatment effects during the 120 min infusion period were analyzed by two-way ANOVA with $P < 0.05$ considered significant.

Infusion of saline or BIBP 3226 alone did not significantly alter basal RBF (8.0 ± 0.3 vs 7.9 ±0.7 ml/min), urine formation (110 ± 12 vs 114 ± 16 µl/15 min) and sodium excretion (8.5 ± 1.7 vs 10.3 ± 3.2 µmol/15 min). Infusion of NPY significantly reduced RBF with peak reductions of ≈ 1.9 ml/min, followed by a tachyphylaxis, and enhanced diuresis and natriuresis by ≈ 250 µl/15 min and ≈ 40 µmol/15 min, respectively. Concomitant infusion of BIBP 3226 significantly inhibited peak NPY-induced RBF reduction to ≈ 0.8 ml/min. BIBP 3226 reduced NPY-induced diuresis to ≈ 100 µl/15 min and did not significantly alter NPY-induced natriuresis. Systemic infusion of PYY_{3-36} did not reduce but significantly enhanced RBF and enhanced diuresis and natriuresis with the same efficacy as NPY.

We conclude that NPY-induced RBF reductions occur via a classical Y_1 receptor subtype. The NPY effects on natriuresis may occur via the newly discovered Y_5 receptor subtype, while enhancements of diuresis may possibly involve both subtypes. To our knowledge these are the first data implicating the Y_5 receptor subtype, which has previously only been linked with food intake (Gerald et al. 1996), in a peripheral tissues function.

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112P IMMEDIATE PRECURSORS OF NATRIURETIC DOPAMINE AND ANTINATRIURETIC 5-HT SHARE THE SAME UPTAKE TRANSPORTER IN RENAL OK CELLS

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In the kidney, dopamine and 5-hydroxytryptamine (5-HT) have been shown to originate in the same cellular compartment (Soares-da-Silva & Pinto-do-Ó, 1996) - epithelial cells of renal proximal convoluted tubules - and to exert antagonistic actions on tubular reabsorption of sodium and Na^+-K^+ ATPase activity (Soares-da-Silva et al., 1996). The present study was aimed to define the nature of the L-DOPA and L-5-hydroxytryptophan (L-5-HTP) transporter in Opossum kidney (OK) cells and to determine if the two substrates exert upon each other some degree of competition for uptake. OK cells (ATCC 1840-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B and 100 µg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 µg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, OK cells were preincubated (30 min) with Hanks medium with added pargyline (100 µM), tolcapone (1 µM) and benserazide (50 µM). L-DOPA and L-5-HTP were assayed by h.p.l.c. with electochemical detection. Saturation experiments were performed in OK cells incubated for 6 min with concentrations of L-DOPA or L-5-HTP (1 to 2500 µM); non-linear analysis of the saturation curve revealed for L-DOPA and L-5-HTP a K_m of 90 µM (60, 120; 95% confidence limits, n=4-7) and 103 µM (81, 125) and a V_{max} of 13.6±0.5 and 11.2±0.5 nmol mg $protein^{-1}$ 6 min^{-1}, respectively. The kinetic analysis of L-DOPA uptake in the presence of 250 µM L-5-HTP revealed a significant increase ($P<0.05$) in K_m values (179 µM [123, 235]) without changes in V_{max} values (13.3±0.6 nmol mg $protein^{-1}$ 6 min^{-1}). Similarly, L-5-HTP uptake in the presence of 250 µM L-DOPA was accompanied by a significant increase in K_m values (220 µM [126, 313]) without changes in V_{max} values (11.7±0.8 nmol mg $protein^{-1}$ 6 min^{-1}). IC_{50} and K_i values for inhibition of L-DOPA and L-5-HTP uptake by L-5-HTP and L-DOPA, respectively, were determined in the presence of non-saturating (0.25 and 25 µM) or saturating (250 µM) concentrations of the substrates (see table).

L-DOPA	IC_{50} (µM)	K_i (µM)
0.25 µM	251 (116, 545)	250 (115, 544)
25 µM	266 (98, 723)	208 (76, 566)
250 µM	1569 (1318, 1867) *	242 (80, 728)
L-5-HTP	IC_{50}	K_i
0.25 µM	254 (168, 383)	253 (168, 382)
25 µM	220 (109, 447)	177 (87, 360)
250 µM	679 (507, 909) *	220 (164, 295)

(* $P<0.05$ when compared with values for 0.25 and 25 µM)

It is concluded that L-DOPA and L-5-HTP share the same transporter(s) and each compound exerts a competitive type of inhibition upon the other. When competitive inhibition applies, the use of non-saturating or saturating concentrations of substrate does not affect K_i values, whereas IC_{50} values will depend on the concentration of the substrate.

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113P CONTRACTILE EFFECT OF ANGIOTENSIN PEPTIDES IN HUMAN ISOLATED SAPHENOUS VEIN

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The vasoconstrictor effects caused by angiotensin II (AII) have been well studied in various arterial vascular beds. However, limited attention has been paid to its contractile effects in capacitance vessels. Elevated levels of AII may occur under certain clinical conditions, e.g. congestive heart failure (van Zwieten, 1995), during and after cardiopulmonary bypass surgery (Cooper *et al.*, 1985) and antihypertensive treatment with the AT_1-receptor antagonists (Campbell *et al.*, 1995). Therefore, it seems of interest to investigate the effects of AII and its degradation products angiotensin III (AIII), angiotensin IV (AIV) and angiotensin (1-7) in the venous vascular bed.

Human isolated saphenous vein (SV) preparations were obtained from 20 patients who were subjected to aortocoronary artery bypass surgery. After the endothelium had been removed, each piece of SV was cut into 4 mm length rings and set up in a gassed Krebs solution at 37°C, at a tension of 3.5 g, and the isometric force was recorded. The rings were exposed to a 125 mM KCl solution twice and the contraction induced by angiotensin peptides was expressed as percentage of the maximal contraction induced by the second potassium challenge. The effects of cumulative additions of angiotensin peptides were established in the absence or presence of one of the following compounds: the AT_1-antagonist losartan (10 and 100 nM), the AT_2-antagonist PD123177 (1 μM), amastatin (10 μM) and indomethacin (10 μM) on the fresh ring. Tachyphylaxis was investigated by constructing the second series of angiotensin peptide curves after an interval of 60 min on the same ring. The data are presented as mean ± s.e.mean for n observations. Significance ($p < 0.05$) was tested by one way analysis of variance or Student's t test.

In endothelium-denuded SV preparations the cumulative addition of AII (0.1 - 100 nM), AIII (1 nM - 3 μM) and AIV (0.3 μM - 0.1 mM) caused concentration-dependent contractions with comparable maximal responses (AII: 52.7 ± 2.2; AIII: 52.9 ± 2.3; AIV: 57.0 ± 4.4; $p > 0.05$; $n = 5$ - 8). AIII was 16 times less active than AII, whilst AIV was approximately 2700 fold less potent than AII. Angiotensin (1-7) was inactive in this preparation, causing neither contraction nor relaxation. The aminopeptidase-A and -M inhibitor amastatin increased the potencies of AIII and AIV by about 16 and 12 times (pD_2: 8.58 ± 0.05 vs 7.38 ± 0.07 and 6.26 ± 0.07 vs 5.17 ± 0.10, respectively; $p < 0.05$; $n = 5$ - 8), however, no changes in AII potency were observed. Losartan but not PD123177 caused parallel rightward shifts of the curves for AII, AIII and AIV (pA_2: 8.79 ± 0.12, 8.48 ± 0.13 and 8.85 ± 0.05, respectively; $n = 4$ - 5). The cyclo-oxygenase inhibitor indomethacin did not influence the curves for any of the angiotensin peptides studied ($p > 0.05$; $n = 4$ - 5), although AII-induced prostacyclin release has been reported in endothelium-denuded human SV (Barker *et al.*, 1994). Only AII showed strong tachyphylaxis, the maximal response in the second AII curve amounted to 51.3 ± 1.9% of the first one ($p < 0.05$; $n = 4$ - 5). No tachyphylaxis was found for the other angiotensin peptides investigated.

In conclusion, in endothelium-denuded human SV, AIII and AIV are less potent but similarly efficacious vasoconstrictor agents when compared with AII. The contractile responses are mediated by AT_1-receptors but not AT_2-receptors. Endogenous aminopeptidase activity may reduce the effects of angiotensin peptides. Cyclo-oxygenase products are not involved in the contractions induced by angiotensin peptides.

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114P PHARMACOLOGICAL PROFILE OF Z-ETA-1, A NOVEL ORALLY ACTIVE ENDOTHELIN ET_A RECEPTOR ANTAGONIST

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The endothelins (ETs) are a family of vasoactive peptides which have been implicated in a range of cardiovascular disorders (Luscher, 1993). Vasoconstrictor responses to ETs are mediated primarily by the ET_A receptor subtype (Davenport & Maguire, 1994) and thus an orally-active ET_A antagonist has potential for the chronic therapy of cardiovascular disease. The profile of the novel compound N-methyl-2-[4-(2-methylpropyl)phenyl]-3-(3-methoxy-5-methylpyrazin-2-ylsulfamoyl)benzamide (Z-ETA-1) is described.

The affinity of Z-ETA-1 for human ET_A and ET_B receptors was determined in competition binding studies against [^{125}I]-ET-1 using membranes prepared from MEL cells transfected with the recombinant receptor (Whiting *et al.*, 1995). The affinity (mean pIC_{50} [95% confidence limits]) for the ET_A receptor was 8.8 [8.7-8.9] (n=9). No reproducible binding to the ET_B receptor could be demonstrated at concentrations up to 100μM.

Activity *in vivo* after i.v. and oral dosing was assessed against the pressor response to big ET-1 in Alderley Park rats. A partial dose-response curve to big ET-1 (range 0.1-4.0 nmol kg^{-1} i.v.), sufficient to increase mean arterial pressure ≥ 30 mmHg, was obtained prior to, then at various times after, administration of Z-ETA-1 or vehicle (2.5 or 10% DMSO). In pithed rats, the big ET-1 curve was obtained 5 min after i.v. dosing of Z-ETA-1, 0.1 $mgkg^{-1}$ (n=4), or vehicle (n=4). In conscious rats, big ET-1 curves were obtained 30 min, 2h and 4h after oral dosing Z-ETA-1, 2.5 $mgkg^{-1}$ (n=5), or vehicle (n=12). Z-ETA-1, 0.1 $mgkg^{-1}$ i.v., gave a mean dose ratio of 4.5 [2.2-9.2] as compared to vehicle, 0.9 [0.8-1.1] (P<0.05). Oral administration results are shown in Table 1.

Table 1. Antagonist activity of Z-ETA-1 2.5 $mgkg^{-1}$ p.o. in the rat.

	0.5 h post-dose	2 h post-dose	4 h post-dose
Vehicle	1.1 [1.0-1.2]	1.1 [1.1-1.2]	1.0 [1.0-1.2]
Z-ETA-1	6.2 [4.4-8.9] ***	3.3 [1.6-6.6] *	4.0 [2.6-6.3] ***

Results are expressed as mean dose ratios [95% confidence limits] measured at a pressor response of 30 mmHg. *P<0.05, *** P<0.001 vs vehicle, Student's t-test for unpaired data.

The data demonstrate that Z-ETA-1 has high affinity for the human ET_A receptor, has high ET_A : ET_B selectivity, is a potent ET_A receptor antagonist *in vivo* and has a prolonged duration of action after oral administration.

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115P CHARACTERISATION OF ENDOTHELIN RECEPTORS AND CONVERTING ENZYME ACTIVITY IN HUMAN UMBILICAL VEIN *IN VITRO*

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We have characterised the constrictor responses to the endothelin (ET) peptides ET-1, ET-2, ET-3, their precursors big ET-1, big ET-2(1-37), big ET-2(1-38), big ET-3 and the ET_B agonist sarafotoxin 6c (S6c) in human umbilical vein (HUV). The presence of an endothelin-converting enzyme (ECE) was determined by comparing the effect of thiorphan, a specific neutral endopeptidase (NEP) inhibitor and the non-specific NEP/ECE inhibitor phosphoramidon (Turner, 1993) on the response to big ET-1.

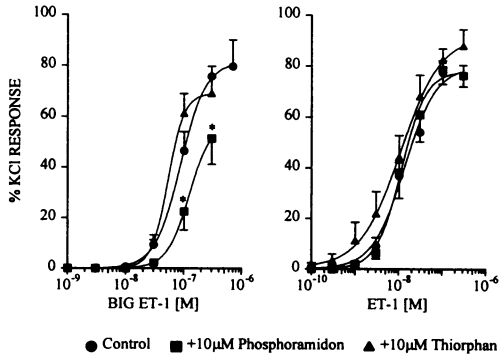
Tissue bath experiments: Endothelium-denuded human umbilical veins were cut into 4mm lengths and suspended in baths containing oxygenated Krebs soln. (37°C). Cumulative concentration-response curves (CRC) were constructed to each peptide (10^{-10}-10^{-6}M) and experiments were terminated by the addition of 50mM KCl. ET-1 and big ET-1 CRCs were repeated in the presence of 10μM thiorphan or 10μM phosphoramidon added 20 minutes earlier.

Table 1. Potency of ET and related peptides in HUV *in vitro*

	EC_{50} (nM)	CI (nM)	n
ET-1	13.9	8.8 - 22.1	9
ET-2	26.5	14.4 - 48.9	7
ET-3	>100	-	7
Big ET-1(1-38)	76.4	53.8 - 108.6	9
Big ET-1(22-38) CTF	inactive	-	3
Big ET-2(1-37)	152.7	75.1 - 310.7	6
Big ET-2(1-38)	89.0	50.0 - 158.8	5
Big ET-3(1-41)	inactive	-	5
S6c	inactive	-	3

n = number of umbilical cords from which veins were obtained.

Figure 1. Effect of inhibitors on big ET-1 and ET-1 in HUV



The lack of potency of ET-3 and S6c compared to ET-1 and ET-2 indicates the presence of vasoconstrictor ET_A receptors on the HUV smooth muscle. The mature peptides were approximately 5 times more potent than their precursors. Big ET-2(1-38) was not significantly more potent than big ET-2(1-37) and the C-terminal fragment of big ET-1 was without constrictor activity. (Table 1). Figure 1 shows the lack of effect of thiorphan or phosphoramidon on the contractile response to ET-1. The big ET-1 response was also unaffected by thiorphan but was significantly (Student's t-test, * $p< 0.05$) reduced in the presence of 10μM phosphoramidon.

The biological activity of big ET-1 is due, at least in part, to its conversion to ET-1 by an ECE, which is not NEP, and which has a non-endothelial location. However an additional direct effect of big ET-1 at ET receptors on the smooth muscle cannot be discounted.

Turner, A.J. (1993) *Biochemical Society Transactions*, 21, 697-701.

116P SUBCELLULAR LOCALISATION OF ET_A AND ET_B RECEPTORS IN HUMAN CORONARY ARTERY

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Endothelin-1 (ET-1) is thought to mediate vasodilatation in animals by activation of endothelial ET_B receptors and vasoconstriction by activation of smooth muscle ET_A and ET_B receptors. The precise location of ET receptors in human coronary arteries is not known. The aim of the present study was to determine the subcellular distribution of ET receptor subtypes in human coronary artery from patients with congestive heart failure.

Human epicardial coronary arteries were removed from explanted hearts of 8 patients (4 male, 4 female, 44-62 years) undergoing cardiac transplantation at the Papworth Hospital, Cambridgeshire. The artery was frozen in liquid nitrogen, transverse sections cut (10 μm) and labelled with [^{125}I]-ET-1 (0.1 nM, 2h, 23°C) in the presence of increasing concentrations of the ET_A selective antagonist, FR139317 (10 pM-300 μM; Aramori *et al.*, 1993) for competition experiments. Sections were labelled with [^{125}I]-ET-1 (0.1 nM) in the absence or presence of BQ3020 (0.5 μM), PD151242 (1μM) or ET-1 (1 μM) to determine the macro-autoradiographic localisation of total binding, ET_A and ET_B receptors and non-specific binding. Sections were washed and either wiped from the slides and counted or dried and apposed to hyperfilm. For electron microscope autoradiography, arteries (2-3 mm in length) were labelled with [^{125}I]-ET-1 (1 nM, 4h, 23°C) with or without BQ3020 (0.5 μM), PD151242 (1μM) or ET-1 (1 μM). Tissues were fixed, embedded in Spurr resin and sections cut (0.1 μm) and apposed to Ilford L4 nuclear emulsion on nickel grids. These were exposed for 3 months, developed and fixed.

FR139317 competed in a biphasic manner (nH=0.38; n=3) for [^{125}I]-ET-1 binding sites (pK_DET_A=9.2±0.1; $BmaxET_A$= 14.4±5.3 fmol/mg protein; pK_DET_B=4.4±0.5; $BmaxET_B$= 0.82±0.19 fmol/mg protein) (mean±SEM). FR139317 was 60,000 fold selective for ET_A over ET_B receptors and the proportion of ET_A to ET_B receptors was 94.6%: 5.4%, respectively. A high density of [^{125}I]-ET-1 binding sites, predominantly the ET_A subtype, was observed in the medial layer of the coronary artery. Localised patches of ET_A and ET_B receptors were detected in adventitial structures, consistent with previous findings in this tissue (Bacon *et al.*, 1996). Binding to the intima and non-specific binding was low.

At the subcellular level a high density of [^{125}I]-ET-1 binding sites was detected over coronary artery vascular smooth muscle cells. The plasma membrane of the smooth muscle cells contained predominantly the ET_A subtype with a lower density of ET_B receptors. Developed silver grains were observed in the nuclear emulsion over cell surface vesicles (60-70 nm diameter) whilst few silver grains were observed intracellularly or over surrounding collagen. Specific binding was not detected in either luminal endothelial cells or in endothelial cells lining small arterioles in the adventitia.

Our results show the predominance of the ET_A subtype in human coronary artery. ET_A and ET_B receptors, detected in medial smooth muscle and localised to the plasma membrane and plasmalemmal vesicles, probably mediate vasoconstriction to ET-1. ET receptors were not detected on endothelial cells suggesting a minor role of the endothelium in ET mediated vasodilatation of the human coronary vasculature.

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117P AT_1-RECEPTOR-MEDIATED SEQUESTRATION OF ANGIOTENSIN II BY THE HEART

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We previously studied the uptake of angiotensin I (AngI) and II (AngII) from plasma in isolated rat hearts (De Lannoy *et al.* 1994) and the generation of AngI and II during combined infusions of renin (R) and angiotensinogen (Ao) (De Lannoy *et al.* 1995), using a modified Langendorff model (De Deckere & Ten Hoor, 1977), which allows separate collection of coronary (CE) and interstitial (ISF) effluent. The aim of the present study was to investigate the role of the AT_1 receptor in the tissue uptake of arterial delivered or generated AngII in this heart model. Infusions of AngII (arterial conc. 5-10 nM, n=6) or R+Ao (arterial conc. resp. 1-4 nM AngI/min and 8-12 nM, n=4) were given in the presence or absence of the AT_1 receptor antagonist losartan (10^{-6} M). AngI and AngII were determined in CE and ISF, collected during steady state, and in cardiac tissue, collected within 1 min after stopping the perfusion. AngI and AngII were measured by RIA after SepPak extraction and HPLC separation.

AngII infusions: AngII steady state levels in CE were two fold higher than in ISF (Table 1). Losartan did not affect these levels. Cardiac tissue levels were 5% of AngII steady state levels in CE without losartan and < 1% with losartan, indicating that AngII is rapidly metabolized after stopping the perfusion and that any remaining cardiac AngII is most likely receptor-bound.

R+Ao infusions: AngI and AngII in ISF (81 ± 34 and 3.4 ± 2.1 pM, mean and s.d.) were 2-3 fold higher than in CE (29 ± 11 and 1.1 ± 0.5 pM). Tissue AngI (18 ± 6 fmol/g, mean and s.d.) could be attributed to its presence in ISF and CE, whereas tissue AngII (4.8 ± 1.8 fmol/g) was higher than expected on the basis of its localisation in these compartments. Consequently, the AngII/I ratio in tissue was 5-10 fold higher than in CE and ISF. Losartan did not affect the levels of AngI and AngII in CE, ISF or tissue, nor the AngII/I ratios reached at these sites.

Conclusions: Despite rapid metabolism, AngII levels are maintained longer after discontinuation of R+Ao infusions than after AngII infusions, presumably due to ongoing AngI generation by trapped R+Ao. AngII, but not AngI, is concentrated at cardiac tissue sites, most likely through receptor binding. Receptor-bound AngII appears to be protected from rapid metabolism.

Table 1. AngII levels (mean ± s.d.) in the absence and presence of the AT_1 receptor antagonist losartan during AngII infusion.

	AngII (pmol/mL or pmol/g)		
Losartan	CE	ISF	cardiac tissue
-	4.6 ± 1.4	2.5 ± 1.5	0.23 ± 0.14
+	4.9 ± 1.8	1.8 ± 0.5	0.03 ± 0.01*

* Significantly different from the level in the absence of losartan ($p<0.05$).

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118P CARDIOVASCULAR EVENTS FOLLOWING MICRO-INJECTION OF ANGIOTENSIN II, ENDOTHELIN-1 OR L-NAME TO THE SUPERIOR COLLICULUS OF RATS

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The superior colliculus (SC) of the brain, in addition to integrating visual inputs, takes part in the control of cardiovascular function via descending neuronal pathways originating in its superficial, intermediate and deep layers. Autoradiographic and immunohistochemical studies show receptors for angiotensin II (ANG II) and endothelin-1 (ET-1) within the SC (Steckelings *et al.*, 1992; Kohzuki *et al.*, 1991). However, there has been no study of the cardiovascular consequences of stimulating these receptors. Thus, using anaesthetised rats we have determined the systemic and regional cardiovascular events following micro-injection of ANG II or ET-1 to the superior layer of the SC. Moreover, as it has been demonstrated that the enzyme nitric oxide synthase (NOS) is located within the SC (Bredt *et al.*, 1990), we have also examined the cardiovascular consequences of micro-injecting locally the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME).

Male Wistar rats (250-300 g) were anaesthetised with urethane ethyl carbamate (1.2 g kg^{-1}, i.p.) and their femoral arteries cannulated to measure mean arterial blood pressure (MAP) and heart rate (HR). The spontaneously breathing rats were then placed in a stereotaxic head frame and the dorsal surface of the brain exposed by a craniotomy to allow intracerebral micro-injections, positioned according to the co-ordinates of the atlas of Paxinos and Watson (1986) (mm from the bregma: posteriorly, -8.0; laterally, 0.5; vertically, 3.2). Regional vascular resistances, total peripheral resistance (TPR) and cardiac output (CO) were evaluated by administration of ^{57}Co-labelled microspheres into the left ventricle. Renal blood flow (RBF) was also measured by an ultrasonic flow probe placed around the left renal artery.

Injection of ANG II (0.1, 1 and 10 nmol) into the SC caused dose-dependent increases in MAP (e.g. 1 nmol, 43±6 mm Hg, n=5) and TPR (control, 2.36±0.1 mm Hg ml^{-1} min 100 g^{-1} body weight; 1 nmol ANG II, +130±10 %, n=5) but decreases in HR (e.g. 1 nmol ANG II, -13±2 %, n=5, $p<0.05$) and CO (control, 99.8±1.3 ml min^{-1}; 1 nmol ANG II, -51±3 %, n=5). The increases in TPR were associated with elevated vascular resistances in many organs, such as the left and right kidneys (+390±15 % and +352±12 %, respectively, n=4), the skeletal muscle (+91±7 %, n=4), the stomach (+43±2 %, n=4), the colon (+50±3 %, n=4) and the caecum (+65±5 %, n=4). Injection of L-NAME, but not its inactive enantiomer N^{ω}-nitro-D-arginine (both 1 µmol), into the SC increased the mean arterial blood pressure (31±4 mm Hg). This effect was lessened (-25±3 mm Hg) by pre-injection (10 min) of L-Arg (1 µmol), the substrate for NOS. D-Arg (1 µmol) was without effect. Similarly, injection of L-Arg, but not D-Arg (7.5 µmol for both) at the peak of the pressor response to L-NAME (1 µmol) caused a fall (-17±4 mm Hg) in MAP. In contrast to ANG II and L-NAME, micro-injection of ET-1 (1-100 pmol) into the SC caused dose-dependent decreases in MAP (e.g., 10 pmol, -16±2 mm Hg) and falls in RBF (control, 7.7±0.4 ml min^{-1}; 10 pmol ET-1, -27±2 %, n=4, $p<0.01$).

In conclusion, injection of ANG II or ET-1 into the superficial layer of the SC induces markedly different cardiovascular and haemodynamic changes. These different profiles of activity suggest that the systemic effects recorded cannot simply be explained by ANG II and ET-1 causing local vasoconstriction within the SC. In addition, our data also show that endogenously produced nitric oxide acts within the SC to modulate MAP.

TDW holds a British Heart Foundation Lectureship (BS/95003)

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119P PHARMACOLOGICAL PROFILE OF HUMAN 5-HYDROXYTRYPTAMINE 5-HT7 RECEPTORS EXPRESSED IN INSECT CELLS USING THE BACULOVIRUS SYSTEM

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The pharmacological profile of recombinant human h5-HT_7 receptors (Lovenberg et al, 1993) expressed in SF9 insect cells was determined using [^{3}H]5-CT (5-carboxamidotryptamine) and [^{3}H]LSD with a range of standard drugs known to interact with 5-HT_7 receptors.

A recombinant baculovirus stock transfected with h5-HT_7 receptor was used to infect SF9 cells, grown in roller bottle cultures in medium TC 100 + 10 % FCS. 48 h following infection cells were harvested by centrifugation, resuspended in culture medium and kept at -70 °C. For binding studies SF9 cells transfected with baculovirus were washed twice with warm PBS and resuspended in standard binding buffer (50 mM Trizma pH 7.7, 4mM $CaCl_2$, 0.1% ascorbate), homogenized and membranes pelleted. After two additional washes, the final pellet was resuspended at 30-50 µg/assay. [^{3}H]5-CT and [^{3}H]LSD binding was performed at 37 °C for 30 min. Non-specific binding was determined with 10 µM 5-HT. Table 1 lists pKd values (-log mol/l, n = 3-14) derived from monophasic competition curves.

The pharmacological profile of h5-HT_7 receptors expressed in SF9 cells for about 70 compounds determined with either radioligand was in good agreement ($r = 0.954$, $P < 0.001$) and compared well with affinity values at r5-HT_7 receptors (R= 0.90-0.94). However, table 1 shows that differences exist in pKd's suggesting that affinity values determined in binding studies may be radioligand-dependent.

Table 1

Drug	[^{3}H]5-CT pKd	[^{3}H]LSD pKd
5-HT	8.45±0.08	8.29±0.14
5-CT	8.59±0.04	9.02±0.12
lisuride	8.14±0.02	9.13±0.21
methiothepin	7.48±0.08	8.84±0.02
risperidone	8.03±0.07	8.78±0.12
fluphenazine	7.25±0.10	8.09±0.19
clozapine	6.88±0.04	7.92±0.13
fluperlapine	6.95±0.12	7.71±0.06
loxapine	6.03±0.13	7.24±0.03
amoxapine	6.31±0.12	7.15±0.15
mCPP	5.75±0.09	7.10±0.16
ritanserin	7.51±0.20	6.98±0.17
olanzapine	6.22±0.04	6.38±0.26
haloperidol	5.42±0.06	6.63±0.12

In addition, saturation experiments produced different Bmax values depending on the radioligand used: [^{3}H]5-CT, [^{3}H]LSD and [^{3}H]5-HT, Bmax = 858±32, 1132±140 and 2405±83 fmol/mg, pKd = 9.66±0.01, 8.75±0.13 and 8.44±0.10 (n = 2-3, mean ± s.e.mean or range). Together the data suggest that caution be used when comparing affinity values for the same receptor binding site obtained using different radioligands.

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120P THE UNUSUAL ACTIONS OF d-LSD AND OTHER ERGOLINE DERIVATIVES AT RAT 5-HT_6 RECEPTORS STABLY EXPRESSED IN CHINESE HAMSTER OVARY (CHO) CELL LINE

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The recently cloned rat 5-HT_6 receptor couples to adenylyl cyclase via stimulatory G-proteins and thereby increases intracellular cyclic AMP production. It is located within the CNS with high mRNA expression levels in the striatum, olfactory tubercle, nucleus accumbens and hippocampus. Although no direct role has yet been attributed to this receptor, it has been implicated in the etiology of some neuropsychiatric disorders including schizophrenia.

In the present study, we used *in vitro* receptor binding techniques and cyclic AMP measurements (Nordstedt & Fredholm, 1990) to pharmacologically characterise the rat 5-HT_6 receptor stably expressed in CHO cells. The radioligand binding experiments were performed in buffer of 50 mM Tris-HCl (pH 7.6), 1 mM EDTA and either 4 mM $MgCl_2$ for [^{3}H]5-HT binding (0.2 mg homogenate/2 ml) or 120 mM NaCl and 0.1 mM Gpp(NH)p (nonhydrolysable GTP analogue) for [^{3}H]-LSD binding (0.1 mg homogenate/0.5 ml). The incubation was carried out for 60 min at 30°C and terminated by rapid filtration over Whatman GF/B filters. Data was analysed using the LIGAND program (Munson & Rodbard, 1980) and is represented here as mean ± s.e.mean.

We found that the agonist radioligand [^{3}H]5-HT bound in a biphasic manner with the high affinity component (K_d = 1.78 ± 0.14 nM; B_{max} = 161 ± 20 pmol/g protein; n =13) being reduced in the presence of sodium and Gpp(NH)p. Conversely, [^{3}H]-LSD bound in a monophasic manner (K_d = 2.41 ± 0.30 nM; B_{max} = 422 ± 28 pmol/g protein; n =6) and was unaffected by sodium and Gpp(NH)p. These receptor binding properties suggest that 5-HT is an agonist while *d*-LSD is an antagonist at the rat 5-HT_6 receptor. However, signal transduction studies clearly demonstrated that *d*-LSD is an even more potent and efficacious agonist than the natural agonist 5-HT. Thus, it was able to increase cyclic AMP levels to 135 ± 23 % (n=6) which was greater than observed for maximum stimulation with 10 µM 5-HT (designated as 100 % intrinsic activity).

Using [^{3}H]-LSD and [^{3}H]5-HT under low and high affinity agonist binding conditions, respectively, we investigated the correlation between the ratio $K_{I\ LOW}$ / $K_{I\ HIGH}$ of various compounds and correlated it with the intrinsic activity as determined by cyclic AMP measurements. We found that 5-HT had the highest ratio (23.5). Other reference compounds showed varying ratios that correlated with the intrinsic activity (r=0.78). However, similarly to *d*-LSD, other ergoline derivatives tested had a narrow range of ratios (1 to 7) that did not correlate with their broader range of intrinsic activities (23 to 135 % of the stimulation achieved with 10 µM 5-HT). The molecular mechanism underlying these peculiar receptor binding properties of *d*-LSD and other ergoline derivatives remains to be elucidated.

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Nordstedt C. and Fredholm B.B.(1990) *Anal. Biochem.* 89 :231-237

121P *IN VITRO* VISUALIZATION OF 5-HT RECEPTORS IN RAT BRAIN USING [^{35}S]GTPγS BINDING

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Assays to measure agonist-induced [^{35}S]GTPγS binding using membranes from cells transfected with the receptor of choice have now been established for a number of G protein-coupled receptors and offer a convenient means of obtaining measures of agonist potency and efficacy, and also antagonist affinity. Such cell lines, depending on their levels of receptor reserve, do not, however, necessarily reflect the situation seen in native tissue. For example the level of receptor expression may have marked effects on the ability of agonists to activate secondary messenger systems. Boddeke et al. (1992) found the ability of 5-HT to stimulate phospholipase C in a human 5-HT$_{1A}$ expressing cell line to be dependent on the density of receptor expression. An ability to monitor secondary messenger activation *in situ*, therefore, offers advantages over recombinant cell lines where levels of receptor expression or altered signal transduction (depending on availability of endogenous G-proteins, signaling enzymes and effector systems) can affect agonists ability to activate secondary messenger systems.

Here an autoradiographic technique described by Sim et al. (1995) has been modified to investigate [^{35}S]GTPγS binding, in the presence of excess GDP, to native 5-HT receptors in 10μm thick coronal rat brain sections. Slides were pre-incubated in assay buffer (20mM HEPES, 10mM $MgCl_2$, 100mM NaCl, 0.01% ascorbate, 0.2mM EGTA and 10μM pargyline, pH 7.4 at RT) for 10 minutes at RT followed by a 15 minute incubation at RT in the same assay buffer containing GDP (300μM or 1mM). [^{35}S]GTPγS (75pM) and test drug (± methiothepin) or buffer (basal) were then added and sections incubated for 2 hours at RT before being washed in ice cold wash buffer (20mM HEPES, pH 7.4 at RT) for 5 minutes, dipped briefly in ice-cold water, dried and exposed to Amersham Hyperfilm βmax for 24 hours and analysed.

Using this technique dose dependent agonist-induced binding was demonstrated. Selective agonists enhanced binding only in areas rich in the appropriate receptor subtype (i.e. 5-HT overall enhancement, 8-OH-DPAT and buspirone mainly raphe and hippocampus, CP93,129 mainly substantia nigra). In hippocampus 8-OH-DPAT, 5-HT and buspirone yielded pEC_{50} values of 7.49 ± 0.05, 7.28 and 6.28 ± 0.13 (mean ± S.E.M., n = 2-15) respectively. Buspirone was 54 ± 8% efficacious with respect to 8-OH-DPAT (mean ± S.E.M., n = 6). (-)UH 301 displayed no intrinsic activity. Methiothepin was shown to dose dependently blockade the 8-OH-DPAT response yielding an apparent pA_2 value of 8.80 ± 0.17 (mean ± S.E.M., n = 4). In substantia nigra the agonists CP93,129 and 5-HT yielded pEC_{50} values of 7.34 ± 0.14 and 7.20 (mean ± S.E.M., n = 2-10) respectively whilst methiothepin displayed inverse agonism. This confirms the findings of Thomas et al. (1995) who reported this inverse agonism of methiothepin in a human 5-HT$_{1D}$ receptor expressing cell line.

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122PIMMUNOLOGICAL CHARACTERISATION OF THE 5-HT$_3$ RECEPTOR COMPLEX PURIFIED FROM PIG BRAIN

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The technique of Western blotting has been widely used for the immunological characterisation of proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). However, some highly glycosylated proteins bind weakly to nitrocellulose, making their transfer and subsequent analysis difficult (Thornton et al. 1994). In the present study we employ another technique for the immunological characterisation of proteins separated by SDS-PAGE, and apply this to the purified 5-HT$_3$ receptor preparation.

Porcine 5-HT$_3$ receptors were purified as described previously (Fletcher & Barnes 1996). Purified receptor protein was precipitated using acetone and subsequently solubilised in either reducing or non-reducing sodium dodecyl sulphate (SDS) buffer (2% SDS, 50 mM Tris, 3% sucrose, 0.02% bromophenol blue, containing 5% 2-β-mercaptoethanol for the reducing buffer, pH = 7.0). The preparation was then boiled for 3 min, before an aliquot (100 μg protein) was subject to SDS-PAGE (5% stacking gel, 10% separating gel). Gels were polymerized using N,N'-diallyl-tartardiamide (DATD) as a crosslinker, rather than the more commonly used cross-linking agent N,N'-methylene bisacrylamide. DATD contains a 1,2 diol structure that can be oxidised by periodic acid, this allows gels polymerized using DATD to be solubilised by incubation with 2% periodic acid (Hames 1990).

SDS-PAGE of the affinity purified protein in reducing buffer resulted in 3-6 silver stained bands at apparent molecular masses of 37, 44-46, 51-53, 61, and 66 - 70 kDa (n = 8). Corresponding samples in non-reducing buffer failed to enter the separating gel, indicating a molecular mass for the receptor complex of > 200 kDa. Further investigation of the non-reduced purified protein using a 7.5% separating gel gave a mass for the complex of approximately 279 kDa.

In a dot-blot procedure, 2-5 μg (2 μL) of purified receptor preparation was applied onto a nitrocellulose membrane and allowed to dry at 37°C for 1 hour. The membrane was blocked with 2% Marvel in PBS/0.1%Tween (PBS mM:NaCl, 136.8; Na_2HPO_4, 10.1; KH_2PO_4, 1.5; KCl, 0.3) overnight at 4°C. The membrane was probed with a 1/500 dilution of either pre-immune serum or an antiserum specific for the cloned subunit of the 5-HT$_3$ receptor (Turton et al., 1993). Bound antiserum was detected using a peroxidase-conjugated secondary antibody and an ECL detection system (Amersham). The purified receptor preparation gave a positive reaction with the 5-HT$_3$-A antiserum. No reaction was detected with pre-immune serum.

In a modified version of the dot blot procedure, bands were excised from DATD gels using a scalpel, dissolved in 5 volumes of 2% periodic acid at room temperature for 2h, dotted (2-5 μL) onto nitrocellulose membrane and analysed as described above. Several bands excised from gels gave a postive signal with the 5-HT3 receptor antiserum (e.g. 44, 46, 61 and 66 kDa) but the band corresponding to a protein of ~53 kDa gave no reaction (n=3).

The results of this study suggest that the purified 5-HT$_3$ receptor preparation from pig brain may contain a non-5-HT$_3$-A subunit protein with an estimated molecular weight of ~ 53 kDa.

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123P CYP2C13 AND TYROSINE HYDROXYLASE EXPRESSION IN THE 6-HYDROXYDOPAMINE LESIONED (6-OHDA) RAT

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Environmental or endogenous neurotoxins are implicated in nigral cell loss in Parkinson's disease (PD). Brain P450 enzymes may be important in their formation and/or metabolism. Recently we found that CYP2C13 is widely distributed in rat midbrain including the substantia nigra pars compacta (SNpc). CYP2C13 participates in the 6-hydroxylation of testosterone. The CYP2C subfamily can metabolise the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Narimatsu *et al.*, 1996) but its role in PD and the cellular localisation of CYP2C13 in nigra are unknown. We now investigate the association with dopaminergic neurones in the substantia nigra and its expression following 6-hydroxydopamine (6-OHDA) lesioning of the nigrostriatal tract in rat.

Naive male and female Wistar rats (150-200g; n=6) were anaesthetised, transcardially perfused with 0.1M phosphate buffered saline (PBS), 4% paraformaldehyde and brains post-fixed in 4% paraformaldehyde. Cryosections of midbrain (30μm) were taken at the level of the exit of the third nerve and incubated overnight with both rabbit anti-rat CYP2C13 (1:1000) [A gift from A.R. Boobis] and mouse anti-rat glial fibrillary acidic protein (GFAP) (0.1μg/ml) or sheep anti-rat tyrosine hydroxylase (TH) (0.2μg/ml) antibodies. Second layer antibodies consisted of biotinylated goat anti-rabbit IgG and either rhodamine (LRSC) donkey anti-mouse or LRSC donkey anti-sheep F(ab')2. All sections were incubated in streptavidin-FITC and examined under a laser scanning confocal microscope. In further experiments, female Wistar rats were injected stereotaxically into the left medial forebrain bundle with either 6-OHDA (8μg free base, n=4), or were sham-lesioned with vehicle (n=4). Four weeks later, rats were tested for amphetamine and apomorphine induced rotational behaviour. Those showing >6 rotations/min were terminally anaesthetised 19 weeks post-surgery, sections prepared as before and incubated overnight with rabbit anti-rat CYP2C13 (1:1000), mouse anti-rat GFAP (0.1μg/ml) and sheep anti-rat TH (0.2μg/ml). Second layer antibodies consisted of biotinylated goat anti-rabbit IgG, LRSC donkey anti-mouse and Fluorescein (DTAF) donkey anti-sheep F(ab')2. Third layer consisted of Streptavidin Cy5. Eight optical sections for each tissue slice (15μm) were scanned. The mean signal intensities of 6 dopaminergic neurones from four rats in the medial compacta from each tissue section were calculated.

CYP2C13 signal was detected in all TH positive neurones in the SNpc. CYP2C13 was also detected in the reticulata, but in cells which were negative for both TH and GFAP immunoreactivity. No CYP2C13 signal was detected in any of the GFAP positive cells. There was a complete loss of CYP2C13 and TH signal in the 6-OHDA lesioned nigra with proliferation of astrocytes as shown by GFAP immunoreactivity. On the contralateral side there was a 44% decrease in TH signal intensity compared to the sham-lesioned group (133±30 and 75±30 respectively, $p<0.05$, two-tailed Student's t-test), while no change in CYP2C13 or GFAP signal. These results indicate that CYP2C13 is localised in dopaminergic neurones and may play a specific role in their function or in the neurodegenerative process leading to PD.

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124P QUANTITATIVE AUTORADIOGRAPHIC MAPPING OF μ-, δ- AND κ-OPIOID RECEPTORS IN THE BRAIN OF μ-RECEPTOR KNOCKOUT MICE

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We have disrupted the mouse μ-opioid receptor (MOR) gene (Matthes et al., 1996) and shown that animals deficient in this receptor have no major compensatory changes in opioid peptide expression, but exhibit complete loss of *in vivo* responses to morphine. Further, no behavioural responses to morphine mediated by δ- or κ-opioid receptors could be detected although homogenate binding studies indicated no changes in the Bmax for these receptors. These data raise the question that the absence of the μ-receptor may produce subtle changes in the expression of other opioid subtypes and also possibly alter their discrete distribution. We have therefore carried out a complete quantitative autoradiographic mapping, using radioligands selective for the μ- δ- and κ-opioid receptors. Brains from mice deficient in the MOR gene were processed for autoradiography (Kitchen et al., 1995). 20μm coronal sections were cut (300μm apart) and adjacent sections used for determination of total binding for μ- δ- and κ labelled with 4nM [^{3}H]-D-Ala^{2}MePhe^{4}Gly-ol^5 enkephalin (DAMGO), 7nM [^{3}H]-D-Ala^2 deltorphin I (DELT I) and 2.5nM [^{3}H]-CI-977 respectively. Non-specific binding was determined with naloxone (1μM for DAMGO, CI-977, and 10μM DELT I). Binding was carried out as previously described (Kitchen et al., 1995) and sections apposed to [^{3}H]-Hyperfilm for three (μ- and δ-) and four (κ-) weeks. Slides from wild-type, heterozygous and homozygous brains were laid down against the same film and developed and analysed in parallel.

In brains from knockout mice there were no detectable μ-receptors in any brain regions and no evidence for μ-receptors derived from another gene. In mice deficient in one copy of the MOR gene (+/-) μ-receptors were detectable at about 50% compared to wild-type brains. In mutant mice, δ- and κ-binding was present in all brain regions where binding was detected in wild-type mice and there were no major quantitative differences in δ- or κ-binding although there was greater interanimal variation in levels in some regions.

Table 1. μ-, δ- and κ-opioid receptor binding (fmol/mg tissue) in selected brain regions from wild-type (+/+) heterozygous (+/-) and homozygous (-/-) mice.

	Caudate Putamen	Frontal-Parietal Cortex	Thalamus	Basolat. Amygdala	Nucleus Accumbens
μ (+/+)	66 ± 4.1	30 ± 3.4	105 ± 8	59 ± 7.3	136 ± 4
μ (+/-)	32 ± 5.0	11 ± 2.3	48 ± 11	20 ± 4.5	58 ± 6.8
μ (-/-)	n.d.	n.d.	n.d.	n.d.	n.d.
δ (+/+)	89 ± 6.8	49 ± 3.2	18 ± 1.8	53 ± 4.8	31 ± 2.7
δ (+/-)	85 ± 9.2	43 ± 3.7	16 ± 2.3	44 ± 7.2	27 ± 3.9
δ (-/-)	82 ± 7.8	48 ± 3.4	17 ± 2.8	49 ± 7.8	23 ± 1.7
κ (+/+)	19 ± 2.5	23 ± 2.6	13 ± 1.2	20 ± 2.1	41 ± 6.3
κ (+/-)	16 ± 3.9	22 ± 5.4	12 ± 1.3	21 ± 3.5	35 ± 6.4
κ (-/-)	15 ± 1.6	19 ± 2.0	10 ± 1.7	17 ± 1.7	31 ± 3.8

Values are mean ± s.e. mean, n = 5-6. n.d. = not detectable.

The results show there are no major compensatory changes in δ- or κ- binding throughout the brains of animals deficient in μ-receptors.

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125P COINCIDENT SIGNALLING BETWEEN δ-OPIOID AND MUSCARINIC RECEPTORS IN SH-SY5Y CELLS

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The δ-opioid ligand DPDPE elevates intracellular calcium ($[Ca^{2+}]_i$) in SH-SY5Y cells only when applied in the continued presence of carbachol (Connor & Henderson 1996). The elevation of $[Ca^{2+}]_i$ by DPDPE was pertussis toxin sensitive, was caused by release of Ca^{2+} from intracellular stores and required ongoing muscarinic receptor activation not simply an elevation of $[Ca^{2+}]_i$.

In the present experiments $[Ca^{2+}]_i$ was measured in confluent monolayers of undifferentiated SH-SY5Y cells using Fura 2 as previously described (Connor & Henderson 1996). Data are expressed as mean ± s.e.m.

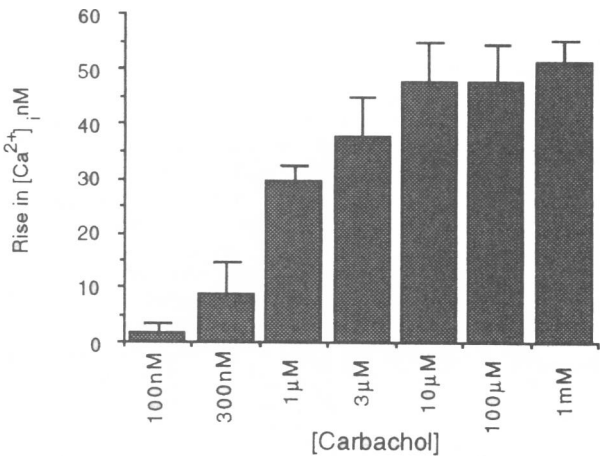
We have sought to determine whether carbachol and DPDPE release Ca^{2+} from the same store. In the absence of extracellular Ca^{2+} (including 10μM EGTA) the elevation of $[Ca^{2+}]_i$ evoked by a near maximal concentration of carbachol (1mM) was not maintained but declined to pre-drug levels presumably because the Ca^{2+} stores had been emptied. Emptying the stores in this way significantly reduced the elevation of $[Ca^{2+}]_i$ by bradykinin (acting at the Gq-coupled B2 bradykinin receptor) and DPDPE (acting at the pertussis toxin-sensitive Gi/Go-coupled δ-opioid receptor). After carbachol-induced store depletion the elevation of $[Ca^{2+}]_i$ evoked by bradykinin in the presence of 1mM carbachol was 11±1% of the control response (n=3) and by DPDPE was 9±6% of control (n=5). These results indicate that DPDPE is releasing Ca^{2+} from a carbachol-releasable store..

DPDPE (30nM) was applied for 60s in the continued presence of 1μM carbachol, either 0min, 1min, 2min, or 10 min after the beginning of the carbachol application. The responses seen at 0min, 1min, and 2min were 96.15±16%, 120±14%, and 106.7±12.27% of the response seen when DPDPE was applied after 10min. This indicates that the increase in $[Ca^{2+}]_i$ is independent of the time of DPDPE application.

The responses seen to DPDPE (30nM) in the presence of increasing concentrations of carbachol are shown in Figure 1. The response evoked by DPDPE increased with increasing carbachol concentration, reaching a maximum in 10μM carbachol. This is in contrast to the elevation of $[Ca^{2+}]_i$ by carbachol alone which does not reach a maximum until 1-3mM.

These data indicate that DPDPE causes release of $[Ca^{2+}]_i$ from a carbachol-releasable store and that the response evoked by DPDPE is independent of the concentration of carbachol and the time of appliaction.

Figure 1. Elevation of $[Ca^{2+}]_i$ by DPDPE (30nM) in the continued presence of various carbachol concentrations.



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126P FURTHER EVIDENCE FOR ALLOSTERIC INTERACTIONS OF PURINOCEPTOR ANTAGONISTS WITH THE $P2X_4$ PURINOCEPTOR

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The binding of [^{35}S]ATPγS to the $P2X_4$ purinoceptor can be allosterically regulated by purinoceptor antagonists (Miller et al., 1996). In this communication we have examined the effect of NaCl on [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor and on the interaction of antagonists with this receptor.

The rat $P2X_4$ purinoceptor was expressed in CHO-K1 cells using the Semliki forest virus expression system and membranes were prepared from these cells as described previously (Michel et al., 1996). Binding assays using [^{35}S]ATPγS (0.15nM) were conducted as described previously (Michel et al., 1996) except that a low ionic strength assay buffer 5mM Hepes; 1mM EDTA, 5 mM N-methyl-D-glucamine (HEN buffer) of pH 7.4 at 4°C was used. Incubations were for 3hr at 4°C. Data are means±s.e.mean of 3-5 separate experiments.

In HEN buffer, in the absence of NaCl, [^{35}S]ATPγS bound with relatively low affinity to the $P2X_4$ purinoceptor (pK_D=9.6±0.1, B_{max}=54±4 pmol.mg.protein^{-1}). In the presence of 150 mM NaCl there was an increase in radioligand affinity and the density of sites detected (pK_D=10.1±0.1, B_{max}=113±5 pmol.mg.protein^{-1}).

The interaction of purinoceptor antagonists with the $P2X_4$ purinoceptor was also affected by NaCl. In the absence of NaCl the purinoceptor antagonists only inhibited a proportion of the specific binding of [^{35}S]ATPγS. Thus, 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS), evans blue and coomassie blue G, respectively, inhibited 21±8, 62±2 and 29±4% of [^{35}S]ATPγS binding, with pIC_{50} values of 6.0±0.1, 6.8±0.2 and 6.5±0.1. In the presence of 150 mM NaCl, the proportion of [^{35}S]ATPγS binding inhibited by DIDS (63 ± 2%; pIC_{50} 5.4±0.1), and evans blue (75±5 %; pIC_{50} 6.8±0.1), but not coomassie blue G (33±6, pIC_{50}=6.3± 0.2),differed when compared to that determined in the absence of NaCl. The interaction of the antagonists with the $P2X_4$ purinoceptor was studied to determine if coomassie blue G and evans blue interacted with the same population of sites. Optimal conditions for studying this interaction were in HEN buffer containing 30mM NaCl. Under these conditions, coomassie blue G inhibited [^{35}S]ATPγS binding maximally by 20±3%, whereas evans blue inhibited binding maximally by 64±3%. When co-incubated, the effects of the two compounds were not additive. Instead coomassie blue G was able to antagonise the ability of Evans blue to inhibit [^{35}S]ATPγS binding (pIC_{50} values for evans blue in the presence of 0, 1, 3, 10 and 30μM coomassie blue G were 6.5±0.2, 6.0±0.1, 5.2±0.3, 4.6±0.1 and 4.3±0.2, respectively). Coomassie blue G did not affect the maximal inhibition of [^{35}S]ATPγS binding produced by evans blue.

These results demonstrate that NaCl can modulate the binding of [^{35}S]ATPγS to the $P2X_4$ purinoceptor and can differentially affect the interaction of purinoceptor antagonists with this receptor. Under similar assay conditions purinoceptor antagonists inhibit [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor to differing extents. The finding that coomassie blue G maximally inhibited [^{35}S]ATPγS binding to a lesser extent than evans blue, but could antagonise the effects of evans blue, suggests that these compounds are not acting as simple competitive antagonists and instead they may be acting as allosteric regulators of [^{35}S]ATPγS binding to the $P2X_4$ receptor. It is possible that both compounds interact at the same site on the $P2X_4$ purinoceptor to affect [^{35}S]ATPγS binding, but that coomassie blue G possesses lower efficacy as an allosteric regulator of [^{35}S]ATPγS binding than evans blue.

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127P PHARMACOLOGICAL CHARACTERISTICS OF MICROGLIAL P2X, PURINOCEPTORS

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The cytolytic P2Z is a member of the P2X family of ligand-gated channels (Surprenant *et al.*, 1996). This receptor, termed $P2X_7$, differs from other P2X receptors, notably in biophysical properties, where in certain circumstances, application of agonist causes formation of large ligand-gated "pores" in the cell membrane.

In this study, we investigated the pharmacological profile of this receptor in an immortalised (using wild type SV40 T-antigen oncogene) mouse cell line (NTW8), whose properties are very similar to native microglial cells.

Whole-cell recordings were made using Cs-aspartate containing electrodes (2.5-4 MΩ), at 24-27°C. In a HEPES-buffered extracellular medium containing 1mM Mg^{2+} and 2mM Ca^{2+} (normal divalent ion medium), application of ATP or benzoylbenzoyl ATP (BzATP) produced concentration-dependent inward currents (holding potential -90 mV). At the highest concentration of agonist tested (3mM and 300 μM, respectively), currents were 644±198 and 549±191 pA. Concentration-effect curves did not reach a maximum. In medium containing zero Mg^{2+} and 0.5 mM Ca^{2+} (low divalent ion medium), short applications (0.5s) of these agonists produced much larger currents at the highest concentrations tested (1818±218 and 909±138 pA, respectively), and inward currents recorded for second concentration-effect curves were larger than those observed for the first concentration-effect curve (2347±310 and 1304±378 pA, 3mM ATP and 300 μM BzATP, $P<0.05$, respectively).

In separate experiments in low divalent ion medium, repeated applications for 1s of 1mM ATP resulted in successively larger inward currents, reaching a plateau of 464±48% of the first current after 10 applications. We used this protocol to monitor pore formation. Data are expressed as a percentage of the current induced by a first application of ATP (1mM) alone. Following this first application, ATP applied in the presence of hexamethylene amiloride (HMA; 100 μM) caused an immediate increase of 439±34% of the current elicited by the first application of ATP alone. HMA had neither agonist nor antagonist effect when tested on cells in which pore formation had already been induced. Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; 30 μM) prevented current augmentation when applied during second concentration-effect curves to ATP (443±69 vs. 213.5±50 pA, 3mM ATP, absence and presence of PPADS, respectively), slowed pore formation (increase in current of 228±46% after 10th ATP application in the presence of PPADS) and antagonised responses to ATP in cells following pore formation in a non-competitive manner (maximum response 66±6% of response to 3mM ATP in the absence of PPADS). Suramin (100 μM) had no effect on any of these parameters.

In low divalent ion medium, following pore formation, ATP, BzATP and 2-methylthio-ATP produced full concentration-effect curves with EC_{50} values of 279 [253-308], 62 [47-82] and 479 [387-594] μM respectively (geometric means with 95% confidence intervals, n=25, 12 and 6). The agonist α,β-methylene ATP was without significant effect at concentrations up to 1mM.

We conclude that in the NTW8 mouse microglial cell line, a ligand gated ion channel receptor with properties resembling those described for the $P2X_7$ receptor can be distinguished, with characteristics that differ markedly from other known P2X subtypes. Its presence in these microglial type cells may implicate this receptor in the pathophysiology of certain CNS diseases.

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128P CHARACTERISATION OF PURINOCEPTORS MEDIATING RESPONSES TO ATP ON RAT DORSAL ROOTS

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Adenosine 5'-triphosphate (ATP) elicits inward currents in dorsal root ganglion (DRG) cells (Bean, 1990) and depolarises dorsal roots (Phillis & Kirkpatrick, 1978). However, of the six subtypes of the P2X receptor ($P2X_1$-$P2X_6$) found to be expressed in DRG neurones, it is unknown which subtypes contribute to these responses (Collo *et al.*, 1996). Recently functional studies have suggested that the $P2X_3$ purinoceptor subtype mediates the ATP activated inward currents seen in DRG neurones (Robertson *et al.*, 1996) although results from other studies suggest additional P2X subunits may be involved (Bean, 1990). The aim of the present study was to characterise the purinoceptors present on dorsal roots using an extracellular 'grease-gap' recording technique.

Isolated lumbar dorsal roots, L3-L6, from male Sprague-Dawley rats (~50g), were placed across a grease-gap and recordings were made using silver-silver chloride wick electrodes. One compartment was continuously perfused with Krebs solution containing calcium and magnesium at 1-2mls min^{-1}, 20-25°C and gassed with 95% O_2/ 5% CO_2. Agonist depolarising responses were measured as the change in the d.c potential between the two compartments. An initial depolarising response to potassium chloride was used to assess the viability of the preparation. If the response did not exceed 0.1mV or if the baseline recordings were unstable then the preparation was rejected. In the remaining preparations application of KCl (1mM) produced a mean depolarising response of 0.22 ± 0.01mV, n=66.

Applications of ATP (100μM) to the preparation for 120 secs evoked a depolarising response which rose rapidly and faded in the continued presence of the drug and was 16.0±6.4% of the response to KCl (1mM); concentrations below this did not elicit measurable responses. The ATP analogue, αβmethylene ATP (αβmeATP), evoked concentration-related depolarising responses of the dorsal roots and was effective at much lower concentrations than ATP (EC_{50} value of 19[4-97]μM), with a maximal response at 100μM of 34.6 ± 6.3% of the response to KCl; n=5). After a two hour incubation with the P2 receptor antagonists, suramin (100μM), pyridoxal-5-phosphate (100μM) or PPADS (1μM), responses to αβmeATP (30μM) were reduced to 18 ± 6, 38 ± 3 and 32 ± 11%, respectively, of the initial response to αβmeATP(30μM) (n=4-6). The effects of all the antagonists were reversed upon washout. Neither pyridoxal-5-phosphate, suramin or PPADS, significantly reduced depolarising responses to GABA (30μM). To assess whether responses to αβmeATP underwent tachyphylaxis, repeated applications at decreasing time intervals revealed that responses were only significantly decreased when less than 10 mins was left between applications. The compounds ADP, AMP, adenosine, UTP and ADPβS, all at 100μM, were without effect.

In conclusion, these data suggest that P2 purinoceptors are present on dorsal roots and mediate the depolarising responses to ATP and its analogues. The lack of depolarising responses to AMP and adenosine precludes the involvement of P1 receptors, and the high agonist potency of αβmeATP suggests involvement of ionotropic P2X receptors sensitive to PPADS, suramin and pyridoxal-5-phosphate. Not unexpectedly responses to ATP were small and this is probably due to agonist breakdown by ectonucleotidases (see Trezise *et al.*, 1994). The sensitivity to αβmeATP suggests that the $P2X_1$ and/or the $P2X_3$ subunits are involved in the action of ATP. The desensitisation observed in the presence of αβmeATP was not as rapid as would be expected if the receptors involved were homomultimers of $P2X_1$ or $P2X_3$, and further study is required to investigate whether the $P2X_2/P2X_3$ heteropolymer, as previously described in sensory neurones, is involved (Lewis *et al.*, 1995) or whether the ionotropic purinoceptors in the dorsal roots represent a novel subunit composition or a mixed pop ulation.

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129P CHARACTERISATION OF A FUNCTIONAL SPLICE VARIANT OF THE $P2X_2$ PURINOCEPTOR

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Recently, cDNAs encoding seven members of the P2X family of ligand-gated ion channels have been isolated (Surprenant *et al.*, 1995). We used the reverse transcriptase-polymerase chain reaction (RT-PCR) on neonatal rat cerebellum to amplify $P2X_2$ purinoceptor related cDNAs. Using specific primer pairs, the RT-PCR reaction always produced several partial cDNAs of different sizes, one of which was about 200 bp smaller than the expected product for the $P2X_2$ purinoceptor (Brake *et al.*, 1994). We obtained the corresponding full length cDNA with the deletion by recloning the PCR fragment into the original $P2X_2$ clone. This construct, which we have termed RC604, is different from the variant cloned by Housley *et al.* (1995). We investigated the pharmacological properties of the RC604 clone in *Xenopus* oocytes or in transiently transfected HEK-293 cells by cRNA injection or lipofectamine transfection, respectively.

Using two electrode voltage-clamp of ooctyes, the properties of RC604 were investigated using a range of agonists and antagonists. The pharmacological properties of RC604 did not appear to differ markedly from the larger, original $P2X_2$ clone. Both ATP and 2-methylthio-ATP produced concentration-dependent inward currents with EC_{50} values of 28 [21-38] and 25 [14-46] μM ($P2X_2$) and 18 [11-28] and 21 [6-82] μM (RC604), respectively (n=5, geometric mean with 95% confidence intervals). The agonist, α,β methylene-ATP, had little effect (≤ 10 % of ATP maximum) up to 500μM (n=3) at either clone. The P2 antagonists, suramin (30μM) and pyridoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS; 3μM), reduced the ATP (30μM) induced current in both $P2X_2$ and RC604 injected cells to 4.6±2.4 and 3.5±0.7% ($P2X_2$, n=5 and n=6) and 7.2±2.4 and 6.0±0.5% (RC604; n=5 and n=6) of the current elicited by application of ATP in the absence of antagonist.

A striking difference in the desensitization profile between $P2X_2$ and RC604 was observed. In oocytes, with a holding potential of -60mV, in response to sustained application of 300 μM ATP, RC604 currents decayed along a single exponential time course with a time constant of 26.0 ± 2.1 s (n=10). This was more rapid than that observed for $P2X_2$ currents which decayed with a time constant of 112.0 ± 10.7 s (n=5). Similar experiments were performed with HEK-293 cells expressing either RC604 or $P2X_2$. RC604 currents decayed significantly ($P < 0.01$) more quickly than $P2X_2$ currents, with residual currents after a 10s application of 100μM ATP of 34.6±4.7% (n = 11) for RC604 and 71.4±3.9% (n = 13) for $P2X_2$. Application of 300μM ATP gave similar results, with residual currents of 41.1±3.4% (n = 11) and 63.8±2.3% (n = 11) for RC604 and $P2X_2$, respectively ($P < 0.01$).

We conclude that the $P2X_2$ splice variant, RC604, forms ATP-gated cation channels with similar agonist and antagonist sensitivities to the original $P2X_2$ receptor. However, these channels have a distinct desensitization profile compared to that of the $P2X_2$ receptor.

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130P POTENTIATION OF ATP-RESPONSES AT A RECOMBINANT $P2X_2$ RECEPTOR BY NEUROTRANSMITTERS AND RELATED SUBSTANCES

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An ATP-gated ion-channel ($P2X_2$ receptor) has been cloned from rat phaeochromocytoma PC12 cells (Brake *et al.*, 1994). Distribution studies have shown the $P2X_2$ receptor transcript to be found widely throughout the central and peripheral nervous systems (Collo *et al.*, 1996) including sensory (DRG) ganglion cells. Agonist affinity for the recombinant $P2X_2$ receptor is modulated by extracellular pH (King *et al.*, 1996), as are ATP-responses via a native P2X receptor in rat nodose ganglion cells (Li *et al.*, 1996). We have investigated the modulatory actions of a series of neurotransmitters and related substances on $P2X_2$ receptors, taking into account their effect on extracellular pH (pH_e). Attention was paid to neurotransmitters and paracrine substances that are contained in or found near to sensory neurons and their nerve endings.

ATP-evoked responses were recorded under voltage-clamp conditions, from $P2X_2$ receptors expressed in defolliculated *Xenopus* oocytes. Nicotine, 5-HT, noradrenaline, adenosine, bradykinin and histamine (100μM, n=4) significantly potentiated responses ($P<0.05$) to submaximal concentrations of ATP (3μM), but acetylcholine, GABA and dopamine (100μM, n=4) showed no effect. The rank order of potency was: bradykinin> adenosine > 5-HT = noradrenaline > histamine > nicotine, with bradykinin and nicotine showing a 55±20% and 20±10% enhancement, respectively. Active compounds caused a small acidic shift in pH_e (0.03-0.06 pH units); on restoring pH_e to original levels, all active substances failed to enhance ATP-responses. Inactive substances (acetylcholine, dopamine and GABA) caused very little change in pH_e (0.00-0.02 pH units). Arachidonic acid and met-enkephalin (100μM, n=3) potentiated ATP-responses significantly (42±13% and 56±17% enhance-ment, respectively; $P<0.05$), largely through a pH-independent effect since readjusting pH_e failed to reduce potentiated ATP-responses. NGF (50ng/ml) also caused an increase in ATP-responses (12±5%; $P<0.05$) without altering pH_e. Substance P and CGRP (both 100μM, n=1) strongly potentiated ATP-responses and, after adjusting pH_e, still enhanced by 92% and 113%, respectively. A positive correlation was seen between the mean pH shift caused false-positive potentiators, and the amplitude of ATP-responses altered by HCl (0.1M) and NaOH (0.5M), revealing that small acidic and alkaline shifts of 0.03 pH units could enhance or diminish ATP-responses. It was noted that pH-independent and pH-dependent potentiation caused no change in maximal ATP-responses (100μM).

In conclusion, substance P, CGRP, arachidonic acid, NGF and met-enkephalin all modulated ATP-responses at the $P2X_2$ receptor in a pH-independent manner and may play a role in sensitising sensory nerves via the $P2X_2$ receptor under physiological and, perhaps, pathophysiological conditions. Other tested substances proved to be potentiators only under acidic conditions, or because they are weak acids.

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131P ARL 67085 AND ARL 66096 ARE SLOWLY DISSOCIATING COMPETITIVE P2T-PURINOCEPTOR ANTAGONISTS

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The P_{2T}-purinoceptor antagonists, ARL 66096 and ARL 67085, are potent and selective inhibitors of ADP-induced platelet aggregation (Humphries *et al.*, 1994,1995). In these reports, the nature of the antagonism was not always consistent with simple competition, with varying degrees of depression of the concentration/effect (E/[A]) curve asymptote observed. In the present study, we investigate the mechanism of this depression.

Aggregation of human washed platelets was assessed turbidometrically as a decrease in absorbance (650 nm), read 5 and 30 min after the addition of ADP to 150 µl aliquots of platelet suspension in 96-well microtitre plates (Fratantoni *et al.*, 1990). E/[A] curves to ADP (0.03 -1000 µM) were constructed in the absence and presence of antagonist (3 -100 nM) added 2 (ARL 66096) or 5 (ARL 67085) min before, or simultaneously with ADP.

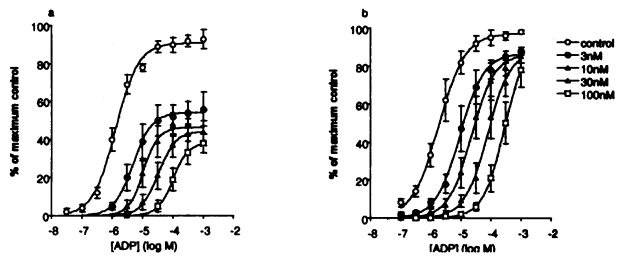

Figure 1: The effect of ARL 67085 on ADP-induced aggregation read after a) 5 min b) 30 min. Values are means ± se (n=4).

When aggregation was measured 5 min after addition of ADP, ARL 67085, added 5 min before ADP, caused a concentration-dependent rightward displacement of the E/[A] relationship and depressed the asymptote (Figure 1a). Less depression was observed when responses were allowed longer to plateau (30 min reading, Figure 1b). No depression was observed when agonist and antagonist were added simultaneously and responses read at 5 min. Affinity estimates for both antagonists were not influenced by the different protocols (Table 1).

Table 1 Analysis of antagonism under different incubation conditions. Values are mean ± se (n = 4-6).

Antagonist	Incubation (min)	Asymptote (%) [ARL] 30nM	$pK_B(pA_2)$	Schild slope
ARL 66096	0	95 ± 1	(9.13 ± 0.09)	1.13 ± 0.03
	2	73 ± 2	(8.98 ± 0.03)	0.96 ± 0.03
ARL 67085	0	94 ± 8	9.14 ± 0.07	1.03 ± 0.06
	5	44 ± 5	8.94 ± 0.09	0.93 ± 0.05

Insurmountable antagonism can occur when an antagonist dissociates slowly from its receptor, exhibits irreversible kinetics or when the receptor exists in two states (Robertson *et al.*,1994). The present study indicates that the major factor determining the effects of the antagonists is slow off-rate from the receptor relative to the on-rate for ADP. When ADP and antagonist are co-incubated for sufficient time for equilibrium to be reached, the asymptote depression disappears. Under these conditions, antagonism is consistent with simple competition and pK_B estimates can be measured with confidence.

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132P ANTAGONISM OF A NOVEL P1 RECEPTOR IN FOLLICULAR *XENOPUS* OOCYTES

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A novel P1 receptor, present on the follicle cell layer enveloping *Xenopus* oocytes, couples to G_s to activate adenylate cyclase and, in turn, a cAMP-dependent non-rectifying outward K^+-current in oocytes connected electrotonically by gap junctions to follicle cells (Miledi & Woodward, 1989). Both adenosine and ATP activate the oocyte P1 receptor; their EC_{50} values are similar (1.9±0.3µM (n=6) and 1.7±0.3µM (n=3)) and, although adenosine exceeds the maximal activity of ATP, only adenosine-activated currents are inhibited by adenosine deaminase (0.1U/ml) (King *et al.*, 1996). Here, we identify competitive and non-competitive antagonists of this novel P1 receptor and compare their potency (in terms of IC_{50} values; given as mean±s.e.mean, n=4).

Follicular oocytes (stages V and VI) were studied under two-electrode voltage-clamp conditions (V_h = -20mV) and outward K^+-currents were evoked by superfused adenosine (10µM for 60s every 20 min.). Five antagonists were selected on the basis of preliminary results (King *et al.*, 1996) and data on a similar adenosine/ATP receptor (the so-called P3 receptor) on sympathetic nerve terminals (Todorov *et al.*, 1994); α,β-meATP, 8-(*p*-sulphophenyl)theophylline, theophylline, 2,2'-pyridylisatogen tosylate (PIT) and suramin. Each compound was added in cumulative concentrations (0.01-100µM) to the superfusate. The most potent test compound was α,β-meATP (IC_{50} value: 0.19±0.03µM) which reversibly antagonised adenosine responses. The slope of its inhibition curve was close to unity (-0.83±0.13). 8-(*p*-sulphophenyl)theophylline (8-SPT) (IC_{50} value: 0.21±0.01µM) reversibly antagonised adenosine responses and was as potent as α,β-meATP. The slope of its inhibition curve was steep (-1.71±0.13). Theophylline (IC_{50} value: 4.8±3.3µM) also reversibly antagonised adenosine responses, but was 25-fold less potent than α,β-meATP. The slope of its inhibition curve was shallow (-0.57±0.06). PIT (IC_{50} value: 26.1±4.5µM) was an irreversible antagonist of adenosine responses and 137-fold less potent than α,β-meATP. The slope of its inhibition curve was steep (-1.47±0.09). PIT is a strong alkylating agent and its irreversible antagonism is probably related to its chemical activity. Suramin was the least effective and, at 100 micro-molar, caused a small reduction (8±2%) of adenosine responses. Thus, suramin was >500-fold less potent than α,β-meATP.

In summary, a novel P1 receptor on follicular *Xenopus* oocytes shows a unique profile for agonist (adenosine = ATP) and antagonist activity (α,β-meATP = 8-SPT > theophylline > PIT >> suramin) which distinguishes this receptor from other known P1 and P2 receptor subtypes, but not from the so-called P3 receptor.

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133P MICE LACKING THE APOLIPOPROTEIN E GENE DO NOT SHOW ANY MARKED CHOLINERGIC DEFICITS

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ApolipoproteinE genotype (ApoE) is a major risk factor for Alzheimer's disease (Corder et al, 1993), however little is known about how ApoE isoforms influence this disease. Recent studies by Gordon et al (1995) suggested that mice deficient in ApoE may show impaired central cholinergic function. Because this neurotransmitter system has traditionally been implicated in the pathogenesis of AD, we decided to investigate this further.

Female ApoE knockout mice (29.9±0.8g) and wild type littermate controls (28.5±0.9g), approximate age 12 months and derived from the colony established by Maeda and coworkers (Zhang et al, 1992) were used throughout. At the end of the experiment, genotype was confirmed by measuring plasma cholesterol levels, which typically are 5x higher than control. Behavioural studies consisted of testing the mice for their sensitivity to elicit cholinomimetic signs following pretreatment with the cholinesterase inhibitor, E2020. Mice also underwent testing in the Morris water maze. At the completion of these studies, central choline acetyltransferease (ChAT) activity and acetylcholinesterase (AChE) staining was performed.

Pretreatment with E2020 (2.5-5mg/kg; i.p route) produced a significant fall in body temperature and induction of tremor in both wild type and ApoE ko mice. The magnitude of change did not differ between the groups. Watermaze testing revealed that both wild type and ApoE ko mice could learn the location of a hidden platform with similar rates of acquisition and accuracy as determined by a subsequent 60s probe test (% time in island quadrant: WT: 43±2% ApoE ko: 50±4%). The protocol used was previously shown to be sensitive to the disruptive effects of scopolamine (2mg/kg ip; but not N-methyl scopolamine). ChAT activity was measured in the hippocampus, frontal and entorhinal cortex and striatum. In each case there was no difference between the groups (see Table 1). Fifteen micron coronal sections of striatal, frontal cortical and anterior hippocampal regions showed similar patterns of AChE staining, with no obvious group difference. Finally, analysis of plasma cholesterol levels confirmed ApoE genotype (see Table 1).

Table 1. Summary of results

	plasma cholesterol	Δ BT(°C)	ChAT (hippocampus)	ChAT (frontal cortex)
Wild type	1.6±0.1	-7.5±0.8	12.8±1.4	11.6±0.5
ApoE ko	8.8±1.4*	-6.6±0.8	11.7±0.8	10.5±0.7

*p<0.05 vs. wild type controls. Plasma cholesterol expressed in mMole/l, ChAT expressed in nmoles/min/mg protein. Body temperature change (BT) expressed as the peak difference from baseline following E2020 5mg/kg treatment.

In conclusion, using a combination of behavioural, histochemical and biochemical measurements, we have failed to observe any significant differences in central cholinergic activity between wild type and ApoE ko mice. The differences between the present study and that of Gordon et al (1995) may be due to the actual transgenic line used, or selection of wild type controls (see Gerlai, 1996).

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134P PHENOTYPE OF SPONTANEOUS BEHAVIOUR IN TRANSGENIC MICE WITH D_{1A} DOPAMINE RECEPTOR 'KNOCKOUT'

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The family of D_1-like dopamine receptors [D_{1A-D}] plays a fundamental role in the regulation of psychomotor behaviour but, to date, drugs selective for this family are unable to distinguish between individual members thereof (Waddington et al., 1995). Targeted gene deletion has been applied to generate mice having 'knockout' of D_{1A} receptors; however, there is contradictory evidence on spontaneous behaviours in these animals: both decreased rearing with normal locomotion (Drago et al., 1994) and 'hyperactivity' (Xu et al., 1994) have been reported. We describe here the ethological evaluation of spontaneous behaviour therein.

Homozygous D_{1A} 'knockout' [$D_{1A}^{-/-}$] and wildtype controls [$D_{1A}^{+/+}$] were genotyped, using a PCR technique, among the offspring of heterozygous [$D_{1A}^{+/-}$] matings; young adult 'knockouts' showed reduced body weight [black females, ≈ 8 weeks: $D_{1A}^{+/+}$ 19 ± 3g; $D_{1A}^{-/-}$ 13 ± 5g, N=9-11; -34%, P <0.02], as noted previously (Drago et al., 1994; Xu et al.,1994), in the absence of gross neurological deficit and with survival rates comparable to their wildtype counterparts. Spontaneous behaviour was evaluated using an ethological approach (Waddington et al., 1995) so as to resolve and quantify all behaviours manifested over an initial 1h period.

Relative to wild-types, D_{1A} 'knockout' was associated with: reductions in sniffing [-11%, P<0.01], rearing free [-87%, P<0.01], and sifting [-60%, P<0.01] and chewing [-63%, P<0.05] of cage bedding/faecal pellets; increases in locomotion [+31%,P<0.05], grooming [+66%, P<0.01] and intense grooming [+78%, P<0.01]; there were no significant alterations in total rearing, rearing against cage walls, rearing from a sitting position, climbing, eating or vacuous chewing. There were very few correlations between body weight and individual behaviours, none of which could account for these differences in behaviour.

Though abnormalities of spontaneous behaviour in D_{1A} 'knockout' mice were evident, they could not be encapsulated as either hypo- or hyper-activity; rather, there were significant shifts between multiple individual elements of behaviours which may account for apparent contradictions in the existing literature. It remains to be established whether alternative D_1-like receptors, [over]compensatory processes subsequent to developmental absence of D_{1A} receptors or other mechanisms, perhaps related to the complex genetic background of these animals, contribute to these effects.

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135P THE ETHANOL PREFERENCE OF LOW DRINKERS OF THE C57 STRAIN IS INCREASED BY SALINE INJECTIONS: CHANGE PREVENTED BY A CCKB ANTAGONIST

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When given a free choice, the majority of the inbred C57/Bl6 strain of mice drink a large percentage of their fluid as ethanol (Unwin and Taberner, 1982). We have previously demonstrated the existence of C57/Bl6 mice with low voluntary alcohol intake (Watson et al., 1996). We now report the effects of repeated saline injections and of the CCKB antagonist CAM1028 (Hughes et al., 1990) on the alcohol intake of these mice.

Female C57/Bl6 mice (25-30g), bred exclusively in-house at Bristol University were used. Prior to the study they were given a choice between water and 8% ethanol (available 24h), every 2 days for 6 days, for their preference. The amount of 8% ethanol drunk, as a proportion of total fluid intake, was calculated and the animals identified as high or low preference mice (high = ratio > 0.8, low = ratio < 0.4.)

The following studies were carried out on single housed animals with either low or high preference. Baseline preference was checked for at least two days, then intraperitoneal injections of either saline vehicle or CAM1028 were given between 4pm and 5pm each day. In the first study, CAM1028 was administered at 1 mg/kg for 8 days (n values = 8). In the second study, CAM1028 was given at 100 ug/kg for 4 days, then 1 mg/kg for 7 days (n values 7-9). The amount of water or 8% ethanol consumed was measured between 8am and 9am every morning; measurements were continued for several days after cessation of injections. Statistical comparisons were made by Student's t-test.

The ratios of ethanol to water consumed by the low drinkers increased during the saline administration schedule; this was significant for the last two days ($P < 0.05$) in both studies. Mice receiving CAM1028, 1 mg/kg, did not show this change in either study. There were no changes in the ethanol intake of the high preference mice, or in the total fluid intake of either group, in either experiment. The results suggest the effects of CCKB antagonists in stress-induced alcohol intake warrant further investigation.

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Table 1: Ratios of ethanol/total fluid consumed, in the first study. Values are mean ± s.e.m; *P < 0.05 compared with saline values

Treatment	Low drinkers given saline	Low drinkers given CAM1028	High drinkers given saline	High drinkers given CAM1028
Baseline	0.22 ± 0.03	0.22 ± 0.04	0.83 ± 0.04	0.84 ± 0.02
Baseline	0.26 ± 0.03	0.3 ± 0.02	0.78 ± 0.03	0.79 ± 0.04
7th treatment day	0.55 ± 0.08	0.32 ± 0.04*	0.73 ± 0.08	0.64 ± 0.08
8th treatment day	0.57 ± 0.01	0.3 ± 0.07*	0.86 ± 0.03	0.77 ± 0.11
7 days after treatment	0.58 ± 0.1	0.45 ± 0.1	0.86 ± 0.02	0.76 ± 0.08
8 days after treatment	0.62 ± 0.11	0.42 ± 0.1	0.83 ± 0.03	0.76 ± 0.09

136P PROLONGED CHANGES IN ACTIVITY OF VENTRAL TEGMENTAL NEURONES AFTER CHRONIC ETHANOL TREATMENT

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Long-term changes in brain function may be responsible for the frequent relapse back into drug taking which is a major problem in addicts. We have previously demonstrated prolonged increases in the effects of amphetamine and cocaine on locomotor activity after chronic ethanol administration (Manley and Little, 1995; 1996). We now investigate the effects of chronic ethanol administration on the activity of neurones in ventral tegmental area (VTA), an area of the brain thought to be involved in both drug dependence and motivation. Recordings were made at a time when all signs of behavioural withdrawal hyperexcitability had disappeared

Male TO mice (20-25g) were given ethanol by liquid diet. Ethanol-treated mice received control diet for 3 days, followed 3.5% v/v ethanol diet for 2 days, rising to 5%, then 8% ethanol, each for 9 days (average daily intake was 22-30 g/kg). Controls were pair-fed equi-calorific control diet. At the end of the ethanol treatment, laboratory chow was given to all animals for 24h. Midbrain slices (350μ) containing the VTA were then prepared, after cervical dislocation. Recordings were made under perfusion with aCSF at 2.3 ml/min (one slice per mouse, one neurone per slice;) after an incubation period of 60 min. The n values were 6 - 12 per treatment group.

Single unit recordings were made from dopamine sensitive-cells with firing frequencies less than 5 Hz and action potential duration over 2 ms. Initial studies showed that very few active cells could be detected in slices prepared after ethanol treatment (active cells were found in 1/7 slices after ethanol treatment and in 5/5 control slices; $P < 0.05$, Fisher's exact probability test). N-methyl-DL-aspartate (NMDLA), 5 μM, was therefore added to all subsequent slices, enabling recordings to be made. Firing frequency was recorded for 10 min, then dopamine (DA) was added to the aCSF perfusion medium at 50 μM, then at 100 μM. After 25 min washout, amphetamine (Am) was added at 1, 5 and then 20 μM. The drugs were applied for 10 min, recordings being obtained during the last 1 min. Firing rate compared by Student's t-test; its variability (calculated as interspike interval standard deviation/mean x 100) by the Mann-Whitney U-test and the effects of the drugs by two-way analysis of variance.

The mean firing frequency was significantly ($P<0.0001$) lower in slices prepared after ethanol treatment (Table 1), and the variance was increased ($P<0.05$). Firing rates after addition of either dopamine or amphetamine were significantly lower in slices prepared after ethanol treatment (DA $P<0.001$; Am $P<0.0001$). The results indicate that major alterations in firing rate and pattern can be seen in dopamine-responsive cells in the VTA at time when the acute behavioural signs of ethanol withdrawal have subsided. These may be related to the prolonged psychological changes seen during abstinence.

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Table 1. Values are mean ± s.e.m. *P<0.05; **P<0.0001 compared with controls

Chronic treatment	Baseline firing rate (spikes/min)	Variance of firing rate	% of basal firing rate DA 50 μM	% of basal firing rate DA 100 μM	% of basal firing rate Am 1 μM	% of basal firing rate Am 5 μM	% of basal firing rate Am 20 μM
Control diet	231 ± 18	4.5 ± 1.1	69 ± 12	38 ± 14	98 ± 2	78 ± 17	60 ± 14
Ethanol diet	101 ± 9 **	17.1 ± 6.8 *	38 ± 12	4 ± 4	68 ± 19	13 ± 11	0 ± 0

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These experiments tested the prediction (Stephens, 1995) that repeated withdrawal from chronic treatment with benzodiazepines will result in an intensification of the withdrawal signs relative to a single withdrawal from an equivalent drug exposure. Male mice of a C57Bl x SV129 derived strain, were treated chronically with diazepam (DZP, 15 mg/kg/day) given s.c. in sesame oil, or with vehicle (Steppuhn et al, 1993). The following 3 treatment groups were used: DZP for 21 days; vehicle for 21 days; three 7-day periods of DZP treatment interspersed with 3 days vehicle. Withdrawal intensity was assessed 72 hours following the last DZP or vehicle treatment, to allow complete elimination of the drug and metabolites (Steppuhn et al 1993). Convulsant threshold to pentylenetetrazole (PTZ; 4.55 mg/ml) infused into the tail vein (0.25ml/min) was assessed as time to the first clonic twitch (n=7-13).

The behavioural measures of withdrawal in an elevated plus maze were total number of open and closed arms entered, head dips from the security of the closed arms, or from the open arms of the maze during five minute tests (n=17-18). The data were analysed using a one way ANOVA; *post hoc* analysis was by Newman-Keuls (N.K.) tests with significance levels set at 0.05. Table 1 shows lowered convulsant thresholds following repeated *vs* single withdrawal, but the reverse effect in the plus maze.

These data support the prediction that repeated withdrawal from a benzodiazepine will result in an increase in intensity of abstinence signs when sensitivity to convulsions is taken as the end point. Nevertheless, the observations using the plus maze suggest that a similar sensitisation does not take place in measures thought to relate to anxiety.

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Steppuhn KG *et al* (1993) *J. Pharm. Exp. Ther.* 264: 1395-1400.

Table 1 Salient behavioural measures in withdrawal

Group	PTZ (mg/kg)	Plus Maze: Entry open	Plus Maze: Head dip open	Plus Maze:Head dip mid
Vehicle 21 days	42.6 ± 5.0	4.58 ± 0.8	12.11 ± 2.3	10.11 ± 1.0
DZP 21 days	36.9 ± 3.3	1.11 ± 0.4*	3.47 ± 1.3*	3.58 ± 0.5*+
DZP 3x7 days	28.7 ± 3.5	2.44 ± 0.6*	5.94 ± 1.5*	5.78 ± 0.9*+
	F(2,25)=3.23	F(3,65)=4.49	F(3,65)=5.9	F(3,65)=14.7
	$p<0.05$	$p<0.05$	$p<0.001$	$p<0.0001$

Data given are means ± s.e.mean. * N.K. $p<0.05$ compared to vehicle; + N.K. $p<0.05$ Repeated withdrawal compared to 21 day drug

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138P EFFECTS OF SB 204070A AND SB 207266A, SELECTIVE 5-HT$_4$ RECEPTOR ANTAGONISTS, IN THREE RAT MODELS OF ANXIETY

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Costall and Naylor (1993) have suggested that 5-HT$_4$ receptors may be involved in the control of anxiety. In the present study, the actions of two highly selective 5-HT$_4$ receptor antagonists, SB 204070A (Wardle et al., 1994) and SB 207266A (Wardle et al., 1996) were studied in three rat models of anxiety.

Male Sprague-Dawley rats (200-250 g) were held under a 12 h light cycle (lights on 0700 h) with free access to food and water in groups of six. Rats were singly housed on day 1 and given SB 204070A (s.c. 30 min pre-test in 1 ml/kg saline), SB 207266A (p.o. 1 h pre-test in 2 ml/kg 1% methyl cellulose) or chlordiazepoxide (CDP) in the appropriate vehicle on day 5. Thirty min or 1 h later, pairs of like-treated rats were placed in a brightly lit, white perspex, test arena for 15 min and their social interaction (SI) (grooming, following, sniffing, crawling over or under, boxing, biting) was scored by an observer, blinded to treatments, via a video monitor (see Kennett et al., 1996). Alternatively, group housed rats were administered drugs 30 min or 1 h prior to placing on an elevated X-maze of black perspex, lit by white light for 3 min (see Kennett et al., 1996). In the Geller-Seifter (GS) test, rats (400-500 g) fed a restricted diet, were trained to press a lever for a food reward, and to associate a light cue with both a high level of reward and a contingent footshock, in 5 x 3 min periods with rewards at variable intervals (see Kennett et al., 1996). Results are cited as means ± s.e.m. and analysed by 1-way ANOVA and Dunnett's test (SI) Kruskal-Wallis ANOVA and Mann-Whitney U test (X-maze) or 2-way ANOVA (treatments x subjects) in the GS test.

SB 204070A and CDP s.c. increased time (secs) spent in SI (saline 59.0 ± 4.8, 0.01 mg/kg 124.8 ± 7.2, $p<0.01$, 0.1 mg/kg 110.3 ± 8.7, $p<0.01$, CDP 5 mg/kg 145.2 ± 12.5 $p<0.01$) as did SB 207266A p.o. (vehicle 51.0 ± 6.1, 1 mg/kg 96.5 ± 9.2, $p<0.05$, 10 mg/kg 99.3 ± 15.9 $p<0.01$, CDP 5 mg/kg 112.9 ± 17.3 $p<0.01$) without affecting locomotion. SB 204070A also increased % time spent on the open arm of the elevated X-maze (saline 16.5 ± 2.8, 0.1 mg/kg 24.8 ± 3.1 $P<0.05$, 1 mg/kg 27.8 ± 3.0 $p<0.01$, CDP 5 mg/kg 41.7 ± 3.7 $p<0.01$), but SB 207266A had no significant action on this parameter despite a trend in the same direction (vehicle 17.7 ± 3.7, 0.1 26.5 ± 3.3 ns, 1 mg/kg 25.8 ± 4.8 ns, CDP 5 mg/kg 47.4 ± 5.5 $p<0.01$). In the GS procedure, neither compound at 0.1 or 1 mg/kg affected punished or unpunished responding.

In conclusion, SB 204070A and SB 207266A have anxiolytic-like actions in the rat SI test, but have more modest actions in the elevated X-maze test compared to CDP and no effect in the rat GS procedure. This profile is similar to that reported for 5-HT$_3$ receptor antagonists (Blackburn et al., 1993).

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139P A SINGLE AMPHETAMINE TREATMENT IS ABLE TO DISRUPT LATENT INHIBITION IN THE RAT BY AN ACTION AT CONDITIONING ALONE

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d-Amphetamine (AMP) disrupts latent inhibition (LI), the effect of pre-exposure (PE) of a stimulus in impairing subsequent conditioning (CON) to that stimulus, both in rat and human subjects. This has been taken to provide a plausible link between the dopamine (DA) theory of schizophrenia and the attentional deficits and the (positive) symptoms of the disorder. Accordingly, studies of the mode of action of AMP in disrupting LI in rats could help to identify critical mechanisms in the neurobiology of schizophrenia. Some differences have emerged however between the animal and human studies, in that while a minimum of two i.p. injections of d-AMP sulphate (1 mg/kg, 15 min before PE and CON on successive days) were necessary to disrupt LI in the rat, a single oral administation (5 mg, 90 min before combined PE and CON) was sufficient in humans. Following a report (McAllister, 1996) that a single dose of AMP 45 min before combined PE and CON could disrupt LI in rats, we have re-examined the effect of varying the time of AMP administration before CON only.

Groups of 6-8 male Sprague-Dawley rats, 250-300g, were used in a procedure based on that of Moran *et al.* (1996). In untreated animals, conditioning of a tone - mild footshock association (in the control, or NPE group) is prevented (latently inhibited) by 40 pre-exposures to the tone stimulus in 35 min (PE group); this is indexed by the ability of the tone to suppress licking for water the next day (TEST). AMP was given at the indicated dose and time prior to CON (i.e. no drug given at TEST). Analogous to the results of McAllister (1996), we found that strong LI (block of formation of the tone-shock association) in saline treated rats, using a combined PE-CON session, was significantly attenuated ($p<0.05$) by d-AMP, 0.5 mg/kg, and abolished ($p<0.01$) by 1 mg/kg, given 45 min earlier. When PE and CON were separated by 24 h, a single dose of AMP, (1 mg/kg, 15 min before CON) was ineffective, confirming earlier results from our group and others. However, the same dose given either 45 min, or 90 min, before CON abolished LI ($p<0.05$ and $p<0.01$ respectively).

These results confirm that AMP, like other drugs modulating DA function (nicotine, haloperidol), has its critical action on LI at CON. The effectiveness after longer time delays appears to result from a greater proportion of calcium dependent DA release, a phenomenon also accompanying rapid (24 h) sensitisation to AMP (Warburton *et al*, 1996). We hypothesise that this reflects a requirement for impulse-dependent, rather than impulse-independent, DA release to disrupt LI. This is consistent with an impact of the released DA on stimulus information processing, rather than on indiscriminate stimulation of receptors.

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140P IMPORTANCE OF 5-HT$_{1B}$ SELECTIVITY FOR 5-HT TERMINAL AUTORECEPTOR ACTIVITY: AN *IN VIVO* MICRODIALYSIS STUDY IN THE FREELY-MOVING GUINEA-PIG

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The 5-HT$_{1B/1D}$ receptor inverse agonist, methiothepin, and the partial agonists, GR 127935 and GR 125743, have been reported to increase extracellular 5-HT when perfused into the frontal cortex of the freely-moving guinea-pig (Roberts *et al.*, 1994). However, all 3 compounds when administered systemically decrease extracellular 5-HT (Roberts *et al.*, 1994). 5-HT autoreceptors are reported to be of the 5-HT$_{1B}$ subtype in terminal areas (Buhlen *et al.*, 1996) but 5-HT$_{1D}$ subtype in the raphe (Pineyro *et al.*, 1996). These compounds have equal affinity for 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors, therefore, the aim of this study was to determine whether selective 5-HT$_{1B}$ partial agonists, antagonists and inverse agonists also decrease extracellular 5-HT, following systemic administration.

Male guinea-pigs (300-400g) were anaesthetised with methoxyflurane and dialysis probes implanted in the frontal cortex (AP+4.5mm; ML+2mm, V-3mm from Bregma). Animals were allowed to recover overnight before probes were perfused with a.CSF at 2µl/min. After 2 hours perfusion, samples were collected every 20 min into 10µl of 0.4M PCA. 5-HT was separated by HPLC and detected by ECD (electrode potential of 0.65V).

The 5-HT$_{1B}$ selective partial agonist, SB 216641 (n-[3-(2-dimethylamino)eth-oxy-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-(1,1'-biphenyl)-4-carboxamide, 2.5mg/kg ip), antagonist, SB 220272 (n-[3-(2-dimethylamino) ethoxy-4-chloro-phenyl]-2'-meth-yl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl -4-carboxamide hydrochloride, 1.5mg/kg ip), and inverse agonist, SB 219085 (n-[3-(3-dimethylaminopropyl)-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-carboxamide oxalate 2mg/kg ip) had no effect on extracellular 5-HT on systemic administration, with values for mean AUC of 125±10 (n=5), 131±7 (n=6) and 139±15 (n=6) % of basal respectively. SB 219085 produced a small but significant increase in extracellular 5-HT ($p<0.05$). A dose response study for this compound revealed a maximum 5-HT increase of 342±24% of basal (n=4) at 8mg/kg ip. However, this increase was not attributed to an action at the 5-HT$_{1B}$ receptor but rather to a non-specific releasing effect of the compound, demonstrated by *in vitro* [^{3}H]5-HT release. In confirmation of this finding, SB 224289 (2,3,6,7-tetrahydro-1'-methyl-5-{2'-methyl-4'-[(5-methyl-1,2,4 - oxadiazole-3-yl)biphenyl-4-yl] carbon- yl} furo[2,3-f]indole-3-spiro-4'-piperidine oxalate, 2-16mg/kg ip), a selective 5-HT$_{1B}$ inverse agonist, had no effect on extracellular 5-HT.

In summary, compounds which display selectivity for 5-HT$_{1B}$ receptors over 5-HT$_{1D}$ receptors, do not decrease extracellular 5-HT in the frontal cortex. Therefore, the earlier reported decrease in 5-HT levels produced by non-selective 5-HT$_{1D/1B}$ compounds can be attributed to antagonism at 5-HT$_{1D}$ receptors in the raphe to increase 5-HT locally, decrease cell firing through activation of raphe 5-HT$_{1A}$ receptors and hence decrease extracellular 5-HT in terminal areas.

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141P THE NEUROMODULATORY ACTIONS OF SOMATOSTATIN IN THE RAT STRIATUM *IN VIVO*

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Immunoreactivity for the tetradecapeptide somatostatin (SRIF) is found throughout the central and peripheral nervous systems (see Epelbaum, 1986) Somatostatin mediates its multiple actions via a family of at least five G-protein linked receptor types whose genes have been cloned. The localisation of SRIF and its receptors is the subject of much research, yet the *in vivo* actions of SRIF have been largely ignored. The work described here investigates the effects of exogenously applied SRIF in the striatum of the anaesthetised rat using a microdialysis technique.

Rats were anaesthetised with 25% w/v urethane (2.0ml kg^{-1} i.p.) and allowed to breathe spontaneously. Microdialysis probes (CMA/12, CMA/Microdialysis, Sweden) were placed in the dorsal region of the right striatum of male wistar rats (250-300g) and perfused with Krebs Ringer solution (pH 7.2). Sampling (15 min intervals) commenced one hour after probe placement. The perfusing solution was changed to SRIF for 15 min, 90 min after the start of sampling, at a concentration of either 10nM, 50nM, 100nM or 1μM, via a liquid switch (CMA-110, CMA/Microdialysis, Sweden). Samples were frozen and taken for analysis of monoamines and amino acids by high performance liquid chromatography. A concentration of 100nM SRIF produced significant increases ($P<0.05$, determined using a two-tailed Student's t-test, n=6) in dopamine (mean ± s.e.mean=2486±1575%), glutamate (647±387%), aspartate (437±125%), GABA (2281±1164%) and taurine over basal levels (425±118%). A dose of 50nM (but not 10nM or 1μM) also significantly increased dopamine (292±74%), glutamate (607±283%) and GABA (580±210%). No significant change in levels of DOPAC, 5-HIAA, 5-HT, citrulline, arginine, glycine and acetylcholine were seen at any dose of SRIF tested. A second 15 min application of SRIF 135 min after the first did not significantly increase levels of any compound measured at any dose tested.

Pharmacological investigations were performed to determine whether other transmitter systems were involved in mediating the actions of SRIF. Local retrodialysis infusion of the NMDA glutamate receptor antagonist, APV (200μM), for 1 hour significantly attenuated the actions of a 15 min infusion of SRIF (100nM) when compared to untreated animals. SRIF-induced increases in dopamine were dramatically reduced (only 128±15% of basal values) by APV implicating the NMDA receptor in mediating the actions of SRIF. Similar retrodialysis infusions with the $GABA_A$ receptor antagonist, bicuculline (10μM), significantly increased the levels of dopamine (457±12.5%), glutamate (281±17%), and GABA (251±12%) in the absence of exogenous SRIF. A subsequent application of SRIF (100nM) had no effect on any of the compounds measured. This effect of bicuculline on striatal neurotransmitter release has been observed by others (Leviel *et al.*, 1990).

We speculate that, in part, SRIF mediates its neuromodulatory actions by acting on its receptors located on glutamatergic terminals releasing glutamate. The glutamate released could then act on NMDA receptors to promote dopamine release from dopaminergic terminals and GABA from GABAergic neurones It remains to be determined which SRIF receptor type(s) are involved in such a possible mechanism.

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142P EFFECT OF PROBENECID ON DEPOLARIZATIONS EVOKED BY *N*-METHYL-D-ASPARTATE (NMDA) IN THE RAT STRIATUM

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Inhibition of organic anion transport by probenecid increased brain extracellular levels of endogenous kynurenic acid, a competitive antagonist of the NMDA-receptor glycine site (Russi *et al.*, 1992). Accordingly, we have examined whether intracerebral application of probenecid reduced depolarizations evoked at the same tissue site by NMDA.

Field (d.c.) potential was recorded with microdialysis electrodes (Obrenovitch *et al.*, 1994) implanted into the striatum of 250-350 g, male rats anaesthetized with halothane (concentration reduced to approximately 1 % in 1:1 $N_2O:O_2$ after surgery). Ten separate, local depolarizations were induced by perfusion of 200 μM NMDA through the microdialysis probe for 2 min, each followed by 20 min of recovery, except for the 8th challenge (see below). The first two stimuli were used as individual controls. Ten min after the 2nd NMDA application, normal ACSF was replaced by a medium containing 1 mM probenecid in ACSF, and two further NMDA-stimuli applied with NMDA dissolved in probenecid-medium. This procedure was repeated with 5 and 20 mM probenecid. As 20 mM probenecid, by itself, altered the d.c. potential and gradually reduced NMDA-responses, the last two NMDA-stimuli were used to test whether this inhibitory effect was reversible. They were applied 30 and 52 min after switching from 20 mM probenecid to normal ACSF. Matching experiments without probenecid application were also performed.

Figure 1. Representative effect of probenecid on depolarizations evoked in the rat striatum by NMDA (microdialysis application).

At 1 mM probenecid had no effect. At 5 mM, the slight decrease in depolarization amplitude did not reach significance, but the hyperpolarization which consistently followed NMDA-responses was abolished (Fig. 1) suggesting that sustained, high extracellular concentrations of probenecid reduce the capacity of the tissue to recover from a depolarizing stimulus. At 20 mM, probenecid inhibited NMDA-evoked depolarization by approximately 60 % (from 4.7 ± 0.7 mV to 2.1 ± 0.2 mV; mean ± s.e.mean; $n = 6$, $p < 0.005$ by Student's *t*-test). This effect was more marked 30 min after returning to perfusion with normal ACSF, suggesting that probenecid may have irreversible effects at this concentration.

These data suggest that increasing extracellular levels of kynurenic acid by inhibition of organic anion transport may not, by itself, be sufficient to protect against neurological disorders involving excessive NMDA-receptor activation.

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143P EFFECTS OF SB 216641 AND BRL 15572 (SELECTIVE h5-HT_{1B} AND h5-HT_{1D} RECEPTOR ANTAGONISTS, RESPECTIVELY) ON GUINEA-PIG AND HUMAN 5-HT AUTO- AND HETERO-RECEPTORS

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The subclassification of inhibitory presynaptic 5-HT auto- and heteroreceptors, which belong to the 5-$HT_{1B/1D}$ subfamily, has been hampered by the lack of selective ligands. Such compounds have now become available: SB 216641, a selective h5-HT_{1B} receptor ligand and BRL 15572, a selective h5-HT_{1D} receptor ligand (Price et al., 1996). The aim of the present study was to investigate the effects of these compounds on human and guinea-pig (G-P) presynaptic 5-HT autoreceptors in the cerebral cortex and on human presynaptic heteroreceptors on the sympathetic nerves of atrial appendages.

Human cortical synaptosomes and G-P cortical slices were preincubated with [^{3}H]5-HT and human atrial appendages were preincubated with [^{3}H]noradrenaline(NA). Subsequently they were superfused with Krebs' solution and [^{3}H]transmitter release was induced either electrically (G-P slices, 3Hz; human atria, 2Hz) or by 25mM K^+ (human synaptosomes). The study was approved by the local ethics committee.

The electrically evoked [^{3}H]5-HT release from G-P cortical slices was reduced by the 5-HT receptor agonist 5-carboxamidotryptamine (5-CT; pIC_{30} = 7.74). This effect was not modified by BRL 15572 (2 and 20μM; concentrations 100 and 1000 times higher than the K_i at h5-HT_{1D} receptors), but was antagonised by SB 216641 (0.1 and 1μM; concentrations 100 and 1000 times higher than the K_i at h5-HT_{1B} receptors), apparent pA_2 = 8.45. SB 216641 alone (0.1μM) facilitated evoked release by 95% ± 8% (P<0.05), whereas BRL 15572 (2 and 20μM) was ineffective.

In human cortical synaptosomes, 5-CT (1μM) reduced K^+ evoked [^{3}H]5-HT release by 30% ± 6% (P<0.05). The inhibitory effect of 5-CT (100nM) was not changed by BRL 15572 (300nM) but abolished by SB 216641 (15nM); drug concentrations 15 times higher than their K_i at cloned h5-HT_{1D} and h5-HT_{1B} receptors, respectively.

In human atrial appendages, the electrically evoked [^{3}H]NA release was inhibited by 5-HT (1μM) by 33% ± 5% (P<0.005). This effect was antagonised by BRL 15572 (300nM; 15 times its K_i at h5-HT_{1D} receptors) but not by SB 216641 (30nM; 30 times its K_i at h5-HT_{1B} receptors).

In conclusion, SB 216641 is a preferential antagonist at native h5-HT_{1B} receptors, whereas BRL 15572 is a preferential antagonist at native h5-HT_{1D} receptors. We confirm that the human 5-HT terminal autoreceptor is a h5-HT_{1B} receptor and the heteroreceptor on human atrial appendages is a h5-HT_{1D} receptor.

Price G.W., Burton M.J., Roberts C. *et al.* (1996) Br. J. Pharmacol. 119, 301P.

144P THE ECTO KINASE INHIBITOR K-252B BLOCKS THE INDUCTION OF LONG-TERM POTENTIATION IN THE PERFORANT PATH OF THE ANAESTHETISED RAT

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Extracellular kinase (ecto kinase) activity is present on the surface of neural cells (Ehrlich *et al.*, 1986) and is required for the expression of Long-Term Potentiation (LTP) in the CA1 region of the hippocampal slice preparation (Fujii *et al.*, 1995).

Since, at present, there is no evidence for ecto kinase modulation of LTP *in vivo*, we have investigated the effect of K-252b, a protein kinase inhibitor which is confined to the extracellular space (Nagashima *et al.*, 1991) on LTP in the anaesthetised (urethane 1.8g/kg i.p.) rat (male Lister-Hooded 250-300g). Stimulating electrodes and glass recording micropipettes were positioned bilaterally in medial perforant path, and molecular layer of the dentate gyrus respectively and stable extracellular recordings achieved. Recording micropipettes (1-5MΩ, 10μm tip diameter) contained either drug or vehicle (0.01% DMSO in artificial cerebro-spinal fluid (ACSF)). Drugs were delivered from the recording micropipette to the extracellular domain of the recording site under constant, low positive pressure (1-5psi) for the entire experiment. Vehicle was applied to the ipsilateral recording site as a control. Test stimuli were given to both sides of the brain with a current sufficient to produce 75% of maximal slope of the evoked epsp. LTP was induced using a tetanus (4 trains of 8 pulses at 400Hz) or theta-burst (10 trains at 5Hz of 5 pulses at 100Hz) at 2 x test stimulus amplitude. In control experiments, where micropipettes contained ACSF, similar increases in e.p.s.p. slope were obtained on the two sides of the brain following either tetanic or theta-burst stimulation.

In order to validate the micropipette administration method and characterise the LTP induction protocols, the selective NMDA receptor antagonist D-APV (1mM), was delivered via the recording micropipette to one side of the brain, and produced complete blockade of theta burst and tetanically induced LTP on that side, whilst on the control side normal potentiation was obtained.

K-252b (50μM) consistently (6/6 experiments) blocked the induction of theta-burst induced LTP while 5μM K-252b caused a variable (1/6) blockade. Interestingly, K-252b (50μM) produced a similarly variable blockade of tetanically induced LTP (2/6) (see table 1).

These findings suggest that phosphorylation of extracellular sites has a significant role to play in the induction of LTP in the perforant path *in vivo*. Therefore, such extracellular sites could include structures important for the processes associated with the induction of LTP including NMDA / AMPA receptors. At present, it is unclear why the inhibition of ecto kinase activity has a more profound effect on theta-burst induced LTP than tetanically induced LTP.

Drug Treatment	% increase in EPSP slope (• s.e.mean)			
	Theta-burst		Tetanic	
	Control	Drug	Control	Drug
Control	29±5	32±3	41±3	38±2
K-252b (5μM)	29±2	25±6	-	-
K-252b (50μM)	39±16	*7±3	37±15	19±8
APV (1mM)	30±4	*3±1	39±11	*1±3

Table 1. Summary of results. Data expressed as % increase in EPSP slope ± s.e.mean 10 mins post LTP induction; n=6. Statistical significance determined using unpaired Students' t-test. (*p<0.05)

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