51P TACHYKININ RESPONSES IN DIFFERENT TYPES OF NEURONES OF GUINEA-PIG SUBSTANTIA NIGRA PARS COMPACTA *IN VITRO*

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Recently, a suggestion was made by Whitty *et al.* (1995) about the selective expression of different tachykinin receptors in different neuronal populations in the substantia nigra pars compacta (SNc), based on the results obtained by the mRNA hybridisation method. We have tested this hypothesis *in vitro* by studying the effects of selective tachykinin agonists and antagonists on the electrical activity of nigral neurones. Coronal slices containing SNc were prepared from the brains of 150-200 g male Hartley guinea pigs, as previously described (Nedergaard & Greenfield, 1992). A total of 79 neurones located in SNc were intracellularly recorded at 33-34°C with glass microelectrodes in a current clamp mode. Three types of neurones were electrophysiologically identified. Type I neurones (71% of total recorded cells) possessed properties which were typical of dopaminergic (DA) neurones while type II cells (11%) corresponded to non-DA, probably GABA-ergic interneurones (Yung *et al.*, 1991); cells of type III (16%) were similar to the neuronal groupe of unidentified biochemical nature, described in rostral SN (Nedergaard & Greenfield, 1992). In 23/26 tested type I neurones, the selective NK_3 receptor agonist senktide concentration-dependently increased the spontaneous firing rate (average maximal response (mean ± s.e.mean) from 1.7 ± 0.2 to 3.4 ± 0.7 Hz at the concentration of 30 nM; EC_{50} = 14.7 nM; n=6) while the selective NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$] Substance P and the NK_2 receptor agonist [Nle^{10}] NeurokininA (4-10) were without any effect (both up to 100 nM). Excitatory responses to senktide (30 nM) were reduced by 87.3±1.1% by the selective NK_3 antagonist SR 142801 (100 nM, 1 h; n=4), but affected neither by the selective NK-2 antagonist SR 48968 nor by the selective NK_1 antagonist SR 140333 (both at 100 nM, 1 h; n=3). Responses to senktide persisted in the low-Ca^{++} medium (n=3). As for the type II neurones, 6/9 tested cells were excited with [Sar^9,$Met(O_2)^{11}$]Substance P in a concentration-dependent manner (average response was from 14.3 ± 2.1 to 18 ± 3.2 Hz at the concentration of 100 nM; EC_{50}=41.2 nM; n=5); no effect of [Nle^{10}]Neurokinin A (4-10) was observed up to the concentration of 100 nM, while senktide (30-100 nM) excited only one type II cell. Responses to [Sar^9,$Met(O_2)^{11}$]Substance P (100 nM) were decreased by 75.7±4.9% by SR 140333 (100 nM, 1 h; n=4), but affected neither by SR 48968 nor by SR 142801 (both at 100 nM, 1 h; n=3). Concerning the type III neurones, no effect was observed in the 7 cells tested with the three NK agonists (all at 100 nM). Our results correlate well with the data concerning the localisation of tachykinin receptor mRNA in SNc (Whitty *et al.*, 1995), and confirm the selective presence of NK3 receptors on type I DA neurones and of NK1 receptors on type II non-DA cells, which may be GABA-ergic neurones.

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52P INHIBITION OF K^+-EVOKED [^{3}H]NORADRENALINE RELEASE AND Ca^{2+} CURRENTS BY ANGIOTENSIN II IN SH-SY5Y CELLS TRANSFECTED WITH THE RAT ANGIOTENSIN (AT_{1A}) RECEPTOR

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Stimulation of muscarinic receptors evokes release of [^{3}H]noradrenaline ([^{3}H]NA) from human neuroblastoma (SH-SY5Y) cells (Murphy *et al.*, 1991), and also inhibits K^+-evoked [^{3}H]NA release via inhibition of voltage-gated Ca^{2+} channels (McDonald *et al.*, 1994). We have recently shown that angiotensin II (AII) also stimulates [^{3}H]NA release from SH-SY5Y cells stably expressing the recombinant rat AT_{1A} receptor (McDonald *et al.*, 1995a). Here, we have investigated whether AII modulates K^+-evoked [^{3}H]NA release and Ca^{2+} channel currents in SH-SY5Y cells.

Exposure of SH-SY5Y cells transfected with the rat AT_{1A} receptor to 100mM K^+ at 37°C evoked [^{3}H]NA release, measured as previously described (Murphy *et al.*, 1991). Mean release (with basal values subtracted) was 11.8±0.7% (mean ± s.e.mean) of total cellular ^{3}H content (n=6). Pre-treatment of cells for 4 min with AII (1-50nM) reduced K^+-evoked [^{3}H]NA release in a concentration-dependent manner (IC_{50} 2nM), with maximal inhibition of 67.3±4.8% (n=6) seen at a concentration of 10nM. Since K^+-evoked [^{3}H]NA release is dependent on Ca^{2+} influx through voltage-gated L- and N-type Ca^{2+} channels (McDonald *et al.*, 1994), we also investigated whether AII could inhibit such channels using whole-cell (conventional and perforated-patch) patch-clamp recordings. To enhance current amplitudes, 10mM Ba^{2+} was used as charge carrier (21-24°C; see McDonald *et al.*, 1995b, for further details). Currents were evoked by 200ms step depolarizations to 0mV applied at 0.2Hz from a holding potential of -80mV. Bath application of AII (3-300nM) reversibly inhibited currents. For example, at 100nM currents were inhibited by 59.4±5.5% (n=10 cells, $p<0.03$, paired Student's t-test). At concentrations > 10nM, responses to AII were often transient i.e. current amplitudes decreased then recovered partially during continued AII exposure. Ca^{2+} current inhibition was abolished by 2μM losartan (n=3) and in untransfected cells AII was without effect (n=4).

Our results indicate that recombinant rat AT_{1A} receptors couple functionally to the inhibition of Ca^{2+} channels, an effect which may account for the ability of AII to inhibit K^+-evoked [^{3}H]NA release in these cells.

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53P INHIBITION OF HIGH-VOLTAGE ACTIVATED Ca^{2+} CHANNEL CURRENTS IN NG108-15 CELLS BY THE κ AGONIST U50488H DOES NOT INVOLVE OPIOID RECEPTOR ACTIVATION

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Recent studies have demonstrated that U50488H, a κ-opioid receptor agonist, can inhibit cardiac L-type and Purkinje neurone P-type Ca^{2+} channels without acting at κ receptors (Utz *et al.*, 1995; Kanemasa *et al.*, 1995). Here we have investigated, in differentiated NG108-15 neuroblastoma x glioma cells, the actions of U50488H on high-voltage activated (HVA) Ca^{2+} channel currents which are attributable to N- and L-type Ca^{2+} channels (e.g. Kasai, 1992).

Whole-cell patch clamp recordings were made from cells perfused and dialysed with solutions designed to minimize currents flowing through channels other than voltage-gated Ca^{2+} channels (20-24°C), using 10mM Ba^{2+} as charge carrier (see Peers & Green, 1991). Cells were voltage-clamped at -70mV and step depolarized to 0mV for 100ms at 0.1Hz. Evoked currents were measured for amplitude over the last 10-15ms of the step depolarization.

Bath application of [D-Ser^2, Thr^6]Leu-enkephalin (DSLET; 0.1-1μM) rapidly inhibited HVA currents in NG108-15 cells. For example, 0.5μM DSLET produced an inhibition of 23.9±3.9% (mean ± s.e. mean, n=17 cells, $p<0.001$, paired Student's t-test), and the actions of 0.5μM DSLET were always fully blocked by 30μM naloxone (n=6), indicating that δ opioid receptors were coupled to HVA Ca^{2+} channel currents, as previously reported (Kasai, 1992).

Bath application of U50488H at concentrations of 0.1 to 1μM was without effect on HVA Ca^{2+} channel currents, but at higher concentrations produced a slowly-developing inhibition of currents. When applied for 4min at concentrations of 5, 15 and 50μM, currents were inhibited by 14.8±1.4% 29.2±1.9% and 58.9±3.8% respectively (n=8-10, $p<0.001$ at each concentration). In the presence of 2μM nifedipine to block L-type channels fully and selectively (Caulfield *et al.*, 1992), 15μM U50488H produced an inhibition of 23.7±2.6% (n=3) of the remaining (N-type) current. The inhibitory action of 15μM U50488H was not significantly affected in 5 cells which were simultaneously exposed to 30μM naloxone (25.6±2.8% inhibition).

These findings suggest that U50488H inhibits N-type Ca^{2+} channel currents in NG108-15 cells via a mechanism which does not involve κ opioid receptor activation, as has been shown for neuronal P-type and cardiac L-type Ca^{2+} channels (Utz *et al.*, 1995; Kanemasa *et al.*, 1995). We cannot at present discount an additional inhibitory action on L-type channels in NG108-15 cells.

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54P CYCLIC AMP AND CYCLIC GMP MODULATE METHACHOLINE-INDUCED CALCIUM INFLUX IN BOVINE TRACHEAL SMOOTH MUSCLE

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Tracheal and bronchial diameter, and thereby airway resistance, are largely controlled by the contractile state of airway smooth muscle (ASM). Although the mechanisms underlying the initial phase of contraction (IP_3 generation and release from internal Ca^{2+} stores) are well characterised, those underlying the maintained phase of agonist-induced contraction have been less studied. Previous studies have shown that although dihydropyridine-sensitive Ca^{2+} channels whose activity is modulated by contractile agonists exist in ASM (Tomasic et al, 1992) they do not seem to be important in the maintainance of ASM tone (Ahmed et al, 1985). Histamine has been previously shown to activate a dihydropyridine-insensitive calcium influx pathway in bovine tracheal smooth muscle that is negatively modulated by both cyclic AMP and cyclic GMP (Boyle et al, 1994) but no data exist on the influx pathway activated by cholinergic agonists. Therefore, we have studied, using a functional assay, the Ca^{2+} influx pathway activated by methacholine (MCh) in strips of bovine tracheal smooth muscle.

Bovine tracheal smooth muscle strips were set up for the isometric recording of tension in Krebs-Henseleit buffer (KHB) at 37°C. After 30min equilibration the tissues were washed into Ca^{2+}-free KHB containing 2mM EGTA and 1μM nifedipine and challenged with 200nM MCh. The contractile response was allowed to decay to resting levels, the tissue washed with Ca^{2+}-free KHB and then challenged with 200nM MCh again. The tissue was then washed into Ca^{2+}-free KHB containing 1μM nifedipine and 200nM MCh and a Ca^{2+}concentration-effect curve constructed by the addition of $CaCl_2$ to the bath. The maximum concentration of Ca^{2+} added was 12.8mM as higher concentrations cause irreversible contracture.

The addition of Ca^{2+} caused a concentration-dependent contraction of BTSM (EC_{50} 0.5±0.1mM) this response was well maintained and repeatable, with time matched controls either superimposing on the first curve or being shifted to the left. This contraction was abolished by pretreatment of the strip with atropine (10nM). Atropine did not modify the Ca^{2+} contraction curve under depolarizing conditions or when histamine rather than MCh was the spasmogen. Isoprenaline (100nM) caused a rightward shift of the Ca^{2+} curve relative to time matched control (EC_{50} 2.0±0.3mM) and a reduction in the maximim response to Ca^{2+} (63% of control). Sodium nitroprusside (SNP; 1μM) caused a rightward shift of the Ca^{2+} curve (EC_{50} 7.9±5.0) and a depression of the maximum response (44% of control). At higher concentrations SNP further depressed the maximum response to 12.8mM Ca^{2+} (18% of control maximum) but caused no further significant shift of the curve. Amiloride (30μM) antagonised the contractile response to added Ca^{2+} (sixfold shift to the right) suggesting that Ca^{2+} influx may be through a non-selective cation channel.

These data suggest that muscarinic receptor activation leads to Ca^{2+} influx in BTSM via a non-dihydropyridine sensitive pathway possibly a non-selective cation channel. Calcium influx via this pathway can be inhibited by increases in intracellular cAMP or cGMP. Further work is required to characterise the muscarinic receptor subtype involved, and also the coupling mechanism between the muscarinic receptor and ion channel.

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55P SODIUM NITROPRUSSIDE INHIBITS THE NON-SELECTIVE CATION CURRENT ACTIVATED BY INTRACELLULAR CALCIUM STORE DEPLETION IN MOUSE ANOCOCCYGEUS CELLS

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We have been using preparations of the mouse anococcygeus to study the cellular mechanisms by which nitrovasodilators (NVDs) relax non-vascular smooth muscle. In this tissue, contractions to receptor agonists are dependent upon calcium entry *via* a pathway, distinct from voltage-operated calcium channels, and activated following depletion of intracellular calcium stores (Gibson *et al*, 1994). Recently we have described the properties of a non-selective cation conductance which appears to underlie the calcium entry process (Wayman *et al*, 1996). The aim of the present series of experiments was to determine whether NVDs affect this current, and thus calcium entry in response to store depletion.

The methodology for the preparation of single smooth muscle cells is described elsewhere (Wayman *et al*, 1996). Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique; the extracellular solution bathing the cells contained *inter alia* tetraethyl-ammonium chloride (TEA; 30mM), $CaCl_2$ (10mM) and nifedipine (1μM). The intracellular solution contained (in mM) CsCl 130, TEA 20, HEPES 10 and was supplemented with ATP 0.5 and GTP 0.5 (pH 7.20).

As reported previously, in cells held at a membrane potential of -40 mV, intracellular calcium store depletion using caffeine (10mM) produced an initial, large transient inward current - as a result of activation of calcium-dependent chloride channels - followed by a smaller (4.8 ± 0.3pA; mean ± SEM; n = 57) sustained inward current. This second, sustained current - a non-selective cation current thought to underlie store-regulated calcium entry - was inhibited by 86.5 ± 4.1% (n=30) following the application of sodium nitroprusside (SNP; 10 μM). This response to SNP was mirrored by the extracellular application of 8Br-cGMP (200μM) which inhibited the caffeine-induced non-selective cation current by 95.0 ± 3% (n = 5). When caffeine (10 mM) was applied in the continued presence of either SNP (10 μM) or 8Br-cGMP (200μM) it was unable to activate the non-selective cation current (n = 26 and 5 cells respectively). These data suggested that the inhibitory action of SNP was mediated by a rise in the intracellular concentration of cGMP, and we examined this possibility further using ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one) an inhibitor of soluble guanylyl cyclase (Garthwaite *et al*, 1995). In the presence of ODQ (1 μM), the inhibitory effect of SNP (10 μM) on the caffeine-induced cation current was significantly reduced, such that the NVD only inhibited the current by 5.7±5.6% (n = 7). The current was however readily reversed on washing with caffeine-free solution. Furthermore, caffeine (10 mM) was still able to activate the non-selective cation current (8.6 ±1.0pA; n = 5) in cells pre-treated with a combination of SNP (10μM) and ODQ (1 μM). ODQ had no effect on the ability of 8-Br-cGMP (200μM) to inhibit the caffeine-induced non-selective cation current (92.5 ± 4.8% inhibition; n = 4).

These results suggest that SNP inhibits store regulated calcium influx into single smooth muscle cells isolated from the mouse anococcygeus, a response mediated by activation of soluble guanylyl cyclase and the subsequent rise in the intracellular concentration of cGMP. This cellular action of SNP may underlie, in part, its relaxant effects in this tissue.

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56P EFFECTS OF ENDOTHELIN (ET) RECEPTOR SELECTIVE AND NON-SELECTIVE ANTAGONISTS ON ET-1-INHIBITED L-TYPE Ca^{2+} CURRENTS IN VENTRICULAR CARDIOMYOCYTES ISOLATED FROM RABBIT MYOCARDIUM

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Ventricular cardiomyocytes possess both ET_A and ET_B receptor subtypes (Molenaar *et al.*, 1993). ET-1 was found to increase the L-type Ca^{2+} current (I_{Ca}), at a concentration of 1nM, whereas the peptide decreased I_{Ca} at greater concentrations (Kelso *et al.*, 1996). The aim of the present study was to establish whether specific receptor subtypes are responsible for mediating the effects of ET-1 on the I_{Ca} using ET-receptor antagonists, including the ET_A receptor-selective antagonist, PD155080 (Doherty *et al.*, 1995), ET_B receptor-selective antagonists, BQ-788 and RES-701, and the non-selective antagonist, PD145065 (Doherty *et al.*, 1995).

Ventricular cells were isolated by enzymatic dissociation from adult, male New Zealand White rabbits (2.5-3kg) as previously described (Kelso *et al.*, 1995). Cells were perfused with a solution of the following composition (mM): NaCl 137; KCl 5.4; $CaCl_2$ 3; $MgCl_2$ 1.2; HEPES 5; Glucose 10 (pH 7.4, 35°C), and the I_{Ca} was recorded using a whole-cell patch clamp technique. In voltage-clamp mode, cells were clamped at -40mV and voltage steps of 200ms duration were applied every 5s. Following stabilisation, recordings were made from depolarizing pulses applied in 10mV increments from -40 to +60mV, in the absence and presence of the various ET-1 antagonists (1μM-10μM) in combination with ET-1 (10nM). Peak I_{Ca} values were expressed as mean±standard error, and comparisons were made using analysis of variance followed by a multiple range test (Duncan's), and values of $P<0.05$ were taken as significant.

ET-1 (10nM) decreased ($p<0.05$) the I_{Ca} to -1.86±0.18nA from a control value of -2.89±0.18nA (n=6); this effect was reversed upon washout (-2.46±0.30nA). The decrease in I_{Ca} produced by ET-1 was blocked by PD155080 and PD145065 (1-10μM); however, the I_{Ca} was increased upon washout of PD155080 (Table 1). The negative effect of ET-1 was partially blocked by the ET_B-selective antagonist, BQ-788 (Table 1). However, RES-701, in combination with ET-1, produced a similar decrease in I_{Ca} to that produced by ET-1 alone (Table 1).

It is clear that the decrease in I_{Ca} produced by ET-1 is mediated by the ET_A receptor subtype, since PD155080 completely inhibited this negative response. The negative effect of ET-1 is not mediated by an RES-701-sensitive ET_B receptor subtype, but is partially sensitive to a BQ-788-sensitive receptor subtype. Furthermore, the increase in current amplitude following washout of PD155080, in combination with ET-1, may result from a residual effect of ET-1 acting at ET_B receptors.

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Table 1. Effects of the various receptor selective antagonists alone, and in combination with ET-1 (10nM), on peak L-type Ca^{2+} current amplitude; #p<0.05 with respect to ET-1 alone; *p<0.05 with respect to control values.

	PD155080(n=6)	BQ-788(n=6)	RES-701(n=10)	PD145065(n=8)
Control	-2.65±0.15nA	-2.57±0.27nA	-2.65±0.24nA	-2.45±0.28nA
ET-1 + antagonist (1μM)	-2.53±0.13nA#	-2.13±0.20nA#*	-1.63±0.13nA*	-2.31±0.26nA#
ET-1 + antagonist (10μM)	-2.58±0.14nA#	-2.28±0.20nA#*	*missing data*	-2.40±0.30nA#
washout	-2.88±0.13nA	-2.56±0.23nA	-2.26±0.24nA	-2.34±0.29nA

57P INOSITOL 1,4,5-TRISPHOSPHATE PERFUSION PRODUCES QUANTAL CALCIUM UPTAKE AND RELEASE IN SH-SY5Y NEUROBLASTOMA CELLS

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D-*myo*-inositol 1,4,5-trisphosphate [IP_3]-induced Ca^{2+} mobilisation (IICM) exhibits biphasic kinetics, whereby sub-optimal concentrations of IP_3 induce a rapid release of only a fraction of the total IP_3-sensitive Ca^{2+} pool, followed by a prolonged "leak" phase of slow Ca^{2+} efflux. This phenomenon has been labelled "quantal Ca^{2+} release" (QCR) or "incremental detection". Here IP_3 perfusion of permeabilised SH-SY5Y human neuroblastoma cells was used to investigate QCR under conditions where prolonged Ca^{2+}-dependent feedback on the IP_3-receptor is reduced.

SH-SY5Y cells were harvested and washed in a cytosolic-like buffer (CLB), electropermeabilised and perfused on to glass wool packed into a perfusion cell where they were immobilised. The cells were then perfused with CLB supplemented with 1 μM fluo-3 (adjusted to 150-250 nM free Ca^{2+} using EGTA 0-1 μM). Downstream the perfusate passed through a 200 μl fluorometric cell where the Ca^{2+} dependent changes in fluo-3 were continuously monitored. The fluorescence intensity of the fluo-3-CLB perfusate was recorded at 1 sec intervals with excitation at 505 nm (5 nm slit-width) and emission collected at 530 nm (5 nm slit-width) using a LS-50β fluorimeter (Perkin-Elmer, UK). The fluorescence intensity values were calibrated as described [1].

Electropermeabilised SH-SY5Y cells (basal free Ca^{2+} 150-250 nM) were perfused with each increasing concentration of IP_3 (0.1-10 μM) for 30 min, then allowed to recover for 20 min between doses. The cells exhibited clear concentration-dependent IP_3-induced Ca^{2+} mobilization associated with a proportional ATP-dependent Ca^{2+} uptake when cells were allowed to recover in IP_3-free fluo-3-CLB. This release was IP_3 receptor specific exhibiting all the stringent positional and stereospecificity requirements of this binding site [2].

The Ca^{2+} release peak rose rapidly and fell more slowly recovering to the basal Ca^{2+} even in the presence of continuous perfusion with the same IP_3 concentration. However stimulating with supra-maximally effective concentrations of IP_3 (5-10 μM) yielded a unique profile, consisting of rapid onset calcium release (1000-1500 nM above basal), the slower recovery phase, but also a unique sustained Ca^{2+} release plateau. Naive cells exposed only to maximal IP_3, without prior exposure to lower doses, exhibited an identical sustained Ca^{2+} release plateau. Although the plateau was relatively small (50-150 nM above basal) it was not due to Ca^{2+} contamination in the IP_3 since this was stringently controlled to < 10 nM using chelex-100 or BAPTA-sponge. When cells were allowed to recover from a maximal IP_3 concentration (5-10 μM) in IP_3-free CLB, the initial uptake was rapid (2-3 mins) and within 15 mins stabilised the Ca^{2+} to basal levels.

We hypothesise that at sub-maximally effective concentrations of IP_3 the incremental inactivation resistant "leak" phase of IICM can be effectively accomodated by the activity of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. However at supra-maximal IP_3 the SERCA pumps fail to fully accommodate the more significant "leak" phase, resulting in the small but sustained Ca^{2+} release plateau. We provide evidence to support our hypothesis that the secondary leak phase of IICM which follows quantal inactivation of IP_3-induced Ca^{2+} release is significant at supra-maximally effective IP_3 concentrations.

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58P INTRACELLULAR Ca^{2+} RESPONSES TO BRADYKININ, METHACHOLINE OR K^+ DEPOLARIZATION ARE GRADED ACCORDING TO STIMULUS STRENGTH IN SINGLE SH-SY5Y HUMAN NEUROBLASTOMA CELLS

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Following activation of a variety of cell surface receptors, intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) elevation occurs by release from intracellular stores and/or Ca^{2+} influx. In cell populations the size of $[Ca^{2+}]_i$ elevation is related to stimulus strength. In some cases this may represent progressive recruitment of cells responding in an "all-or-none" manner but having variability in the initiating stimulus intensity. This phenomenon may be cell and/or agonist dependent. Thus, for agonists acting via the second messenger, $Ins(1,4,5)P_3$, all-or-none $[Ca^{2+}]_i$ responses may be a consequence of a slow $Ins(1,4,5)P_3$ generation rate often associated with high agonist affinity (eg. many peptides) (Chiavaroli et al, 1994). Using ratiometric fluorimetry we examined $[Ca^{2+}]_i$ signalling (at 37°C) to activation of muscarinic M_3- or bradykinin B_2-receptors or K^+ depolarization in single, fura-2 loaded, SH-SY5Y cells (Willars and Nahorski, 1995). Agonists were applied for 30s with 300s between stimuli unless stated. Data are mean±sem of 340/380nM excitation ratios and compared by Student's paired t-test.

In 7 out of 8 (7/8) cells, maximal [methacholine] (MC) (100μM) gave a greater ($p=0.025$) $[Ca^{2+}]_i$ spike (5.65±0.51) than a preceding maximal [bradykinin] (BK) (10μM) (4.26±0.37) indicating BK had not evoked maximal cellular $[Ca^{2+}]_i$ elevation. Furthermore, in all cells (n=4-6), maximal [MC] or [BK] gave greater ($p<0.05$) $[Ca^{2+}]_i$ responses (2.62±0.39 and 2.46±0.28 respectively) than a preceding lower concentration of the same agonist (0.1μM MC or 16nM BK) (1.60±0.18 and 1.67±0.14) consistent with a concentration-dependent $[Ca^{2+}]_i$ elevation. It was possible, however, that graded Ca^{2+} entry masked all-or-none intracellular Ca^{2+} release. In the absence of a trans-plasmalemmal Ca^{2+} gradient (extracellular [Ca^{2+}] 88±5nM (n=5)) to prevent Ca^{2+} entry, following 100μM MC BK was unable to elevate $[Ca^{2+}]_i$. This indicates a Ca^{2+} store shared by the agonists, its full depletion by 100μM MC and a Ca^{2+} influx requirement for refilling. At low extracellular [Ca^{2+}], all cells (n=4-7) did, however, respond to maximal [MC] or [BK] (2.00±0.16 or 1.81±0.26) following a low concentration of the same agonist (1.66±0.23 or 2.22±0.70). Thus, responses to low [agonist] were not all-or-none. K^+ also evoked a rapid $[Ca^{2+}]_i$ spike followed by a lower sustained phase, both dependent upon extracellular Ca^{2+}. Both phases were [K^+] dependent ($20<50<100$mM). Furthermore, in 5/5 cells, 100mM K^+ added during a sustained 50mM K^+ response evoked a $[Ca^{2+}]_i$ spike (2.00±0.15) greater ($p=0.02$) than the initial response (1.62±0.14).

Although it is difficult to exclude an all-or-none release of intracellular Ca^{2+} dependent upon Ca^{2+} influx and masked by graded Ca^{2+} entry, these data suggest that $[Ca^{2+}]_i$ responses in SH-SY5Y are graded with stimulus intensity. We cannot preclude all-or-none responses to other agonists or these agonists in other cells where kinetics of $Ins(1,4,5)P_3$ generation or the Ca^{2+} release elements may differ.

Supported by a Programme Grant from the Wellcome Trust

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59P BLOCKADE OF NEURONAL HYPOXIA-INDUCED [^{45}Ca] UPTAKE BY Ca^{2+} CHANNEL ANTAGONISTS

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It is well documented that when neurones become hypoxic there is both an increase in intracellular calcium concentration (Choi, 1988) and glutamate release which results in cell death (Choi and Rothman, 1990). The effects of hypoxia on [^{45}Ca] uptake in rat cerebellar granule cells in culture have been studied. The effects of drugs acting upon glutamate release and voltage-sensitive calcium channels have been investigated in control and hypoxic cells.

Rat cerebellar granule cells were prepared according to the method of Schousboe *et al.* 1989. Neurones were seeded in 24-well plates precoated with poly-l-lysine (50μgml-1) at a density of 0.25-0.3x10^6 cells cm^{-2}. Cells were maintained in Dulbecco's Minimum Essential Medium / Ham's F-12 (3:1), supplemented by 5% horse serum, 10% foetal calf serum, 2mM L-glutamine, 25mM KCl and 1% penicillin/streptomycin. After 9-11 days *in vitro* the culture-conditioned medium was removed from cells and replaced by either control or hypoxic medium alone or medium containing appropriate drugs. Hypoxic medium was defined as having an O_2 content 3-7% that of control. 0.25μCi [^{45}Ca] was added to each well and cells incubated for 2 h at 37°C. [^{45}Ca] present in granule cells was separated from free [^{45}Ca] by filtration. IC_{50} values were determined using a minimum of four drug concentrations and determined from probit plots.

A time course showed that [^{45}Ca] uptake increased when cells were exposed to hypoxic medium compared with control. This increase was maximal at 2 h, and this incubation time was used to compare the effects of various drugs on hypoxia-stimulated [^{45}Ca] uptake. Dizocilpine and NBQX (6-nitro-7-sulphamoylbenzo(f)-quinoxaline-2,3-dione) both reduced hypoxia-stimulated [^{45}Ca] uptake in cerebellar granule cells (see Table 1). Dizocilpine being the most potent inhibitor of [^{45}Ca] uptake in this system. The glutamate release inhibitor, riluzole, inhibited the hypoxia-induced rise in [^{45}Ca] uptake.

Table 1: The effects of drugs acting upon hypoxia-induced [^{45}Ca] uptake in rat cerebellar granule cells.

Glutamate receptor antagonists and release inhibitors	IC_{50} (μM)	Voltage - sensitive calcium channel antagonists	IC_{50} (μM)
Dizocilpine	0.048±0.012	Verapamil	>100
NBQX	1.58±0.22	Felodipine	>30
Riluzole	63.10±13.58	ω-Conotoxin GVIA	0.316±0.03
Ifenprodil	28.18±5.46	ω-Conotoxin MVIIC	0.398±0.05
		ω-Agatoxin IVA	0.025±0.005

Values are mean ±sem of 6-8 samples

Verapamil and felopdipine both of which act at L-type VSCCs had no effect in [^{45}Ca] uptake in either control or hypoxic cells. ω-conotoxin GVIA reduced hypoxia-induced [^{45}Ca] uptake at high concentrations, as did ω-conotoxin MVIIC, with similar potencies. However, ω-agatoxin IVA (Minz *et al.*, 1992), which blocks P channels was the most potent compound at reducing the rise in intracellular [^{45}Ca] uptake caused by exposure to hypoxic medium.

When rat cerebellar granule cells were exposed to hypoxia in the presence of glucose there was an increase in [^{45}Ca] uptake. This increase was reduced by NMDA receptor antagonists and an AMPA receptor antagonist. Our results suggest also that VSCCs of the P-type are involved in hypoxia-stimulated [^{45}Ca] uptake in this model.

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Minz I M , Venema VJ, Sunderek KM *et al.*, (1992) Nature 355, 827-828.
Schousboe A, Meier E, Drejer J & Hertz L (1989) A dissection and tissue culture manual of the nervous system 203-206.

60P HIPPOCAMPAL MUSCARINIC RECEPTORS MODULATE ANXIETY-LIKE BEHAVIOUR (ALB) IN RATS TESTED IN THE BLACK-WHITE BOX

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Intra-hippocampal administration of the cholinergic antagonist scopolamine (SCOP) 20 min prior to restraint stress in rats results in hyper-secretion of corticosterone and adrenocorticotrophin (Bhatnagar et al. 1994). We reported that peripheral injections of SCOP increased indices of anxiety-like behaviour (ALB) in rats (Smythe *et al*, in press) and, given the role of the hippocampus in the control of stress hormone secretion, we sought to investigate if the ALB response might be due to an effect on the hippocampus. In the present study we have examined ALB following intra-hippocampal infusions of SCOP.

Adult male Lister rats (350-500 g) were implanted bilaterally with 27g guide tubes aimed above the stratum moleculare of the hippocampus (A-P -3.3; M-L ±2.5; D-V -2.3 mm) under pentobarbital anaesthesia (60mg/kg), 3 weeks prior to behavioural testing. On the test day, rats were injected with vehicle (VEH), 15 or 30 ug SCOP, 20 min prior to being placed into the white chamber of the Black-White box (n=7/group). The injection stilette protruded 0.5 mm beyond the tip of the guide tube and the injection volume was 3 ul. Rats were videotaped and the tapes were scored for latency to exit the white chamber, latency to re-enter the white chamber, and amount of intercompartmental crossing. At the conclusion of testing rats were perfused with 10% formalin and guide tube placements were assessed histologically. Data were analysed by analysis of variance (ANOVA) and post hoc testing was performed using a Bonferroni corrected t-test.

ANOVA revealed significant group effects for both time to exit the white arena $F(2,17)=3.60$, $P<.05$, and time to re-enter the white chamber $F(2,17)=4.73$, $P<.03$. As illustrated in figure 1, SCOP at 30 ug significantly reduced time to exit the white arena ($P<.05$), while the 15 ug dose elevated latencies to re-enter the white chamber ($P<.01$). There was no effect of SCOP on crossing behaviour (data not shown). Histological analysis confirmed that guide tubes were all positioned above the stratum moleculare such that injection sites ranged from D-V -2.4 to -2.9mm.

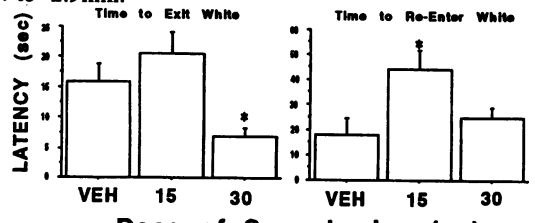

Figure 1. The effects of intrahippocampal infusions of SCOP on time to exit the white chamber (left) and time to re-enter the white compartment (right). Means ±SEM are shown. *significantly different from VEH group ($P<.01$).

Hippocampal ACh systems are important regulators of arousal and attention (Karczmar, 1995; Vinogradova, 1995). Together with our previous report on the effects of SCOP on ALB (Smythe et al., *in press*), it would appear that loss of cholinergic function in this region impairs processing of threatening stimuli that manifests itself as ALB. In conclusion, we contend that hippocampal ACh transmission modulates ALB, but other ACh projections are also involved.

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Karczmar, A.G. (1995) *Prog. Neuro-Psychopharmacol. & Biol. Psychiat.* 19, 187-211.
Smythe, J. W. *et al. Pharmacol. Biochem. Behav.* (in press)
Vinogradova, O. (1995) *Prog. Neurobiol.* 45, 523-583.

61P REPEATED EXPOSURE TO THE BLACK-WHITE BOX DECREASES SENSITIVITY TO SCOPOLAMINE-INDUCED ANXIETY-LIKE BEHAVIOUR (ALB)

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Central acetylcholine (ACh) systems modulate arousal and attention to sensory stimuli (Karczmar, 1995). Recently, we demonstrated that ACh blockade with scopolamine (SCOP) increased anxiety-like behaviour (ALB) in rats tested in the Black-White box (Smythe et al., in press). Repeated presentations of an arousing stimulus produces behavioural habituation and is accompanied by lower ACh activity (Bland, 1986). In the present study, we investigated ALB in rats who were repeatedly exposed to the Black-White box, and how this affected their sensitivity to SCOP.

Adult male Lister rats were assigned to one of two conditions: 1) naive (no prior testing); and 2) habituated (exposed for 5 min/day for the 7 days preceeding the drug evaluation). Rats within each condition were injected with SCOP (0.2 mg/kg ip) or vehicle (VEH; 0.9% saline), 20 min prior to being placed into the white chamber of the Black-White box (n=6/group). Rats were videotaped and the tapes were scored for latency to exit the white arena, latency to re-enter the white chamber, total time spent in the white chamber, and number of intercompartmental crossings. Data were analysed by analysis of variance (ANOVA) and post hoc testing was performed using a Bonferroni corrected t-test.

ANOVA revealed a main effect of condition $F(1,20)=19.6$, $P<.001$, on latency to re-enter the white chamber. Figure 1A shows that habituated rats exhibited significantly delayed latencies compared to the naive rats ($P<.001$). ANOVA on the data for time spent in the white arena showed effects of both drug $F(1,20)=4.84$, $P<.04$, and condition $F(1,20)=21.98$, $P<.001$. Figure 1B demonstrates that SCOP markedly reduced time in the white chamber ($P<.01$) and that habituated rats spent less time in the white compartment ($P<.01$). Figure 1C shows group data for the inter-compartmental crossing variable. ANOVA on these data revealed an interaction between drug treatment and condition $F(1,20)=7.54$, $P<.013$. SCOP decreased crossing behaviour, but only in naive rats ($P<.01$), while habituated rats displayed little crossing behaviour ($P<.001$).

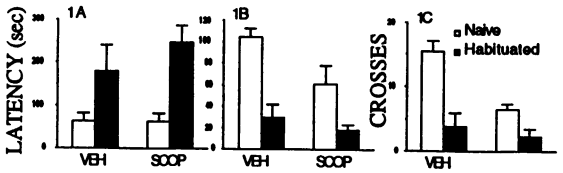

Figure 1. The effects of scopolamine on latencies to re-enter (1A) and time spent in (1B) the white chamber. Panel 1C shows data for amount of intercompartmental crossing behaviour. Means ±SEM are shown. See text for details regarding statistics.

Habituation to the Black-White box produced ALB (reduced time to re-enter the white arena, reduced time spent in the white, and reduced intercompartmental crossings), although these responses probably indicate disinterest rather than anxiety. SCOP elicited minimal ALB in habituated rats, although this may result from the lower control levels of ALB. In summary, these results show that habituation and acute SCOP administration produce behaviours representative of ALB. The lower sensitivity to SCOP in habituated rats suggests that ACh only alters ALB in animals who are already fearful.

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Smythe, J. W. *et al. Pharmacol. Biochem. Behav.* (in press)

62P THE EFFECT OF LITHIUM ON THE HYPERMOTILITY RESPONSE TO THE CENTRAL ADMINISTRATION OF OUABAIN IN THE RAT

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Intracerebroventricular (ICV) administration of ouabain (OB) induces hypermotility in rats which has been suggested to be a model of mania (El-Mallakh et al, 1995). We have characterised the sensitivity of this response to Lithium Chloride (LiCl), a clinically effective antimanic drug (Gelenberg et al, 1989).

Rats (male, Wistar, 275-300g) were implanted under anaesthesia (medetomidine, 0.4 mg/kg s.c., fentanyl 0.45 mg/kg i.p.) with a chronic ICV cannula (El-Mallakh et al, 1995). Twelve days later basal locomotor activity (LMA: defined as the number of transits in a perspex chamber 42(l) x 21(w) x 20(h) cm) was assessed for 30 min in an automated system following an ICV saline injection. Groups of 8-10 rats received either LiCl (50 mg/kg s.c.) or saline (1 ml/kg s.c.) 30 min before the administration of ICV OB (0.25 x 10^{-3} M, all ICV injections were 5 ul in 1 min.) or saline. LMA was monitored for 30 min. Following the LMA monitoring, 0.5ml of venous blood was taken from each rat for measurement of plasma Li^+ levels. LiCl (or saline) was then administered for 6 days (b.i.d.) at 16.7 mg/kg s.c. 18hrs after the last dose of LiCl, the rats received ICV OB as before, LMA was measured and plasma samples taken. In a similar study, hypermotility was induced by dex-amphetamine (AMP) (1 mg/kg s.c.) and the interaction with LiCl (same dosing regime) was investigated.

It was found that OB significantly elevated LMA from 15.4 ± 3.6 to 34.4 ± 8.0 transits (mean ± s.e.m., $p<0.05$, all statistical tests were 2-way ANOVA, Newman-Keuls). Following acute administration, LiCl alone lowered LMA to 8.9 ± 1.4 (NS), and significantly reduced the LMA response to OB to 12.3 ± 2.4 ($p<0.01$). After chronic administration of saline, LMA was again significantly elevated by OB from a basal level of 20.0 ± 4.5 to 80.0 ± 20.2 ($p<0.01$). In contrast to the acute effect, chronic LiCl did not reduce basal LMA below control levels (20.0 ± 2.3), but still significantly reduced the response to OB to 39.0 ± 8.3 ($p<0.05$). Plasma levels of Li^+ determined by atomic absorption spectroscopy were not significantly different following acute or chronic administration (data not shown). AMP hypermotility, (eg 18.0 ± 2.6 to 46 ± 7.4, $p<0.01$), which is not thought to be sensitive to LiCl (Cappeliez and Moore, 1990), was not inhibited by either acute or chronic LiCl.

Thus it can be seen that LiCl selectively inhibited the hypermotility response to ICV OB following acute and chronic administration whilst having no effect on AMP hypermotility. This data thus suggests that ICV OB induced hyperlocomotion may be a viable model of mania.

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63P YOHIMBINE'S ANXIOGENIC EFFECT IN THE BLACK-WHITE BOX CAN BE REDUCED BY CHLORDIAZEPOXIDE: CONFIRMATION OF TEST VALIDITY FOR RATS

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The Black-White box has been used frequently to measure anxiety-like behaviour (ALB) in mice (Costall *et al.*, 1989), but there is comparatively less assurance that the task is valid for rats. We have reported that cholinergic blockade produces increased ALB in rats as measured by the Black-White box (Smythe *et al.*, in press), and it seemed expedient to determine if the task would also prove sensitive to known anxiolytic (chlordiazepoxide; CDZ) and anxiogenic (yohimbine; YOH) agents administered to rats.

Adult male Lister rats were administered CDZ (5.0 mg/kg), YOH (2.5 mg/kg) or vehicle (VEH; 0.9% saline), while a fourth group was treated with YOH followed 40 min later by CDZ (n=6/group). All injections were performed IP. In order to control for the extra injection regimen of the CDZ+YOH group, the other 3 groups were injected with VEH 40 min prior to their respective experimental treatments. Forty min after the drug injections, rats were placed into the white chamber of the Black-White box. Rats were videotaped and the tapes were scored for latencies to exit and re-enter the white chamber, total time spent in the white chamber, and number of intercompartmental crossings. Data were analysed by analysis of variance (ANOVA) and post hoc testing was performed using a Bonferroni corrected t-test.

ANOVA revealed no drug effect on latency to exit the white arena, but there was a significant effect on time to re-enter the white chamber $F(3,20)=9.47$, $P<.001$. As shown in figure 1, YOH delayed re-entry times ($P<.001$) compared to VEH rats, and while CDZ produced no effect on its own, it significantly reduced the YOH effect ($P<.01$). There was an overall drug effect on time spent in the white compartment $F(3,20)= 3.66$, $P<.03$. Post hoc comparisons demonstrated that YOH significantly lowered time spent in white ($P<.01$), an effect that was absent in CDZ+YOH rats. These data are illustrated in figure 1. Intercompartmental crossing behaviour was also affected by drug treatment $F(3,20)=8.00$, $P<.001$. Pairwise comparisons showed that CDZ treated rats exhibited significantly more crosses between chambers relative to all other groups (P's<.01-.001). These data are also depicted in figure 1.

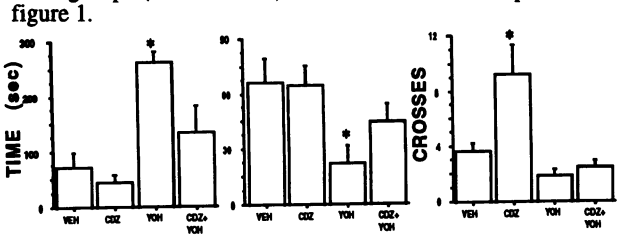

Figure 1. Latencies to re-enter (left) and time spent in (middle) the white chamber following drug treatment. The right hand panel shows crossing behaviour. Means ±SEM are shown. See text for full statistical details.
*significantly different from VEH group (P's<.01-.001)

On the basis of these data, we contend that the Black-White box is a valid model for assessing ALB in rats. In rats, this task may be comparatively more sensitive to anxiogenic drugs and a useful screen for anxiolytic drugs tested against anxiogenic agents. In conclusion, as for mice, the Black-White box is a useful investigative tool for research into ALB in rats.

Costall, B. *et al.* (1989) *Pharmacol. Biochem. Behav.* 32, 777-785.
Smythe, J. W. *et al. Pharmacol. Biochem. Behav.* (in press)

64P CHRONIC MILD STRESS INDUCES A DECREASE IN VOLUNTARY INTAKE OF 10% ETHANOL IN A FOUR BOTTLE CHOICE PARADIGM

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A link between stress and alcohol drinking has long been postulated, but experimental evidence has shown stress to both increase and decrease the intake of ethanol. The stressors used in these experiments were acute and often severe, for example foot shock. Willner (1992) demonstrated that sequential, unpredictable application of very mild stressers decreased both the intake of sweet solutions and the conditioned place preference produced by amphetamine (Papp et al., 1993). The present study determined the effects of such a stress schedule on the intake of different concentrations of ethanol and the influence of continuous availability of alcohol during the stress.

Male Wistar rats (300 - 420g) were housed singly, with normal laboratory food pellets and lights on 10 pm and off at 10 am; a 2% sucrose solution, in addition to water was made available for 48h. All animals were then given a choice of water, or 5%, 10% or 20% ethanol for 22h per day for two weeks, to provide baseline values. Measurements of the amount of each liquid drunk were made over the first 2h of the dark period every day. Rats were then allocated to one of four groups (n = 20 per group): (a) controls (i.e. no stress) (b) chronic mild stress (c) controls with 7.5% ethanol available continuously (in addition to water) and (d) chronic mild stress with 7.5% ethanol, and water, available continuously. The unpredictable stress schedule, including (at different times) cage tilt, food and/or water deprivation, pair housing, restricted space and wet bedding, was carried out for the next 4 weeks. Ethanol intake was measured for 2h, once a week, at the beginning of the dark period.

Both groups of rats (b and d) which received the chronic mild stress treatment showed a significant decrease in the intake of 10% ethanol, compared with corresponding controls, but there were no significant changes in the intake of the other concentrations of ethanol. Water intake was unaffected during the procedure, and both stressed groups showed the expected decreases in sucrose intake during the stress schedule.

The results are comparable with previous findings with regard to mild stress. Wolffgram demonstrated decreased intake of 10% ethanol, but not 5% or 20% ethanol, when rats were socially isolated, compared with group housing. It is possible that intake of 10% ethanol produces different types or levels of reinforcement from the other two concentrations.

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Willner, P. (1992) *Neurosci. Biobehav. Rev.*.16, 525 - 534
Wolffgramm, J. (1990) *Psychopharmacol.*, 101, 233 - 239

Table 1. Values are mean ± s.e.m. for g/kg ethanol drunk per 2h period. Statistical comparisons were by two-way analysis of variance for repeated measures, followed by Student's t-test for daily comparisons with controls; P values presented were from the t-test analysis. *P < 0.05, compared with corresponding control group. CMS = chronic mild stress; Con. = controls; eth. = ethanol available

	Group	Week 1	Week 2	Stress	Week 3	Week 4	Week 5	Week 6
a	Controls	0.32 ± 0.03	0.31 ± 0.02	starts	0.31 ± 0.05	0.25 ± 0.02	0.28 ± 0.04	0.29 ± 0.02
b	CMS	0.34 ± 0.04	0.33 ± 0.03		0.29 ± 0.03	0.22 ± 0.02	0.18 ± 0.01*	0.2 ± 0.01*
c	Con. + eth.	0.33 ± 0.04	0.3 ± 0.02		0.25 ± 0.02	0.24 ± 0.01	0.25 ± 0.01	0.26 ± 0.01
d	CMS + eth.	0.35 ± 0.03	0.31 ± 0.2		0.23 ± 0.01	0.19 ± 0.02	0.19 ± 0.02*	0.2 ± 0.01*

65P IS THERE A CORRELATION BETWEEN THE EFFECTS OF CALCIUM CHANNEL ANTAGONISTS ON ETHANOL WITHDRAWAL HYPEREXCITABILITY *IN VIVO* AND *IN VITRO*?

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The ethanol withdrawal syndrome consists of tremor, anxiety, and convulsions. The calcium channel antagonists, nitrendipine and isradipine, completely prevented withdrawal signs in vivo. Other calcium channel blockers, however, showed different effects, felodipine did not protect against withdrawal (Watson et al., 1994) and diltiazem increased the severity of the syndrome (Watson and Little, 1994). This study compared the effects of these compounds on the withdrawal hyperexcitablity seen in hippocampal slices (Whittington & Little., 1990).

Male C57 mice were given ethanol, 24% as sole fluid for 7 to 9 months; ethanol intake was 12-14 g/kg/day and controls drank tap water. Hippocampal slices were prepared between 9 am and 9.30 am each day without prior withdrawal of the mice from ethanol treatment (lights on 9 am); n=4-6, one slice per mouse. After 105 min equilibration, extracellular recordings were made for 7h from cessation of alcohol treatment (i.e. from slice preparation).The drugs were included in the bathing medium for the whole 7h. Drug concentrations were chosen on the basis of brain concentration measurements (isradipine), comparison of binding affinities (felodipine) and prior electrophysiological studies (diltiazem).Statistical analysis was by Student's t-test for single spike and Mann-Whitney U test for multiple spike thresholds.

Single and multiple spike thresholds for elicitation of population spikes were decreased in slices prepared after the ethanol treament, as shown previously. In the presence of isradipine, 4 μM, these changes were almost completely prevented; the effect was significant. Felodipine, 1 or 10 μM, or diltiazem, 30 μM, had no significant effect on spike thresholds during withdrawal. No effects on thresholds were seen in control preparations at the drug concentrations used; concentrations of diltiazem higher than 30 μM did affect the control responses.

The effects of calcium channel antagonists on ethanol withdrawal hyperexcitability in vitro showed a parallel with their effects on withdrawal hyperexcitability in vivo, in that isradipine was effective and felodipine was not. Diltiazem did not protect against withdrawal in vivo or in vitro, but did not appear to increase hyperexcitability in vitro, as seen in vivo.

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Table 1. Stimulus thresholds (μA) for single (mean ± s.e.m.) and multiple (medians, interquartile ranges, cut off 350 μA) population spikes, 7h after slice preparation. * P<0.01 cf. controls in control medium, † P<0.01 cf. chronic ethanol + control medium. Chr. tr. = chronic treatment; Medium = bathing fluid; Single, multiple = single, multiple spike thresholds; Israd. = isradipine

Chr.tr.	Medium	Single	Multiple	Chr.tr.	Medium	Single	Multiple
Controls	Control	57.5 ± 2.4	350 (350,350)	Ethanol	Felodipine 1μM	34.3 ± 2.9*	44 (44,197)*
Ethanol	Control	34.3 ± 0.5*	47.5 (39.5,68)*	Ethanol	Felodipine 10μM	33.8 ± 1.7*	46.5 (43,50)*
Ethanol	Israd, 4μM	55.7 ± 1.6†	350 (350,350) †	Ethanol	Diltiazem 30 μM	36.0 ± 1.5*	79 (60,156)*

66P ACAMPROSATE HAS ACTIONS IN THE MURINE ELEVATED PLUS MAZE

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Acamprosate is used clinically for decreasing drinking in alcoholics (Chick, 1995) and in animal models it decreases voluntary intake of ethanol. Its mechanism of action is presently unclear, but it appears to have actions at a low-affinity binding site of the dihydropyridine-sensitive calcium channel (Al-Qatari and Littleton, 1995). As there are links between stress, anxiety and alcohol intake, the present study examined the actions of acamprosate on the murine elevated plus maze and on convulsions induced by N-methyl aspartate (NMDA).

Groups of 8 to 10 male TO mice (35-40g) were used in all studies. Acamprosate (50 - 200 mg/kg) or saline vehicle was injected i.p. 30 min before all tests. In the plus maze study, acamprosate was also given at 400 mg/kg and diazepam 2.5 mg/kg included for comparison. Mice were placed on an elevated plus-maze for 5 min and videotapes were analysed by a trained observer, blind to the drug treatment. In the second experiment, the mice were injected with NMDA (300 mg/kg), placed in fresh cages and observed. The latency to the distinct hyperactivity syndrome and the time until the first full convulsion were measured (the mice were then humanely killed). Results are shown as mean ± s.e.m.

Acamprosate had effects in the elevated plus maze, with a U-shaped dose-response curve, producing a mild reduction in anxety-related behaviour. At 200 mg/kg it caused a decrease in the time spent on the closed arms, a decrease in closed arm returns ($P < 0.05$ compared with saline). This profile differed from that of diazepam, which showed an increase in time on the open arms, and a decrease in stretch attend postures.

A U-shaped dose-response curve was again seen in the effects of acamprosate against NMDA. Here, however, the peak effect was seen at 100 mg/kg, when it decreased the time taken for initiation of the hyperactive syndrome ($P < 0.05$ compared with saline).

These results indicate acamprosate has some anxiolytic activity, which might contribute to its effects in alcohol dependence. In contrast, with a different dose relationship, it appears to increase the effects of NMDA, an effect which might not be expected to be associated with anxiolytic action. Further experiments are continuing to examine the actions of this compound following long-term alcohol intake in mice.

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We thank Groupe LIPHA for gifts of acamprosate and the MRC for financial support.

Table 1 Effects of acamprosate on the elevated plus maze. Acamprosate and NMDA

Drug treatment	% time on closed arms	% time on open arms	stretch-attend postures	Closed arm returns	Convulsion Incidence	Latency (s) to hyperactivity	Convulsion latency (s)
Saline	43.3±5.8	13.2±4.2	57.1±5.2	3.5±1.0	6/10	734±185	703±230
Acamprosate 50 mg/kg	35.1±3.6	16.4±4.1	54.0±5.2	2.3±1.1	7/10	697±171	601±104
Acamprosate 100 mg/kg	32.5±2.4	16.6±2.5	52.4±3.6	1.7±0.7	6/10	276±23*	447±32
Acamprosate 200 mg/kg	30.1±3.5*	22.2±4.6	49.9±4.8	1.4±0.4*	7/10	435±37	728±112
Acamprosate 400 mg/kg	40.8±5.4	13.0±3.6	42.7±5.6	2.4±0.9			
Diazepam 2.5mg/kg	42.7±9.7	45.9±10.4*	11.6±3.7*	0.0±0.0*	* P < 0.05 c.f. saline (Student's t-test)		

67P THE EFFECT OF FLUOXETINE ON SOCIAL COMPETITION AND DRINKING BEHAVIOUR IN THE RAT

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Changes in the social hierarchy formed between groups of rats competing for access to palatable fluids has been proposed as a possible animal model of anxiety (Joly & Sanger, 1991). However, as feeding and drinking behaviour *per se* can be altered by anxiolytic drugs, for example the benzodiazepines (Cooper, 1980), drug induced changes in social competition could simply be due to changes in appetite. The aim of the present study was to determine the effect of fluoxetine which is known to modify anxiety (Handley & McBlane, 1992) and decrease feeding behaviour (Clifton *et al.*, 1989), on social competition in triads of rats when administered to dominant and subordinate animals in each hierarchy. In addition, the effect of fluoxetine on the intake of sweetened milk was measured to determine whether any changes in drinking behaviour are accompanied by changes in the rank order of an animal in the social hierarchy.

Baseline levels of competition were established for a 5 week period during 5 min access to sweetened milk in triads (n=7) of male Lister Hooded rats weighing 271.8±10.4g at the start of the study. For each group the social hierarchy was established by observing which animal had access to the spout at 5s intervals throughout the testing period (e.g. 60 observations for one 5 min trial). All groups were tested twice a week. The effect of fluoxetine (2.5, 5.0 and 10.0 mg/kg i.p.) on the access to sweetened milk was assessed 30 min after treatment of either the dominant or subordinate rat. Data were analysed using one factor ANOVA followed by Dunnett's t-test and converted to % drinking for each rat in each group. The effect of fluoxetine on individual drinking rates was obtained by placing individual rats in a test box and measuring the amount of milk consumed (g) after 5 min. Also, during the 5 min drinking period the rats were observed and the time spent at the spout was recorded as described for the competition procedure. Intake data were analysed using one factor ANOVA followed by Dunnett's t-test.

During the initial 5 week studies the triads of rats developed stable hierarchies consisting of dominant, intermediate and subordinate animals which had access to the drinking spout for 39.0±0.9%, 31.0±1.1% and 19.4 ±1.7% of the 5 min testing period respectively. Administration of fluoxetine to either the subordinate rat or the dominant rat in each group did not significantly affect social competition at any of the doses tested. During unlimited access to sweetened milk fluoxetine (10.0 mg/kg) significantly decreased the time spent at the spout by the dominant (70.5 ± 8.9% to 30.2 ± 10.6% $p<0.01$), intermediate (75.2 ± 18.6% to 36.2 ± 10.5% $p<0.01$) and subordinate (77.6 ± 5.5% to 42.2 ± 10.4% $p<0.05$) rats. At the highest dose (10.0 mg/kg) the amount consumed (g) during the 5 min was also significantly decreased in dominant (8.3 ± 1.8g to 3.7 ± 0.7g $p<0.05$), intermediate (8.9 ± 0.9g to 3.7 ± 1.5g $p<0.01$) and subordinate rats (8.1 ± 0.8g to 4.5 ± 0.9g $p<0.01$).

These results show that stable hierarchies of rats measured in terms of access to sweetened milk are not disrupted by administration of fluoxetine at doses which decrease drinking in terms of both time spent at the drinking spout and amount consumed by individual rats.

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68P THE EFFECT OF SOCIAL ISOLATION ON THE BEHAVIOURAL EFFECTS OF COCAINE

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Previous research has demonstrated that social isolation can lead to an enhanced sensitivity to the effects of psychomotor stimulants such as amphetamine (Jones *et al.*, 1992), although results with cocaine have been equivocal (Boyle *et al.*, 1991, Phillips *et al.*, 1994). The aim of the present study was to assess the influence of post-weaning housing conditions on the behavioural effects of cocaine.

Female Hooded Lister rats were housed either in social isolation (isolates), or in groups of 6 (enriched) from weaning for 12 weeks prior to testing. The enriched group had access to toys and were handled daily. Rats (250-300g; n=12 per group) were administered cocaine (5mg/kg i.p.) or vehicle 30min prior to assessment of locomotor activity (LMA) within an automated open field chamber over a 15min period. LMA was also assessed in photocell boxes immediately following daily treatment with cocaine (5 & 10mg/kg i.p.) or vehicle in a 2h test over a 10 day period. Responding for a conditioned stimulus (CS) was compared in 12 food-deprived rats (n=6 per group) maintained at 85% of their free-feeding weight, 30min following treatment with cocaine (5mg/kg i.p.) or vehicle. Responding on one of two introduced levers (CR) resulted in presentation of the previously food paired CS (Smith *et al.*, 1995), whilst responding on the other lever (NCR) had no programmed consequence. LMA data was analysed using a 3-way ANOVA, open field data and lever responding using a 2-way ANOVA, followed by Dunnetts t-test.

Isolates exhibited a significantly greater increase in open field activity from baseline levels following administration of cocaine (5mg/kg) when compared to enriched rats ($F(1,10) = 24.6$ $p<0.001$), total LMA counts increasing by 1457.8±362.8 in isolates, and 491.2±203.2 in enriched rats from baseline counts of 3380.9±344.7 and 4085.6±365.9 respectively. Repeated administration of 5mg/kg cocaine revealed a significant main effect of drug ($F(1,23) = 4.3$ $P<0.001$), but no effects of either housing or time. Following daily administration of 10mg/kg cocaine a significant effect of both drug ($F(1,23) = 29.2$ $P<0.001$), and time ($F(3,20) = 56.0$ $P<0.001$) as well as a drug x time interaction ($F(3,20) = 3.4$ $P<0.05$) were observed, suggesting that both groups of rats sensitised to 10mg/kg cocaine to a similar extent (Table 1). Responding for a CS revealed a significant effect of housing on cocaine sensitivity ($F(1,10) = 11.2$ $p<0.01$), isolates showing a greater increase in CR responding following cocaine treatment (Table 2).

Table 1 The influence of housing conditions on sensitisation to cocaine (10mg/kg)

	ENRICHED	ISOLATED
Saline day 1	562.4±49.4	573.6±38.9
Saline day 9	594.6±57.3	567.9±46.7
Cocaine day 1	1432.0±130.6 **	1826.3±122.4 **
Cocaine day 9	1939.3±106.3 **	2487.6±125.3 **

Data are expressed as mean±s.e.m. photocell counts per 2h test.
Significant effect of cocaine treatment; **P<0.01 (Dunnett's t-test)

Table 2 The influence of housing conditions on cocaine (5mg/kg) induced increases in responding for a conditioned reinforcer.

	ENRICHED		ISOLATED	
	Saline	Cocaine	Saline	Cocaine
CR	6.9±0.7	9.5±1.3**	8.0±0.9	11.6±1.7**
NCR	3.5±0.6	3.3±0.5	3.7±0.5	3.9±0.4

Data represent mean±s.e.m. square root of responses per 30 min.
Significant effect of cocaine treatment;**P<0.01 (Dunnett's t-test)

The data suggest that rats housed in social isolation are more sensitive to the locomotor stimulant and reinforcing properties of cocaine, but that social deprivation does not increase the susceptibility of rats to cocaine induced behavioural sensitisation.

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69P HALOPERIDOL BLOCKS THE ACQUISITION, BUT NOT THE EXPRESSION, OF COCAINE-INDUCED PLACE PREFERENCE IN THE MOUSE

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There is much evidence to suggest that the reinforcing effects of cocaine are mediated via the dopaminergic system (Roberts & Ronaldi, 1995). However, studies using the conditioned place preference (CPP) procedure in the rat have demonstrated a failure of neuroleptics to block the CPP produced by cocaine (Spyraki *et al*, 1982; Mackey & van der Kooy, 1985). The present investigation examined the effects of haloperidol on both the acquisition and the expression of cocaine place preference in mice in order to compare these two aspects of cocaine CPP.

Male adult BKW mice (30-51g) were used. CPP was assessed in a 3 chambered apparatus. Baseline preferences were determined by allowing each subject access to the entire apparatus on 3 separate occasions. The time spent in each outer chamber was recorded for each 15 min session, and the mean of the 3 sessions was taken as the pre-conditioning time (s±sem). For the acquisition experiments mice (n=9-10) received saline or haloperidol (0.125, 0.25 or 0.5mg/kg, i.p.) 45 min prior to conditioning with cocaine (5.0 mg/kg, s.c.) for 30 min and on alternate days saline pretreatment followed by saline conditioning such that each subject received 4 cocaine and 4 saline conditionings. 24 h later mice were preference tested in a drug free state. For the expression experiments mice were conditioned with cocaine (as above) and on the test day each group (n=10) received one of three doses of haloperidol (0.125, 0.25 or 0.5mg/kg, i.p.) or saline 45 min prior to preference testing. 24 h later mice were preference tested again in a drug free state. Data were analysed by 2 way analysis of variance followed by post-hoc t-test analysis.

Saline pretreatment followed by cocaine conditioning produced a significant ($F(2,18)=6.7$, $p<0.01$) increase in the time spent in the cocaine paired chamber from 289.3±11.7 s to 375.5±28.1 s. Haloperidol blocked the acquisition of cocaine induced CPP since conditioning with cocaine failed to modify preference behaviour. For example, the post-conditioning times for 0.125, 0.25 and 0.5 mg/kg haloperidol were 332.9±25.0 s, 304.5±16.0 s and 327.2±23.9 s compared with the pre-conditioning times of 310.9±19.2s, 280.0±14.3 s and 294.8±14.1 s, respectively. In contrast, haloperidol failed to have any significant effect on the expression of the CPP response since the increase in cocaine induced preference behaviour was not affected by any of the 3 doses of haloperidol on the post-conditioning test day. For example, cocaine conditioning and pretreatment with haloperidol (0.25 mg/kg) failed to prevent the increase in time spent in the cocaine paired chamber from 282.5±17.9 s (pre-conditioning) to 399.2±39.2 s (post-conditioning).

The demonstration of a CPP with cocaine in the present investigation is consistent with previous findings in the rat (Mackey & van der Kooy, 1985; Spyraki *et al*, 1982). However, in contradiction to previous reports the present results show a blockade of the acquisition of cocaine CPP by haloperidol and as such implicate a role for dopaminergic neurotransmission in the cocaine CPP acquisition process. Although previous research has not examined the post-conditioning expression of cocaine CPP the failure to block it with haloperidol in the present investigation may suggest that the underlying mechanisms involved in the acquisition and expression of cocaine CPP are different in the mouse and warrants further investigation.

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70P INTRA-ACCUMBENS DOPAMINE INFUSION COMBINED WITH HALOPERIDOL INDUCES BEHAVIOURAL SENSITIVITY

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Withdrawal from chronic intra-accumbens (IACB) infusion of dopamine concomitant with neuroleptic treatment has been shown to induce a rebound hyperactivity response in the rat (Costall *et al.*, 1984). The present studies investigate the ability of clozapine to modify the rebound hyperactivity response.

Sprague-Dawley rats (Bantin & Kingman; female 250±10g, n=5-7) were subject to stereotaxic surgery under pentobarbitone anaesthesia for implantation of chronically indwelling guide cannulae into the nucleus accumbens. Following a 14 day recovery period, rats received bilateral IACB infusions (rate of infusion 12µl/24h per hemisphere) of vehicle (0.1% sodium metabisulphite) or dopamine (50µg/24h) or dopamine (50µg/24h) with haloperidol (1.0mg/kg i.p. b.d.) for 12 days. Locomotor activity was assessed daily for 60 min in individual photocell cages over the 12 day period; data are expressed as mean daily activity counts over the 12 day period. Dopamine/vehicle infusion and haloperidol treatments were withdrawn 14 days before commencement of the acute studies. Acute bilateral IACB injections (1µl/hemisphere) of vehicle or dopamine 25 or 50µg to rats withdrawn from infusion or dopamine 25-200µg to non-infused rats was followed by locomotor activity assessment for 80 min at 10 min intervals; data are expressed as mean total activity counts/80 min. Clozapine (5.0mg/kg i.p.) was given 45min prior to IACB injection of dopamine. Data were analysed by ANOVA with Dunnett's t-test.

The hyperactivity response to dopamine infusion IACB (275±27 counts/day; $p<0.001$ compared to vehicle infusion 82±5 counts/day) was significantly antagonised by haloperidol. (12±1 counts/day; $p<0.001$) with a significant ($p<0.001$) reduction in activity below the level of vehicle-infused animals. Subsequently, dopamine (50µg IACB) induced a significant hyperactivity response in dopamine-infused rats (1115±157 counts/80 min; $p<0.001$) and dopamine-infused+haloperidol rats (1307±206 counts/80 min; $p<0.001$) compared to vehicle-infused rats (399±46 counts/80 min) whilst dopamine (25µg IACB) induced a hyperactivity response only in dopamine-infused+haloperidol treated rats (837±72 counts/80 min; $p<0.001$ compared to 371±106 counts/80 min and 447±39 counts/80 min for dopamine- or vehicle-infused rats respectively), a response which was antagonised by clozapine (235±46 counts/80 min; $p<0.001$). In non-infused rats, dopamine (25 or 50µg IACB) failed to induce a change in locomotor activity (315±37 and 313±44 counts/80 min respectively compared to vehicle 311±43 counts/80 min), although a higher dose of dopamine (200µg IACB) induced a significant hyperactivity response (973±207 counts/80 min; $p<0.001$) which was also antagonised by pretreatment with clozapine (349±183 counts/80 min; $p<0.001$). At the dose used clozapine alone had no effect on behavioural responding.

The results indicate that dopamine infusion IACB, increases sensitivity to a subsequent acute challenge of IACB dopamine and that exposure to haloperidol during the period of dopamine infusion significantly enhances the behavioural response. A peripheral treatment with clozapine completely antagonised the enhanced hyperactivity response to the IACB injection of dopamine. The ability of clozapine to intervene with the behavioural outcome of a chronic dopamine receptor antagonism is of particular interest, since mesolimbic dopamine receptor supersensitivity relating to an ongoing disease process resulting from mesolimbic dopaminergic excess has been hypothesised in schizophrenia (Owen *et al.*, 1978).

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71P 5-HT$_4$ RECEPTOR ANTAGONISM DOES NOT AFFECT DOPAMINE-MEDIATED BEHAVIOURAL EFFECTS IN THE RAT

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5-HT4 receptors are located on GABA neurons in areas with high levels of dopaminergic markers (Patel et al, 1995). We have tested whether blockade of 5-HT$_4$ receptors by a 5-HT$_4$ receptor antagonist SB 204070 (8-amino-7-chloro-(N-butyl-4-piperidyl) methylbenzo-1,4-dioxan-5-carboxylate) alters behavioural effects mediated by dopamine receptor stimulation (at steady state blood concentrations, SB 204070 achieves a 700% brain blood ratio during iv infusion; SB Pharmaceuticals; unpublished).

Male Sprague Dawley rats (200-300g) were used in locomotor activity (LA) and circling experiments; male Lister Hooded rats (200-225g) were used for intracranial self stimulation (ICSS). In LA experiments, rats received SB 204070 (0.03-3.0 mg/kg ip) and were then habituated to the test boxes for 30 min before receiving amphetamine (Amp, 0.55 mg/kg sc). In nicotine (Nic) experiments, a similar protocol was used except that rats were made tolerant by 8 daily injections (0.4 mg/kg sc) prior to tests. In morphine (Mor) experiments, rats received SB 204070 (10-30 mg/kg ip) and were habituated for 20 min prior to Mor (0.5 or 1 mg/kg sc). Activity was monitored for 1 h (Amp & Nic) or 80 min (Mor). In circling experiments, rats received a unilateral 6-OHDA lesion of the left nigrostriatal pathway (Ungerstedt, 1971). Three weeks later, rats received SB 204070 (3.2-10 mg/kg ip) followed by Amp (1.2 mg/kg sc) and ipsiversive circles were counted for 1 h. Data were analysed by one way ANOVA and Dunnett's t test. For ICSS, rats were tested for cocaine (20 mg/kg ip) and SB 204070 (1-10 mg/kg ip)-induced shifts on reward threshold. The frequency at which responding was 50% of maximum (M50) was determined . All results are presented as mean ± 1 sem.

LA, over 1 hour, as measured by total beam breaks was higher after Amp (2030±244;) than after saline (328±81) ($P<0.01$; n=8). SB 204070 (0.1-3 mg/kg) did not alter the Amp effect (range of activity 1807±230 to 2167±162; $P>0.05$; n=8). In tolerant rats, Nic increased total activity from 212±38 (Sal) to 985±210 ($p<0.01$; n=8). SB 204070 did not alter Nic hyperactivity at doses of 0.032-3.2 mg/kg (range of activity 864±144 to 1156±144; $P>0.05$; n=8). Mor (0.5 & 1 mg/kg) increased LA from 80±13 (saline) to 784±132 and 1998±373 respectively ($P<0.01$; n=8). SB 204070 (10 mg/kg) did not antagonise Mor (1 mg/kg) induced LA (2712±171), and SB 204070 (30 mg/kg) did not antagonise Mor (0.5 & 1 mg/kg) induced LA (902±130 and 1942±392 respectively; $P>0.5$; n=7-10). In lesioned rats, saline caused a low rate of ipsiversive circling (23±7); Amp (1.2 mg/kg) increased circling rate to 254±14 ($P<0.01$). SB 204070 (3.2 & 10 mg/kg) had no effect on spontaneous circling (10±3, 9±3 respectively; $P>0.05$) and did not affect Amp-induced circling (293±29 and 256±30 respectively; $P>0.05$; n=8). In ICSS, cocaine (20 mg/kg ip) significantly reduced M50 (53±6%) compared to saline (88±10%; $P<0.05$). M50's after SB 204070 were 87±7%, 88±8% & 92±9% at 1, 3 & 10 mg/kg respectively ($P>0.05$; n=5).

Thus, 5-HT$_4$ receptor blockade did not alter the behavioural effects produced by dopamine receptor stimulation under the conditions described. This suggests lack of interaction between 5-HT$_4$ receptors and dopaminergic mechanisms, or low 5-HT tone.

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72P CHRONIC ADMINISTRATION OF ANTIPSYCHOTICS POTENTIATES DOPAMINE RELEASE INDUCED BY NMDA RECEPTOR BLOCKADE IN RAT FRONTAL CORTEX *IN VIVO*

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The importance of dopamine in the treatment of schizophrenia is well established, with antipsychotics having the ability to block dopamine receptors. The atypical antipsychotic, clozapine, has been reported to be unusually effective in the treatment of negative symptoms of schizophrenia (Coffey 1994). There is evidence to suggest that negative symptoms may be caused by abnormalities that lie in the frontal cortex (Weinberger 1988). This might suggest that clozapine has a preferential effect in the frontal cortex. Post mortem studies indicate that an abnormality of glutamate systems is present in the frontal cortex of schizophrenics (Deakin et al. 1989). In vitro studies have shown that glutamate is able to stimulate dopamine release in various regions including the striatum and the frontal cortex (Jones et al. 1993). However in vivo studies using microdialysis have shown that antagonists at the glutamate NMDA receptor are also able to induce dopamine release (Nishijima et al. 1994). In addition acute administration of clozapine is able to increase glutamate in the prefrontal cortex (Daly & Moghaddam 1993). We therefore investigated the possibility that clozapine acted differentially to classical antipsychotics on dopamine release stimulated by glutamate antagonists in rat frontal cortex.

Using in vivo microdialysis dopamine release from the frontal cortex was shown to be Ca^{2+} dependent and could be stimulated by high concentrations of K^+ in the artificial CSF (data not shown). Male Wistar rats (250g) were pretreated with haloperidol (1.5 mg/kg), clozapine (25 mg/kg) or vehicle for 21 days via daily intraperitoneal injection and after the last dose dialysis probes implanted into the frontal cortex under halothane anaesthesia (coordinates: AP +3.2; L -1.2; V -6.0 from Bregma). 24 hours after the last injection, after the collection of basal samples, the NMDA receptor antagonist 3-((R)-2-piperazin-4-yl)-propyl-1-phosphate (R-CPP) was infused through the dialysis probe at a concentration of 50μM for 1 hour.

In all three groups R-CPP stimulated an increase in extracellular dopamine in the frontal cortex. Dopamine release was expressed as percentage of mean basal release and the response to R-CPP expressed as the sum of this release above basal for the hour after R-CPP infusion. Stimulated dopamine release (mean ± S.e.mean) was 214 ± 48 (n=6), 408 ± 15 (n=4) and 403 ± 53 (n=4) for control, clozapine and haloperidol treated rats respectively. Both clozapine and haloperidol significantly increased the response to R-CPP ($P<0.05$ using Student's t-test). Basal dopamine levels (15.0 ± 2.3, 12.0 ± 1.2 and 13.9 ± 1.5 (fmol/20min) for control, clozapine and haloperidol respectively) were not significantly different. Preliminary results after single administration of either clozapine or haloperidol (not shown) indicate a much smaller increase in stimulated dopamine release, seen as a delay in the return to basal levels of release, compared to control.

This study demonstrates that both typical and atypical antipsychotic drugs, as well as being able to increase dopamine release in the frontal cortex and other brain regions per se, are also able to sensitize dopamine systems to pharmacological stimulation. This effect appears to be much more pronounced after chronic administration of these drugs. With antipsychotic drugs only being effective after prolonged treatment, this chronic sensitization in the frontal cortex may have an important role in the therapeutic efficacy of antipsychotic drugs.

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73P THE GROUP 1 METABOTROPIC GLUTAMATE RECEPTOR AGONIST DHPG ELEVATES STRIATAL DOPAMINE RELEASE IN THE RAT *IN VIVO*

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We have shown previously that direct activation of metabotropic glutamate receptors in the rat striatum by the selective metabotropic glutamate receptor agonist (1S, 3R)-1-aminocyclopentane-1, 3-dicarboxylic acid ((1S, 3R)-ACPD) enhances dopamine (DA) release *in vivo* estimated using the microdialysis technique. These results are consistent with previous studies that showed that intra-striatal injection of (1S, 3R)-ACPD induced contra-lateral turning behaviour in the rat (Sacaan *et al.*, 1991), which was antagonised by the dopamine (DA) receptor antagonist haloperidol (Sacaan *et al*, 1992). It has also been shown that (1S,3R)-ACPD causes a significant increase in extracellular DA level in the nucleus accumbens measured *in vivo* using the microdialysis technique (Ohno and Watanabe, 1995).

The present study investigates the ability of the selective group 1 metabotropic receptor agonist 3,5-Dihydroxyphenylglycine (DHPG) (Schoepp *et al* , 1994) and the selective group 2 metabotropic glutamate receptor agonist (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) (Pin and Duvoisin, 1995) to modulate basal DA release in the rat striatum *in vivo* estimated using the microdialysis technique.

For microdialysis studies, female Wistar rats (150-250 g) were anaesthetised with ketamine and medetomidine before chronic indwelling guide cannulae were stereotaxically inserted. At least 7 days after surgery, a microdialysis probe was inserted (4 mm dialysis tip; A+0.2, V-7.5, L-2.5, relative to Bregma; Paxinos and Watson, 1986) and perfused with artificial cerebro-spinal fluid (aCSF; mM: NaCl 126.6, KCl 2.4, KH_2PO_4 0.49, $MgCl_2$ 1.28 $CaCl_2$ 1.1, $NaHCO_3$ 27.4, Na_2HPO_4 0.48, glucose 7.1, pH 7.4) at 2 µl/min. Dialysate samples collected for at least the first 100 min were discarded and subsequent samples were collected every 20 min into perchloric acid (final concentration 0.1M). A stable baseline was established before drugs were administered for 100 mins via the perfusing aCSF. Dialysate DA levels were analysed immediately using HPLC-ECD (ANTEC working electrode +700 mV versus Ag/AgCl reference electrode).

The basal dialysate DA levels were 17.6±3.2 fmol/20 min, (mean±S.E.M., n=16). Administration of DHPG (0.3-1mM, administered via the microdialysis probe) enhanced the dialysate levels in a concentration-related manner reaching a significant maximal increase (242±53%, mean±S.E.M., n=4 $P< 0.05$,ANOVA, *$P<0.05$, Dunnett's t test) 80mins after perfusion with the agonist. DA levels returned to pre-drug levels 40mins after administration of the drug had ceased. Administration of DCG-IV (3-10µM, administered via the microdialysis probe) failed to significantly modulate dialysate DA levels. The present study indicates that activation of group 1 metabotropic glutamate receptors in the rat striatum enhances DA release.

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74P EFFECT OF DSP4 ON ACQUISITION OF TEMPORAL DISCRIMINATION AND MEMORY FOR DURATION

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The dorsal ascending noradrenergic pathways have been implicated in learning and memory (see Crow & Deakin, 1985). Recently we found that destruction of central noradrenergic neurones by the selective neurotoxin DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine) resulted in retarded acquisition of a temporal discrimination (Ho *et al.*, 1995). Here we report the effect of DSP4 on acquisition and memory for duration in a delayed conditional discrimination task.

14 female Wistar rats (250–300 g) received 2 injections of DSP4 (50 mg kg^{-1}, i.p.) separated by 7 days; 13 rats received injections of the vehicle (0.9% NaCl). The rats were trained in operant conditioning chambers to press levers for a food reinforcer (45 mg food pellets). Experimental sessions took place 7 days a week. In Phase I (56 sessions) each session consisted of 100 trials in which a response on lever A was reinforced following a 2–s presentation of a light stimulus, whereas a response on lever B was reinforced following an 8–s presentation of the same stimulus. In Phase II (50 sessions) delays were interposed between stimulus presentation and the opportunity to respond on the levers in 50% of the trials (2, 4, 8, 16 and 32 s: 10 trials each). At the end of the experiment, the rats were killed and their brains dissected for measurement of noradrenaline, dopamine, 5–hydroxytryptamine (5HT) and 5–hydroxyindoleacetic acid (5HIAA) by high–performance liquid chromatography with electrochemical detection (method: Ho *et al.*, 1995).

During Phase I both groups showed gradual acquisition of accurate discrimination, attaining 90% correct choices by the end of the phase. Analysis of variance showed that the control group learned the discrimination significantly faster than the DSP4–treated group (group: $F[1,25]=6.5$, $P<0.02$; sessions: $F[55,1375]=113.5$, $P<0.001$; interaction: $F<1$). During Phase II, both groups showed decreasing accuracy of performance as a function of the length of the post–stimulus delay. The 'discriminability index' (log d; White, 1991) was calculated for each rat at each value of the delay (t). Exponential decay functions ($\log d = \log d_0.\exp[-mt]$: White, 1991) were fitted to the data, and estimates of the 'initial discriminability', $\log d_0$, and the 'memory decay constant', m, were derived for each rat. The mean (± s.e.mean) values of $\log d_0$, but not those of m, differed significantly between the two groups ($\log d_0$: control group, 1.47 ± 0.10; DSP4 group, 1.21 ± 0.08; $t[25]=2.0$, $P<0.05$; m: control group, 0.072 ± 0.009; DSP4 group, 0.071 ± 0.004; $t<1$). The concentrations of noradrenaline in the parietal cortex and hippocampus of the DSP4–treated group were reduced to 10% and 11% of control values, respectively (ng g^{-1} wet weight of tissue, mean ± s.e.mean: *parietal cortex*, 37 ± 13 [DSP4–treated group], 357 ± 17 [control group]; *hippocampus*, 49 ± 14 [DSP4–treated group], 430 ± 17 [control group]; $P<0.001$ in both cases). The levels of dopamine, 5HT and 5HIAA did not differ significantly between the groups.

The results confirm our previous finding that treatment with DSP4 retards temporal discrimination learning (Ho *et al.*, 1994), but do not provide evidence for an involvement of the noradrenergic projection to the hippocampus and neocortex in temporal working memory.

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75P 7-OH-DPAT INDUCES REVERSAL LEARNING IMPAIRMENTS IN THE COMMON MARMOSET

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The dopamine D_3 receptor has been localised within the limbic system of the marmoset (Hurley *et al*, 1994) but it is not known whether this receptor has a role in cognition. The present studies were designed to investigate the ability of the putative D_3 receptor agonist 7-OH-DPAT [7-Hydroxy-*N*,*N*-di-n-propyl-2-aminotetralin] (Levesque *et al.*, 1992) to induce a cognitive impairment in the marmoset.

Subjects were four (3 female 1 male, >18months old, 290-380g) laboratory-bred marmosets (*C. jacchus*, University of Bradford). Cognitive performance was evaluated by means of the Wisconsin General Test Apparatus (Domeney *et al.*, 1991). Animals were trained to 90% criterion (27 correct object choices in 30 trials) in both the initial and reversal tasks of a serial visual object discrimination task employed in these studies to assess the cognitive effects of 7-OH-DPAT. 7-OH-DPAT (2.5-25 µg/kg) or vehicle (0.9% saline) was administered in a volume of 1ml/kg s.c. 15min prior to testing once weekly according to an experimenter-blind procedure. All animals were tested between 0900h and 1200h. The mean±s.e.m trials to criterion (6 correct consecutive object choices) and mean±s.e.m task errors to criterion in the initial and reversal task (both consisting of 30 serial object presentations) were calculated for each drug treatment and data anlalysed by one way ANOVA with Dunnett's t-test.

Treatment with 7-OH-DPAT (2.5-10 µg/kg) did not impair motor responding or consumption of the food reward and failed to significantly alter mean trials to criterion (9±3.5-11±1.7 compared to vehicle 10±1.8) or mean task errors to criterion (4±1.9-4±1.3 compared to vehicle 5±0.9) in the initial task. Performance of the initial task was thus unaffected by 7-OH-DPAT treatment.

In contrast, 7-OH-DPAT (2.5-10.0 µg/kg) induced significant dose-dependent impairments in reversal task performance as expressed by increases in mean trials to criterion ($F(3,23)=10.1$; $p<0.001$) and increases in mean task errors to criterion ($F(3,23)=20.8$; $p<0.001$). Post-hoc analysis revealed significant ($p<0.05$-0.001) increases in mean trials to criterion following treatment with 7-OH-DPAT 5.5-10 µg/kg (26±2.8-30±0.0 compared to vehicle 16±0.9) and significant ($p<0.001$) increases in mean task errors to criterion following 7-OH-DPAT 6.0 & 10.0 µg/kg (16±0.8 & 19±1.3 compared to vehicle 6±0.6).

Failure to complete both learning tasks following 7-OH-DPAT treatment (25 µg/kg) may correspond with a non-specific inhibition of motor function in the marmoset as reported in the rat (Daly & Waddington, 1993). The impairment of reversal task performance indicates that 7-OH-DPAT can induce a cognitive deficit, which indicates a possible role for the D_3/D_2-like receptors in cognitive behaviour in the marmoset. These studies have successfully extended the findings of ourselves and other workers (Ridley *et al.*, 1980) using a model of amphetamine induced cognitive impairment in performance of a discrimination learning task in the marmoset to demonstrate similar effects for a putative D_3 agonist. Further studies with specific antagonists are necessary to investigate further the nature of the involvement of the D_3 receptor in mediating a disruption of reversal learning processes.

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76P A MICRODIALYSIS STUDY OF THE EFFECT OF FLURAZEPAM ON NORADRENALINE EFFLUX IN THE FRONTAL CORTEX OF FREELY-MOVING RATS AFTER TREATMENT WITH YOHIMBINE

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Both naturalistic stress and an intra-peritoneal injection (i.p.) of the α_2-adrenoceptor antagonist, yohimbine, increase the efflux of noradrenaline (NA) in rat frontal cortex (Dalley & Stanford, 1995b; Mason *et al.*, 1995). Systemic injection of an anxiolytic dose of diazepam reduced NA efflux during stress (Dalley & Stanford, 1995a). The present study used microdialysis *in vivo* to investigate whether the benzodiazepine, flurazepam, also diminishes the increase in NA efflux in the frontal cortex of freely-moving rats caused by an anxiogenic dose of yohimbine (Handley & Mithani, 1984).

The methods for implantation of probes and microdialysis procedures were as previously described (Mason *et al.*, 1995). Male SD rats (260-350 g) were used. An injection of saline (2ml kg^{-1} i.p.) or flurazepam HCl (20 mg kg^{-1} i.p.) was given either alone or 1 h before injection of yohimbine HCl (2 mg kg^{-1} i.p.). Samples were collected for 3 h after treatment. Data were analysed using split-plot ANOVA (Dalley & Stanford, 1995b).

Flurazepam, but not saline, significantly reduced spontaneous ('basal') efflux of NA. The maximum reduction was 40 % and NA efflux remained lower than basal for at least 2 h ($F_{1,18}=5.09$, $P=0.037$). In saline-pretreated rats, yohimbine caused a 2-fold increase in NA efflux; this was sustained for at least 3 h (Table 1: $F_{3,22}=10.11$, $P<0.001$). The NA efflux after yohimbine-injection was significantly reduced by pretreatment with flurazepam (Table 1: $F_{1,11}=5.61$, $P=0.037$). Moreover, flurazepam also reduced the net increase in NA efflux caused by yohimbine ($F_{1,13}=4.77$, $P=0.048$).

The present results indicate that flurazepam blunts the noradrenergic response to yohimbine, a compound with established anxiogenic actions. Previous studies suggest that diazepam also modifies the noradrenergic response to naturalistic stress. These actions of benzodiazepines on noradrenergic transmission could contribute to their anxiolytic effects. Whether benzodiazepines cause these changes in NA efflux through actions in the terminal field, the locus coeruleus, or both, is unknown.

K.Mason is a BBSRC student sponsored by Knoll Pharmceuticals.

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Table 1: NA efflux (fmol 20 μl^{-1}) after treatment with yohimbime 'YOH' and pretreatment with either saline 'SAL' or flurazepam 'FLU'. Mean basal levels were (fmol 20 μl^{-1}): 'FLU+YOH' 29.7 ± 0.7, 'SAL+YOH' 24.0 ± 1.0 ($P>0.05$). Values show mean ± s.e.mean (n=7-8).

Treatment	20 min	40 min	60 min	80 min	100 min	120 min	140 min
SAL + YOH	40.1 ± 2.5	47.2 ± 23.8	47.4 ± 3.7	42.1 ± 4.6	42.5 ± 3.8	46.5 ±4.4	44.4 ± 3.6
FLU + YOH	29.5 ± 3.6	43.1 ± 8.1	35.1± 3.5	32.9 ± 3.8	32.8 ± 5.6	27.3 ± 3.8	29.7 ± 3.7

77P A MICRODIALYSIS STUDY OF THE EFFECTS OF THE SELECTIVE SEROTONIN REUPTAKE INHIBITOR, FLUOXETINE, ON NORADRENALINE EFFLUX IN THE RAT FRONTAL CORTEX

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Recent experiments showed significant inhibition of [^{3}H]-noradrenaline uptake into rat cortical synaptosomes *in vitro* by the selective serotonin reuptake inhibitors fluoxetine and citalopram. The effects of fluoxetine were quantitatively similar to those of the selective noradrenaline (NA) uptake blocker, desipramine (Hughes & Stanford, 1995). One study using *in vivo* microdialysis also inferred that fluoxetine caused an increase in NA efflux but this was not characterised quantitatively (Jordan *et al.*, 1994). The present study investigated the time course of any actions of fluoxetine on NA efflux in rat frontal cortex and compared its effects with those of desipramine.

In the first experiments, NA efflux in the frontal cortex of halothane-anaesthetised SD rats (290-320g) was measured by *in vivo* microdialysis as described by Dalley & Stanford (1995a). 20 µl samples were collected at 20 min intervals. Once stable spontaneous ('basal') efflux was established, fluoxetine or desipramine were perfused *via* the dialysis probe. Three concentrations were perfused consecutively for 80 min each (0.5, 5 and 50 µM). A second series of experiments investigated changes in NA efflux in freely-moving rats (Dalley & Stanford, 1995b). The effects of desipramine and fluoxetine (both at 5 µM) were compared when these drugs were perfused continuously for 3 h. Data were analysed statistically using split-plot ANOVA (Dalley & Stanford, 1995b).

In halothane-anaesthetised rats basal efflux of NA was 36.8 ± 0.9 fmol 20 µl^{-1}. Perfusion of desipramine ($F_{3,15}$=14.31; P<0.001) or fluoxetine ($F_{3,52}$=9.4; P<0.001) *via* the dialysis probe produced a concentration-dependent increase in NA efflux. Perfusion of 5 µM desipramine ($F_{1,7}$=20.13; P=0.003) or fluoxetine ($F_{1,10}$=5.77; P=0.037) caused a significant increase in NA efflux. However, the increase with desipramine was greater than with fluoxetine ($F_{1,9}$=16.3; P=0.003). In freely-moving rats mean basal efflux was 25.6 ± 0.8 fmol 20 µl^{-1}. Continuous perfusion of 5 µM desipramine caused a marked time-dependent increase in NA efflux ($F_{2.27}$=5.57; P=0.006); this reached 77.2 ± 13.7 fmol 20 µl^{-1} (280% *cf* basal) after 3 h. Fluoxetine, at the same concentration, caused a rapid increase in efflux to 38.3 ± 4.8 fmol 20 µl^{-1} (150% *cf* basal) but, after 40 min, there was little further change. Comparison of NA efflux during perfusion of fluoxetine or desipramine showed a significant main effect of drug ($F_{1,17}$=10.13; P=0.005) and a drug *x* time interaction ($F_{2,59}$=5.92; P=0.002).

The results indicate that concentrations of fluoxetine in the dialysis probe of 5 µM, or higher, cause a rapid increase in NA efflux in rat frontal cortex. Inhibition of NA uptake could contribute to this increase. This is because the extracellular concentration of fluoxetine will be within the range of the *Ki* for inhibition of NA uptake *in vitro* (Wong *et al.*, 1974). Since the extraneuronal concentration of fluoxetine after systemic administration of a single low (anticonvulsant) dose is even thought to reach micromolar concentrations (Dailey *et al.*, 1992), increased noradrenergic transmission could contribute to the effects of this drug on behaviour.

Z. Hughes is an MRC PhD scholar

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78P THE BROAD SPECTRUM ANTI-EMETIC ACTIVITY OF THE NOVEL NON-PEPTIDE TACHYKININ NK$_1$ RECEPTOR ANTAGONIST, GR205171

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We have previously reported that tachykinin NK$_1$ receptor antagonists, are effective anti-emetic agents in experimental animals (Bountra *et al.*, 1993; Gardner et al., 1995). We now report on the anti-emetic activity of another novel, potent NK$_1$ receptor antagonist, GR205171, (2-methoxy-5-(5-trifluoromethyl-tetrazol-1-yl)-benzyl)-(2S-phenyl-piperidin-3S-yl)-amine dihydrochloride (Gardner et al., 1996), against various emetic stimuli in the ferret, dog and house musk shrew (*Suncus murinus*).

Ferrets (male, 0.9-1.8kg) received either saline or GR205171 concurrently with cisplatin (200mg m^{-2} i.p.), 30min before cyclophosphamide (200mg kg^{-1} i.p.), morphine (0.5mg kg^{-1} s.c.), copper sulphate (40mg kg^{-1} p.o.) or ipecacuanha (2mg kg^{-1} p.o.), or 90min before exposure to X-irradiation (2Gy). Dogs (beagle, male, 11-15kg) were dosed with vehicle or GR205171 (0.1mg kg^{-1} i.v.) 15min before ipecacuanha (0.7mg kg^{-1} p.o.). Shrews of either sex (36-83g) received vehicle or GR205171 (0.3mg kg^{-1} s.c.) 15min before exposure to linear reciprocating motion (1Hz, 4cm excursion; Ueno *et al.*, 1988). For each species, resulting emesis was recorded. Studies in dog and shrew were of a cross-over design. Results are presented in Table 1.

In vehicle-treated animals, emetic responses were seen to each stimulus, and these responses were significantly reduced in each case by pre-treatment with GR205171 ($p < 0.05$, paired or unpaired t tests). We conclude that GR205171 is a potent anti-emetic with a broad spectrum of action in animals.

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Table 1. Emesis observed in vehicle- or GR205171-treated animals (values are mean and s.e.mean).

Species	GR205171 (mg kg^{-1})	Emetic stimulus	Observation period	Retches Control (n)	Retches GR205171 (n)
Ferret	0.03 i.v.	cisplatin	8½h	86.7±21.38 (3)	10.7±8.21† (3)
Ferret	0.1 s.c.	cyclophosphamide	7h	185.5±31.45 (4)	0.3±0.25† (4)
Ferret	0.1 s.c.	morphine	½h	23.3±1.70 (4)	0† (4)
Ferret	0.1 s.c.	ipecacuanha	3h	54.3±4.64 (4)	0† (4)
Ferret	0.03 s.c.	X-irradiation	2h	72.7±10.72 (10)	1.0±0.58† (3)
Ferret	0.1 s.c.	copper sulphate	2h	114.8±32.08 (4)	2.8±1.89† (4)
Dog	0.1 i.v.	ipecacuanha	1½h	2.5±0.29* (4)	0*† (4)
Shrew	0.3 s.c.	motion	5min	10.1±1.75* (8)	4.3±1.40†* (8)

* emetic episodes †p<0.05, Student's t test.

79P MEASUREMENT OF NITRIC OXIDE RELEASE USING A SELECTIVE MICROELECTRODE FOLLOWING REPEATED WAVES OF CORTICAL SPREADING DEPRESSION

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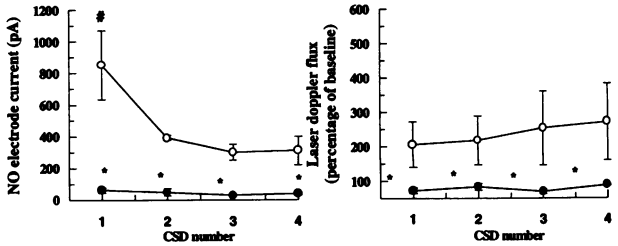
Cortical spreading depression (CSD), is a process characterised by a negative shift in cortical direct current (d.c.) potential and has been implicated in cerebral ischemia and migraine. Previous studies in anaesthetised cats (Goadsby *et* al., (1992) and Wahl *et al.*, (1994)) have demonstrated that CSD induces cerebral artery vasodilation which is inhibited by treatment with nitric oxide synthase blockers. The aim of the present study was to directly measure nitric oxide (NO) release and to assess the effects of L-N^G-nitro-L-arginine (L-NAME) on NO release following CSD.

Anaesthesia was induced in male cats (3-3.9 kg) with halothane and maintained with α chloralose (100 mg kg^{-1}i.v.). The left supersylvian gyrus was exposed and covered with a layer of mineral oil (37°C). From this gyrus, changes in cortical d.c. potential, regional cerebral blood flow ($rCBF_{LDF}$) (laser doppler flux, Oxford Optronics, U.K.) and NO release (NO selective microelectrode Model NO-501, Inter Medical, U.K.) (1000pA = 1μM NO) were recorded. CSD was evoked by placing on the gyrus a KCl pellet (30mg) for 6 min, and recordings continued for a further 25 min. Systemic mean arterial blood pressure (MABP) and derived heart rate (HR) were recorded. Drug (L-NAME, 10 mg kg^{-1} i.v.) or vehicle (10% β-cyclodextrin solution in sterile saline i.v.) were administered over 30 min. prior to CSD initiation. All data is expressed as mean ± s.e.mean. Significance was assessed using Mann Whitney U-test, *p<0.05 or one way analysis of variance (ANOVA) followed by a Dunnett's t test, #p<0.05.

Infusion of vehicle (n=4) had no effect on MABP (76±4 to 76±8mmHg) or HR (148±10 to 148±12 beats per minute (bpm)). L-NAME (n=4) increased MABP from 76±6 to 98±6.6 mmHg but had no significant effect on HR (146±8 to 148±12bpm). Baseline recordings of NO release and $rCBF_{LDF}$ were unaffected by vehicle infusion, however, L-NAME decreased baseline NO electrode current by -20±5 pA and $rCBF_{LDF}$ to 43.5±13.4% of baseline immediately prior to CSD initiation. In both the vehicle and L-NAME treated groups, application of KCl induced 4 transient negative deflections (CSD1-4) in cortical d.c. potential, mean amplitudes 13±2.1, 13.5±5.1, 12.5±4.5, 15.6±2.9 mV and 12±2.5, 13.8±2.8, 15±5.6, 12.7±3.2 mV respectively. In vehicle treated animals d.c. shifts were associated with an increase in $rCBF_{LDF}$ and an increase in NO release (figure 1). The first negative shift in d.c. potential (CSD 1) produced a significantly larger NO electrode current increase (ANOVA) ($p<0.05$, $F=3.55$), when compared to other depolarisations. However, $rCBF_{LDF}$ increases were similar, CSD1-4 (figure1). In L-NAME treated animals, d.c. shifts were not associated with increases in NO electrode current or $rCBF_{LDF}$, both of these variables were significantly (Mann Whitney U-test) inhibited when compared to vehicle (figure1).

Figure 1: Effects of L-NAME and vehicle on NO electrode current (pA) and $rCBF_{LDF}$ (percentage of baseline) increases following induction of CSD (vehicle ○, L-NAME ●). Data shows mean ± s.e.mean (*p<0.05, Mann Whitney U-test, #p<0.05, ANOVA).



These data show that NO release is closely associated with cerebral blood flow (CBF) changes following induction of CSD. However, NO release is not directly proportional to CBF increases.

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80P IS ASCORBIC ACID A BIOCHEMICAL INDEX OF EARLY ISCHAEMIA? AN *IN VIVO* ELECTROPHYSIOLOGICAL AND VOLTAMMETRIC STUDY

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In rats subjected to focal ischaemia by occlusion of the middle cerebral artery (MCAO), analysis by in vitro slices or in vivo cortical microdialysis measurements result in a similar kinetic profile for uric acid (UA) and tryptophan (TRY). Conversely, these techniques generate discrepant data for ascorbic acid (AA) (Hillered et al., 1988; Uemura, Y. et al., 1991).

The aim of the present work was to monitor in vivo, in anaesthetised adult male rats (300g) AA, indole-UA and TRY related oxidation signals using treated carbon fibre micro-electrodes (mCFE) together with differential pulse voltammetry (DPV) and to use the mCFE to make concomitant electrophysiological (EPH) recordings (Crespi et al., 1995). The mCFE was implanted in the rat somatosensory cortical region which was then lesioned by a permanent MCAO (Tamura et al., 1981). To functionally assess the brain damage the somato-sensory evoked potentials were recorded by stimulation of the controlateral hindpaw (Lye et al., 1987). DPV and EPH measurements were performed in parallel every 5 min starting 20 min before (control period), during, and after (up to 280 min) MCAO. DPV analysis indicated an increase of the UA-indole (basal levels: 16.1±1.4μM) and of the TRY related signals (basal levels: 20.2±3.1μM) starting from about 25-30 min after MCAO and reaching within 50-60 min a plateau of approximately 200% and 300% of control values respectively. In contrast, the basal levels of AA (36±5.5μM) were increased to 240±60%, 430±80%, and 510±65% of control values at 5, 10 and 15 min respectively after MCAO. Simultaneously, the EPH recordings of cell firing (basal levels: 420 ± 88μV) were significantly reduced to 16±9%, 22±6% and 26±11% of control values at 5, 10 and 15 min after MCAO (n=6 for all, mean ± S.D., $p<0.05$, student T test). These data show that two independent recording techniques can be combined at a single biosensor so that truly related EPH and DPV analysis can be performed simultaneously at the same ischaemic cerebral site. Furthermore, the improvement in time resolution in comparison to recent biochemical in vivo studies (Langemann et al., 1995), permits the observation of a high correlation between MCAO reduced functional responses of the neurons monitored by EPH and increased levels of AA measured by DPV. This original observation suggests that AA is a biochemical marker of the very early stages of focal ischemia and could be a useful tool for the evaluation of initial ischemic damage.

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81P THE MESULERGINE-SENSITIVE INCREASE IN CORONARY BLOOD FLOW EVOKED BY SUMATRIPTAN IN GR 127935-TREATED GUINEA-PIG ISOLATED HEARTS IS CAUSED BY NO RELEASED FROM CORONARY ENDOTHELIUM

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In guinea-pig heart *in vitro*, sumatriptan (ST) ≥0.01μM reduces coronary flow (CF) without affecting coronary effluent NO content, but at even lower concentrations (≥0.001μM) increases CF and releases NO when combined with the $5HT_{1D}$ receptor antagonist GR127935 (GR) (Ellwood & Curtis 1996). Addition of mesulergine (MS) in the presence of GR renders ST inactive (Ellwood & Curtis 1996). Here we have attempted to confirm that NO released from coronary endothelium mediates the coronary flow increase caused by ST when combined with GR.

Male Dunkin Hartley guinea-pigs (350-400g) were terminally anaesthetised with pentobarbitone (60 mg kg^{-1} i.p.) and heparinised (250 iu sodium heparin i.p.). Hearts were excised and perfused under constant pressure (100cm H_2O) with Krebs solution (pH 7.4, 37°C) modified to contain (mM) KCl 4.0 and $CaCl_2$ 1.4 and paced via the left ventricle (275 beats min^{-1}). Coronary flow (CF; ml $min^{-1}g^{-1}$ of ventricle) was measured by an in-line ultrasonic flow probe (Transonic systems). After 30 min of control perfusion, hearts (n=6-8/group) were perfused for 45 min (pretreatment) with 100 μM L-NAME or a saponin protocol shown previously to ablate coronary endothelium selectively versus smooth muscle (Ellwood & Curtis 1995). This was followed by perfusion with ST (incrementally, 0.1nM-1μM, 6 min per concentration) alone or with GR 10nM, with MS 3μM or with both. Coronary effluent NO was measured as described previously (Ellwood & Curtis 1995). Values are expressed as mean ± s.e.mean.

The effects of ST alone and combined with GR on CF and NO, and the effects of L-NAME alone and saponin alone have been described previously (Ellwood & Curtis 1995,6). Neither saponin nor L-NAME affected the response (pEC_{50} for fall in CF) to ST alone (NO was not changed by ST), nor its abolition by MS+GR combined (Table 1). However, both saponin and L-NAME abolished the increase in CF and NO caused by ST+GR (Table 1).

Table 1. Effects of ST (±MS and ±GR) on NO and CF

Pretreatment	Drugs	[ST] μM	ΔNO	ST pEC_{50}
-	ST	0.1	none	8.6 ± 0.2^b
-	ST+GR	0.01	684±124	9.1 ± 0.2^a
-	ST+MS	0.1	none	8.5 ± 0.1^b
-	ST+MS+GR	0.1	none	no CF change
saponin	ST	0.1	none	8.5 ± 0.1^b
saponin	ST+GR	0.01	none*	no CF change*
saponin	ST+MS	0.1	none	8.5 ± 0.1^b
saponin	ST+MS+GR	0.1	none	no CF change
L-NAME	ST	0.1	none	8.6 ± 0.1^b
L-NAME	ST+GR	0.01	none*	no CF change*
L-NAME	ST+MS	0.1	none	8.7 ± 0.1^b
L-NAME	ST+MS+GR	0.1	none	no CF change

ΔNO=Δpmol $min^{-1}g^{-1}$ for the [ST] shown; $^apEC_{50}$ (-log M) for CF increase; $^bpEC_{50}$ (-log M) for CF decrease; *P<0.05 versus no pretreatment, Dunnett's test. The first 4 lines of data are taken from Ellwood and Curtis (1996).

In conclusion, the ST-induced increase in CF that is unmasked by GR is caused by the release of NO from coronary endothelium.

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82P EVIDENCE THAT THE CENTRAL NERVOUS SYSTEM PLAYS A ROLE IN L-NAME-INDUCED HYPERTENSION

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According to Cunha et al. (1993), an increase in central sympathetic drive plays an important role in the hypertension induced by chronic inhibition of nitric oxide (NO) synthesis. In this study, we have attempted to assess this hypothesis.

Male Sprague-Dawley rats weighing 250-300 g were used. Control rats, and rats chronically treated for 7 or 21 days with the inhibitor of NO synthase N^w-nitro-L-arginine methylester (L-NAME) (100 mg/kg/day in drinking water) were anaesthetized with pentobarbital sodium (50 mg/kg). The carotid artery was cannulated for the recording of arterial blood pressures. The systolic (SBP), and the diastolic (DBP) arterial blood pressure of the anaesthetized animals were measured using a Panlab 4C Datasystem. When stabilized pressures were registered the animals were given a single i.v. dose of 10 mg/kg hexamethonium or pithed by introducing a blunt needle into the spinal canal via the orbit (Shipley and Tilden, 1947), being in the latter case immediately subjected to artificial respiration. The maximal decreases in SBP and DBP caused by hexamethonium or pithing of the rats were measured and compared in control animals and those treated with L-NAME. Results are expressed as mean values ± s.e.mean for a minimum of 8 experiments. Student's t-test was used for comparison of mean values (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

The L-NAME-treated anaesthetized rats had arterial blood pressures significantly higher than those in control anaesthetized rats (table 1). The administration of hexamethonium, or pithing, caused sharp falls in arterial blood pressures of the anaesthetized rats which were greater in pithed than hexamethonium treated animals. The decrease in arterial blood pressure was greater in animals treated with L-NAME and more so in the rats treated for 21 days than those treated for only 7 days (see table 2). The results of this study suggest that a central action could be implicated in the hypertensive effect caused by inhibition of NO synthesis.

Supported by U.C.M. (PR 188/92-4064), and DGICYT (PB93-0065) grants.

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Shipley R.E. & Tilden, J.H. (1947) Proc. Soc. Exp. Biol. Med. 64, 453-455.

Table 1. SBP and DBP (mm Hg) in control and L-NAME-treated anaesthetized rats.

	Control	L-NAME (100 mg/kg/day) 7 days	L-NAME (100 mg/kg/day) 21 days
SBP	77.0 ± 1.0	88.9 ± 0.9*	94.7 ± 7.9**
DBP	61.1 ± 1.8	71.7 ± 2.5**	75.2 ± 4.5**

Table 2. Decrease (mm Hg) in SBP and DBP caused by application of 10 mg/kg hexamethonium or by pithing control and L-NAME-treated anaesthetized rats.

	Control	L-NAME (100 mg/kg/day) 7 days	L-NAME (100 mg/kg/day) 21 days
Hexamethonium			
SBP	37.1 ± 5.1	49.5 ± 2.7	54.4 ± 3.6*
DBP	30.8 ± 3.3	42.2 ± 2.3	45.5 ± 4.3*
Pithing			
SBP	46.1 ± 1.2	63.0 ± 4.5**	75.6 ± 6.1**
DBP	34.0 ± 2.5	50.7 ± 3.2**	59.6 ± 5.7**

83P THE DILATOR RESPONSE TO SENSORY NERVE STIMULATION IN THE MESENTERIC CIRCULATION OF THE RAT IS MODULATED PREJUNCTIONALLY BY NITRIC OXIDE

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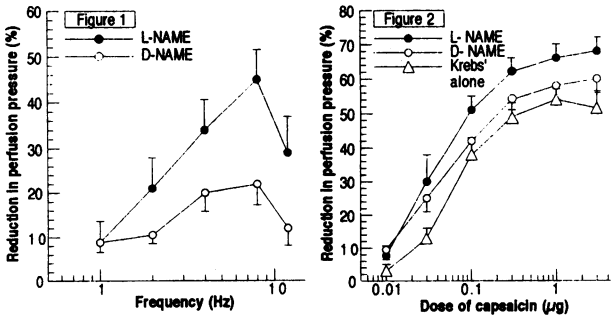
Calcitonin gene-related peptide (CGRP) is present in perivascular sensory nerves and is a potent dilator of rodent and human vasculature. Previous studies by us (Mitchell *et al* 1995) and by Hughes and Brain (1994) suggested that CGRP-induced dilation was nitric oxide (NO) -independent, but the release of CGRP from sensory nerves was modulated by NO. Using the isolated, perfused rat mesentery we have investigated further the involvement of NO in the vascular responses to CGRP and to sensory nerve activation induced by capsaicin (CAPS), or electrical field stimulation (EFS).

Following lethal dosing of sodium pentabarbitone the mesenteric artery of male Sprague-Dawley rats was cannulated, the mesentery was dissected free, and perfused at a constant flow (5ml.min^{-1}) with warmed (37°C), and gassed (95% O_2, 5% CO_2) Kreb's buffer. Perfusion pressure (PP) was recorded via the arterial cannula and in some experiments EFS electrodes were placed on the vessel. PP was raised from 27±8mmHg to 118±11mmHg and adjusted by titration of methoxamine (1-12μM) added to the perfusate. In some experiments the specific NO synthase inhibitor L-NAME (N^Gnitro-L-arginine methyl ester;100μM) or its inactive isomer D-NAME were included in the perfusate. The effects of 1-3μl volume injections of saline or ethanol vehicle, human CGRP (1x10^{-13}-1x10^{-10}moles), CAPS (0.01-3μg in ethanol) or EFS (1-12Hz, 50V, 1ms duration, 30 second stimulation) were recorded.

EFS and CAPS induced dose- and frequency-dependent reductions in PP (Figures 1&2) with maximum effects of 22±5%(n=8) and 60±5% (mean ± s.e.mean; n=9) respectively in D-NAME infused tissues. The response to CAPS given alone and in the presence of D-NAME did not differ significantly. The maximum reduction in PP following EFS was significantly potentiated by L-NAME (Figure 1; p< 0.01 by ANOVA at 8Hz) as were the duration of response (D- vs L-NAME at 8 Hz, 16.8±1.2 min vs 24.5±3.6 min; p<0.01, n=7), and the area under



the curve (D- vs L-NAME at 8Hz; 1192±109 mmHg.min vs 1904±244 mmHg.min; p<0.01, n=7). L-NAME did not significantly alter the maximum reduction in PP (Figure 2) or the duration of response induced by CAPS. However, the area under the curve was significantly increased from 2208±242 mmHg.min to 3450±444 mmHg.min (p<0.01, n=8/9), representing a 56% potentiation that was similar to the 60% increase observed following EFS of 8 Hz. The CGRP dose-response was not altered by L-NAME when compared with D-NAME (maximum reduction in PP at 3x10^{-10} mol CGRP of 56±6% vs 55±5%).

In rabbit coronary (Mitchell *et al* 1995) and rat skin vasculature (Hughes and Brain 1994) NO appears to facilitate CGRP release from sensory nerves. The present study also shows that the dilator action of CGRP is NO-independent and that NO can modulate sensory neuropeptide release prejunctionally. In mesenteric sensory nerves NO has an inhibitory effect, suggesting that its modulatory role varies markedly between tissues.

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84P POSSIBLE PRESYNAPTIC ACTION OF NITRIC OXIDE ON VASCULAR SYMPATHETIC NERVES

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Apart from the direct actions of nitric oxide (NO) on vascular smooth muscle, additional roles for NO in the regulation of cardiovascular functions have been proposed. Possible presynaptic actions of NO on sympathetic nerves have been recently suggested (Chen & Schofield, 1993), and in this study we have tried to clarify whether inhibition of NO synthesis could facilitate catecholamine release from vascular nerve endings. We have studied the effect of the inhibitor of NO synthase N$^{\omega}$-nitro-L-arginine methylester (L-NAME) in pithed rats. The rats were anaesthetized (ether) and pithed (Shipley and Tilden, 1947), and the L-NAME (10 or 100 mg/kg i.v.) was injected into control, reserpine (5 mg/kg)-, prazosin (1 mg/kg)-, yohimbine (1 mg/kg)-, or dimethylsulfoxide (DMSO) (1 ml)-treated rats. DMSO and reserpine were administered i.p. 24 h before, and prazosin and yohimbine were administered i.v. 20 min before L-NAME. Increases in systolic (SBP) and diastolic (DBP) arterial blood pressure, and changes in heart rate were measured using a Panlab 4C Datasystem. Results are expressed as mean values ± s.e.mean for a minimum of 8 experiments. Student's t-test was used for comparison of mean values (*p<0.05 considered statistically significant).

L-NAME increased SBP and DBP in the pithed rats. The increase was dose-dependent, and maximal values were reached 2 min after administration of the drug. Arterial blood pressure then took 15-20 min to return to its basal level. Similar responses were seen in animals in all the treatment groups (table 1). Administration of L-NAME did not change the heart rate of any groups. Control pithed rats had heart rates of 372 ± 16 beats/min before the administration and 366 ± 21 beats/min after the administration of 100 mg/kg of L-NAME. The present results suggest that acutely administered L-NAME does not cause hypertension by altering catecholamine release from vascular nerve endings.

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Table 1. Increase (mm Hg) in SBP, and DBP caused by L-NAME in reserpinized or α-adrenoceptor antagonist-treated pithed rats.

L-NAME	CONTROL	DMSO[a] 1 ml	RESERPINE 5 mg/kg	PRAZOSIN 1 mg/kg	YOHIMBINE 1 mg/kg
10 mg/kg					
SBP	16.1 ± 3.1	17.3 ± 3.0	19.1 ± 3.0	15.9 ± 2.6	15.3 ± 3.3
DBP	16.0 ± 2.9	18.1 ± 3.5	19.1 ± 3.0	15.7 ± 2.3	15.3 ± 3.1
100 mg/kg					
SBP	26.1 ± 3.1	22.3 ± 5.6	22.7 ± 3.5	27.7 ± 6.2	15.2 ± 3.8
DBP	24.9 ± 3.0	22.4 ± 5.3	21.1 ± 3.8	26.0 ± 5.8	15.1 ± 3.7

[a] Reserpine solvent

85P NITRIC OXIDE COMPROMISES THE CONSTRICTOR EFFECTS OF THE NON-CYCLOOXYGENASE-DERIVED PROSTANOID, 8-ISO PROSTAGLANDIN $F_2\alpha$

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Isoprostanes are a newly-described group of prostaglandin (PG)-like compounds formed independently of the cyclo-oxygenase pathway, during conditions of high oxidant stress (Morrow and Roberts, 1996). 8-iso $PGF_{2\alpha}$ is one of the more abundant isoprostanes formed *in vivo* causing constriction of some smooth muscle preparations, through activation of thromboxane receptors (TP) or putative isoprostane-selective receptors. As 8-iso $PGF_{2\alpha}$ is likely to be formed *in vivo* in diseases where oxidant stress is high, such as septic shock, we have assessed the relative potency of this compound in pulmonary arteries from control and lipopolysaccharide (LPS)-treated rats. In addition we have investigated whether endogenous nitric oxide (NO) or cyclo-oxygenase-derived prostanoids, modulate the effects of 8-iso $PGF_{2\alpha}$ under the two conditions. All protocols were also carried out using the thromboxane mimetic, U46619, a TP receptor agonist.

Rats were either left untreated (control) or treated with LPS (20 mg/kg) for 4 hours, killed by cervical dislocation, and the pulmonary arteries removed. Tissues were mounted in 2 ml organ baths filled with Krebs' buffer as previously described (Curzen et al., 1995).

Both eicosanoids tested caused concentration-dependent contractions of the rat pulmonary artery with U46619 being more potent than 8-iso $PGF_{2\alpha}$ (figure 1A,B). The effectiveness and order of potency of the eicosanoids was not changed when tissues were taken from rats treated with LPS (figure 1C,D). In the presence of the NO synthase inhibitor, L-NAME (100 μM) the contractile effects of 8-iso $PGF_{2\alpha}$ but not U46619, on vessels from either control or LPS-treated rats were enhanced. By contrast the cyclo-oxygenase inhibitor, indomethacin (30 μM) did not enhance the contractions evoked by either of the eicosanoids tested.

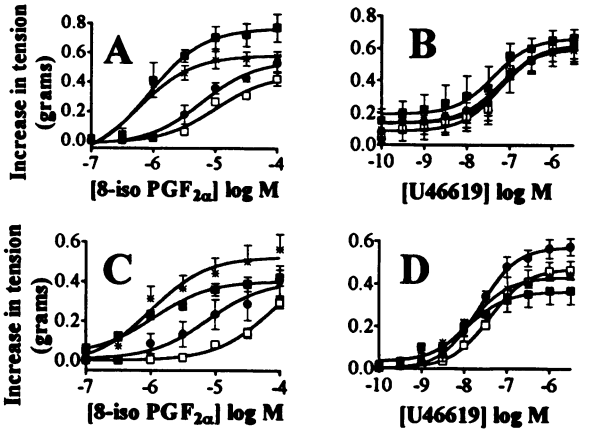

Figure 1. A and B; responses of tissues from control animals, C and D from LPS-treated animals. ●control; □ plus indomethacin; *, plus L-NAME; ■, plus L-NAME plus indomethacin. The figure shows mean ± sem for n=5-6.

This study shows that the 8-iso $PGF_{2\alpha}$ constricts rat pulmonary arteries from control and LPS-treated animals. Thus, isoprostanes may be formed during septic shock and contribute to the associated pulmonary hypertension. Interestingly, contractions evoked by 8-iso $PGF_{2\alpha}$ but not U46619, were functionally antagonised by endogenously released NO. Thus NO formation may contribute to the actions of isoprostanes such as 8-iso $PGF_{2\alpha}$ under physiological and pathophysiological conditions.

This work was supported by grants from the Medical Research Council and British Heart Foundation.

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86P 8-EPI PROSTAGLANDIN $F_2\alpha$ IS A VASOCONSTRICTOR IN THE RAT ISOLATED HEART AFTER LOW FLOW ISCHAEMIA

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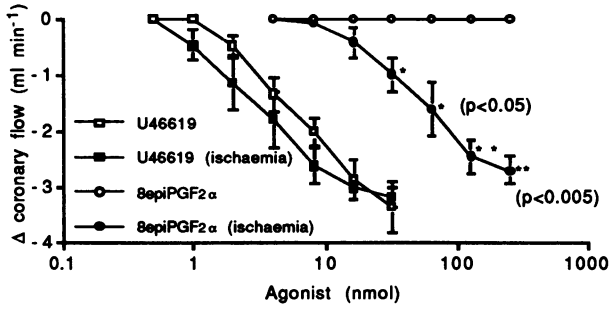
Previously we reported that 8-epi prostaglandin $F_2\alpha$ (8-epi $PGF_{2\alpha}$), an F2-isoprostane, has a vasoconstrictor effect on porcine coronary arteries (Kromer & Tippins, 1995). To assess the role of this compound in oxidant stress, the effects of a thromboxane mimetic (U46619) and 8-epi $PGF_{2\alpha}$ were studied on rat coronary vasculature, using an isolated Langendorff preparation, after low flow ischaemia.

Sprague-Dawley rats (200g) were anaesthetised with hypnorm/ hypnovel/water (2:5:1 v/v/v; 2.7 ml kg^{-1}, i.p.). Hearts were excised and perfused at constant pressure (80 mmHg) and coronary flow (17.5±4.5 ml min^{-1} in control hearts, mean ± s.e.mean, n=6) recorded as an indication of coronary resistance. A latex balloon in the left ventricle allowed the measurement of ventricular rate and pressure, and the calculation of dp/dt. Bolus doses (10μl) of U46619 or 8-epi $PGF_{2\alpha}$ were given into the coronary perfusion cannula. The tissue was left for at least 10 minutes between doses or until a stable baseline had been reached.

U46619 caused a dose-dependent decrease in flow, indicative of coronary constriction, with an ED_{50} of 4.73±2.2 nmol (n=6). 8-epi $PGF_{2\alpha}$ had no effect on normal rat coronary vasculature. After 30 minutes low flow ischaemia (10 mmHg, 2-5 ml min^{-1}) followed by reperfusion, the dose responses were repeated. The U46619 dose response curve shifted slightly to the left to give an ED_{50} of 3.10±2.74 nmol (n=4). This shift was not statistically significant. However, 8-epi $PGF_{2\alpha}$ caused

Figure 1. Effect of U46619 and 8-epi prostaglandin $F_2\alpha$ on coronary flow in the isolated rat heart.



significant coronary constriction (Unpaired t test; n=5, Figure 1).

Thus a dose-dependent response of rat coronary vasculature to 8-epi $PGF_{2\alpha}$ occurs after low flow ischaemia with no response prior to ischaemia. This is of interest, since isoprostanes are formed by free radical peroxidation of membrane phospholipids and have been postulated to be implicated in oxidant injury (Morrow et al, 1990).

We thank the British Heart Foundation (FS/94085) for funding.

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Morrow, J.D., Hill, K.E., Burk, R.F.*et al.* (1990) *Proc. Natl. Acad. Sci. U. S. A.*, 87, 9383-7.

87P ACTIONS OF 8-EPI PROSTAGLANDIN $F_2\alpha$ IN PORCINE CORONARY ARTERY ARE INHIBITED BY THROMBOXANE RECEPTOR ANTAGONISTS

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8-epi prostaglandin $F_{2\alpha}$ (8-epi $PGF_{2\alpha}$), an F2-isoprostane, has been shown to have a vasoconstrictor effect on porcine coronary arteries (Kromer & Tippins, 1995). The effects of a thromboxane mimetic (U46619) and 8-epi $PGF_{2\alpha}$ were studied on porcine coronary arteries in the presence of SQ29548 or BM13505, both selective thromboxane receptor antagonists.

3mm rings of segments 6 ,7 and 8 (AHA Grading Committee, 1975) of the left anterior descending coronary artery from porcine hearts were suspended in Krebs at 37°C. Tension was adjusted by the length/tension relationship for each tissue and responses to 40mM KCl recorded. Cumulative dose response curves for the agonists were obtained in the absence and presence of each antagonist at various concentrations.

SQ29548 and BM13505 caused concentration-dependent rightward shifts in the EC_{50} of U46619 responses (33nM control; 260nM, 457nM and 5165nM in the presence of 10^{-8}, $3x10^{-8}$ and 10^{-7}M SQ29548 respectively; 46nM, 205nM, 237nM and 1113nM in the presence of 10^{-8}, 10^{-7}, $3x10^{-7}$ and 10^{-6}M BM13505 respectively) with no decrease in E_{max} values at any concentration of antagonist. 8-Epi $PGF_{2\alpha}$ responses were also inhibited by both SQ29548 and BM13505 in a concentration-dependent manner. However, their inhibitions were also associated with a decrease in E_{max} values (Figures 1 and 2).

These results suggest that coronary artery contraction induced by 8-epi $PGF_{2\alpha}$ is inhibited in an non-competitive manner by both SQ29548 and BM13505. These data may be interpreted as indicating that 8-epi $PGF_{2\alpha}$ acts at an isoprostane receptor in this tissue, or more probably, that it acts as a partial agonist on the thromboxane TP receptor.

Figure 1. Dose response curves to 8-epi $PGF_{2\alpha}$ on porcine coronary artery in the presence of BM13505

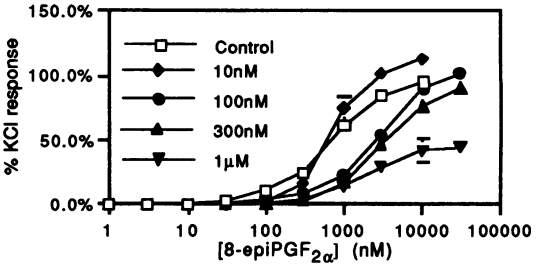
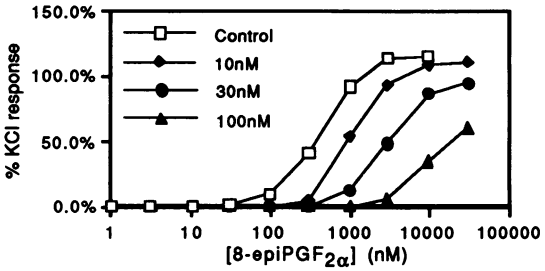


Figure 2. Dose response curves to 8-epi $PGF_{2\alpha}$ on porcine coronary artery in the presence of SQ29548



We thank the British Heart Foundation (FS/94085) for funding.

Kromer B.M. & Tippins J.R. (1995) *Br. J. Pharmacol.* 116, 285P.
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88P COMPARISON OF THE EFFECTS OF PROSTAGLANDIN FP-RECEPTOR MIMICS $PGF_2\alpha$, FLUPROSTANOL AND LARANOPROST ON THE HUMAN ISOLATED UMBILICAL ARTERY

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It has been known for some time that prostaglandins cause constriction of human umbilical artery, but as yet the receptor population has not been defined (Hillier *et al.*, 1968). Previous studies in this laboratory have suggested the presence of a TP-receptor on human umbilical artery (Amin *et al.*, 1995). In the present study an attempt has been made to characterise the FP-receptor on human umbilical artery using the FP-receptor agonists, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), fluprostenol (m-trifluoromethyl-16-phenoxy 17, 18, 19, 20-tetranor-$PGF_{2\alpha}$), and latanoprost (13, 14-dihydro-15(R/S)-17-phenyl-18, 19, 20-trinor-$PGF_{2\alpha}$-IE) (Stjernschantz *et al.*, 1992).

Samples of human umbilical cord were obtained from full term pregnancies (all women gave written consent) and placed immediately into Krebs' physiological solution at room temperature. The cords were then transported to the laboratory within 60 mins. Rings of umbilical artery (with intact endothelium) were suspended in Krebs' physiological solution containing indomethacin (2.79 μM) at 37°C in a 10ml organ bath and oxygenated with 2.5% O_2/ 8% CO_2/ balance N_2 as described previously (Amin *et al.*, 1995). Concentration-effect curves were constructed to $PGF_{2\alpha}$, fluprostenol, and latanoprost. In some experiments in order to investigate the possibility of the FP agonists exerting an effect through the TP-receptor, Bay u3405 (McKenniff *et al.*, 1989) was added to the Krebs' physiological solution at a concentration of 10^{-7} M for at least 30 mins prior to the agonist concentration-effect curves being constructed. In all cases n=5.

All the FP-receptor agonists tested evoked contractile responses.

Table 1:- EC_{50} values for the FP-receptor agonists $PGF_{2\alpha}$, fluprostenol and latanoprost.

FP-agonist	Conc. Range (M)	EC_{50} (M)
$PGF_{2\alpha}$	$1x10^{-9}$-$3x10^{-5}$	6.0×10^{-7}
Fluprostenol	$1x10^{-9}$-$3x10^{-6}$	4.0×10^{-7}
Latanoprost	$1x10^{-9}$-$3x10^{-6}$	6.0×10^{-8}

In the presence of, the selective TP-receptor antagonist, Bay u3405 10^{-7} M, the concentration-effect curve to $PGF_{2\alpha}$ was depressed, maximum tensions were reduced from 1.10±0.15 g to 0.50±0.10 g ($p<0.01$), whereas the concentration-effect curves to fluprostenol and latanoprost were unaffected.

These results indicate that $PGF_{2\alpha}$ is acting through both TP- and FP-receptors, whilst the more selective FP-agonists had no effect at the TP-receptor. The results of this study suggest the presence of a FP-receptor on human umbilical artery. This study is currently being extended to determine the other prostaglandin receptors present on the human umbilical artery.

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89P THE EFFECT OF EICOSAPENTAENOIC ACID UPON CONTRACTURES OF ISOLATED AORTIC RINGS AND INOSITOL PHOSPHATE PRODUCTION IN CULTURED AORTIC CELLS FROM THE RAT

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Eicosapentaenoic acid (EPA) reduces blood pressure and causes vasorelaxation, a mechanism thought to depend upon decreased release of intracellular calcium $[Ca]_i$ (Engler, 1992). In this study, the effect of EPA on contractures of rat aorta, induced by noradrenaline (NA), have been compared with its effect on inositol phosphate (IP) production in cultured aortic cells (AOCs).

Male Wistar rats (200-250g) were stunned and killed by cervical dislocation. Aortic rings devoid of endothelium were suspended in Krebs buffer containing propranolol (PP, 10^{-6}M), EDTA (10^{-5}M) and ascorbic acid (AA, $5x10^{-5}$M) at 37°C, gassed with carbogen, under a resting tension of 2g. Isometric contractures were recorded. After 1hr equilibration, non-cumulative concentration response curves were constructed to NA before and 30min after EPA ($5x10^{-5}$M) or ethanol (ETH, 1%; the EPA vehicle). AOCs were prepared by enzymatic dissociation using papain and collagenase and cultured in MEM D-valine medium, supplemented with 10% foetal calf serum for 10-13 days. AOCs were incubated with myo-[^{3}H] inositol (5μCi ml^{-1}) for 24hr in MEM D-valine. The medium was then replaced with HEPES-buffered physiological saline containing PP (10^{-6}M), EDTA (10^{-5}M), AA ($5x10^{-5}$M) and LiCl (10^{-5}M) and the effects of a 5min incubation with NA (10^{-6}M), ETH (1%), or EPA ($5x10^{-5}$M) on IP production were compared with control (CON). In some experiments, phentolamine (PT 10^{-6}M) or prazosin (PZ 10^{-7}M) was added to the cells 15mins prior to experimentation. Extraction and measurement of IP produced was as described by Jackson *et al.*, 1987.

NA contractures were reduced by EPA. A rightward shift of the concentration-response curve was seen: EC_{50} values; ETH 1.0±0.15 x 10^{-9}M, EPA 3.9± 0.36 x 10^{-9}M (n=6) and a reduction in E_{max}; ETH 4.90±0.30g, EPA 3.81±0.33g. In AOCs; NA, ETH and EPA all caused a significant increase in IP production compared with CON values. The effect of EPA was greater than that of ETH alone. The increase in IP production induced by NA and EPA, but not ETH, was reduced by both PT and PZ (Table 1).

Table 1. The effect of EPA on IP formation in AOCs.

	IP production (cpm mg^{-1} protein)			
(Antagonist)	CON	NA	ETH	EPA.
None	269.8±67.6	*1033.1±91.2	706.4±110.7	#1168.9±197.1
PT	407.6±53.2	463.6±79.0	484.0±98.7	651.1±78.1
PZ	392.8±36.1	461.4±46.1	557.3±107.5	735.6±107.5

Values are mean ± s.e.mean. * indicates significant difference compared with CON; # compared with ETH. $P<0.05$ using Student's unpaired t-test (n≥12).

The results indicate that vasorelaxation induced by EPA is not the result of a decrease in calcium released from stores linked to the IP pathway. The results further suggest that the mechanism of action of EPA is in some way coupled to an α-adrenergic pathway. Clarification of this mechanism remains for future study.

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90P PROSTAGLANDIN E_1 PROTECTS AGAINST REPERFUSION INJURY AND ATTENUATES ENDOTHELIAL P-SELECTIN EXPRESSION IN A RABBIT MODEL OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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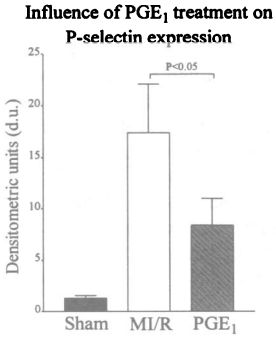
The E-type prostaglandins PGE_1 and PGE_0 reduce infarct size in a rabbit model of acute myocardial infarction (Hide *et al.* 1995a). The mechanism of this cardioprotective effect may involve i) a reduction in afterload, ii) inhibition of platelet aggregation and neutrophil activation and iii) opening of ATP-sensitive potassium channels (Hide *et al.* 1995b). The reperfusion injury of ischaemic myocardium is, in part, due to the adhesion and activation of leukocytes (PMNs) resulting in microvascular plugging (no reflow) and generation of oxygen-derived free radicals. Firm attachment and diapedesis of PMNs are preceded by the rolling of PMNs on the endothelium which is due to the upregulation of P-selectin on the endothelial cell surface. Here we investigate the effects of PGE_1 on i) reperfusion injury and ii) endothelial P-selectin expression in a rabbit model of myocardial ischaemia and reperfusion (MI/R).

Male New Zealand white rabbits 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 the first antero-lateral branch of the left coronary artery (LAL) was occluded for 60 min. Intravenous infusion of either saline (control) or PGE_1 (1 μg/kg/min; 0.05 ml/min) was commenced 10 min prior to reperfusion and continued throughout the reperfusion period. After 120 min reperfusion, area at risk (AR) and infarct size (IS) were determined using Evans blue dye and nitro-blue tetrazolium respectively, as previously described (Hide *et al.*, 1995b). In a second series of experiments PGE_1 was administered as a continuous i.v. infusion starting 10 min prior to LAL occlusion. After 20 min of reperfusion the hearts were excised, frozen in liquid N_2 and cut in 10 μm sections. After fixation (without permeabilization) and staining with monoclonal anti-P-selectin antibody and monoclonal DTAF-conjugated anti-mouse-IgG, DTAF-fluorescence was photographed at 520nm (excitation at 450-490 nm) and analysed using an image analysis system. Densitometric measurement of the specific flourescence was carried out at the luminal surface of endothelial cells.



Infusion of PGE_1 resulted in a significant reduction in infarct size from 62±6% (control; n=7) to 37±4% (n=8) of the AR. AR did not differ between groups. We found P-selectin (CD-62) fluorescence at the luminal surface of endothelial cells within the AR (n=6) and this was significantly reduced by 50%, from 17±5 d.u. to 8±3 d.u., in hearts treated with PGE_1 (n=6).

Thus, the expression of P-selectin on the endothelial cell surface is reduced by PGE_1 which may account for less pronounced neutrophil adhesion and ultimately a reduction in myocardial infarct size. *This work is supported by a project grant from Schwarz Pharma AG, Monheim, Germany. EH is the recipient of a BHF studentship.*

Hide *et al.*, (1995a). *Br. J. Pharmacol.* **112 (Suppl):** 383P
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91P EFFECTS OF IBUPROFEN ON HAEMODYNAMICS, ORGAN INJURY AND DYSFUNCTION CAUSED BY ENDOTOXIN IN THE RAT

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An enhanced formation of prostaglandins has been implicated in the pathogenesis of circulatory shock (Herman and Vane, 1974). Cyclo-oxygenase inhibitors attenuate hypotension and mortality in animal models of endotoxic shock (Jacobs et al., 1982). Here, we investigate the effects of ibuprofen, a cyclooxygenase inhibitor, on haemo-dynamics, multiple organ (liver, kidney and pancreas) dysfunction syndome (MODS) and prostaglandin biosynthesis in rats with endotoxic shock.

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The left carotid artery was cannulated for continous measurement of mean arterial blood pressure (MAP) and the right jugular vein for the administration of drugs. At time 0, rats received either vehicle (saline, n=5) or *E.coli* lipopolysaccharide (LPS, 10 mg kg^{-1} i.v., n=16). At 30 min prior to LPS, LPS-rats were treated with vehicle (0.6 ml kg^{-1} h^{-1} i.v., n=16) or ibuprofen (12.5 mg kg^{-1} i.v. plus 12.5 mg kg^{-1} h^{-1} i.v., n=6) was started and continued for further 2.5 h. The pressure response to noradrenaline (NA; 1 μg kg^{-1} i.v.) was assessed 40 min prior to and every h after LPS. At six h after injection of LPS or vehicle serum samples were taken and analysed for alanine aminotransferase (ALT) and bilirubin (liver function) creatinine (renal function) and lipase (pancreatic function). In addition, the concentration of 6-keto-prostaglandin $F_{1\alpha}$ (6-ketoPGF$_{1\alpha}$) was measured in the plasma by radioimmunoassay.

In the anesthetised rat, LPS caused hypotension, vascular hypore-activity to NA, renal, pancreatic and liver dysfunction as well as an increase in the plasma concentration of 6-ketoPGF$_{1\alpha}$ ($p < 0.05$, table 1). Treatment of LPS rats with ibuprofen attenuated (i) the vascular hyporeactivity, (ii) the increase in the serum levels of ALT, bilirubin, creatinine, (iii) and abolished the rise in 6-ketoPGF$_{1\alpha}$, but has no effect on the delayed hypotension caused by endotoxin (table 1).

Thus, the cyclooxygenase inhibitor ibuprofen attenuates the vascular hyporeactivity to NA, hepatocellular injury as well as the dysfunction of liver and kidney caused by endotoxin in the rat.

HR is a research fellow of the Deutsche Forschungsgemeinschaft (Ru595/1-1)

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Table 1.

Treatment	MAP (at 6 h) (mmHg)	Hyporeactivity (% of control)	ALT (iu l^{-1})	Bilirubin (μM)	Lipase (iu l^{-1})	Creatinine (μM)	6-ketoPGF$_{1\alpha}$ (ng ml^{-1}) -30 min	360 min
saline	116±6	95±6	61±6	3.4±0.6	13±6	33±2	0.19±0.09	0.22±0.08
LPS + saline	68±6	15±3	388±7.7	6.9±1.3	67±9	91±8	0.23±0.15	0.61±0.12
LPS + ibuprofen	73±3	54±14*	128±17*	3.9±0.8*	43±7	57±13*	0.15±0.05	0.15±0.02*

92P EFFECTS OF SELECTIVE INHIBITORS OF ETA- AND ETB-RECEPTORS ON HAEMODYNAMICS AND ORGAN INJURY CAUSED BY ENDOTOXIN IN THE RAT

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An increase in the serum levels of endothelin-1 (ET-1) has been documented in many cardiovascular disorders including circulatory shock (Pittet et al., 1991). This study investigates the effects of the selective ET_A receptor antagonist, BQ-485, and the selective ET_B receptor antagonist, BQ-788, on *(i)* systemic haemodynamics, *(ii)* vascular hyporeactivity to noradrenaline (NA), *(iii)* renal funtion, and *(iv)* liver integrity in a rat model of endotoxic shock.

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The carotid artery was cannulated for the measurement of mean arterial pressure (MAP) and the femoral vein for the administration of compounds. After stabilisation of hemodynamic parameters, animals were challenged with NA (1 μg kg^{-1}, i.v.). Different groups of animals received (5 min later) infusions of vehicle (saline, 0.6 ml kg^{-1} h^{-1}, n=15), or BQ-788 (10 nmol kg^{-1} min^{-1}, n=10) or BQ-485 (10 nmol kg^{-1} min^{-1}, n=10). Fifteen min later, animals received either vehicle (saline, 1 ml kg^{-1}, i.v., n=12) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v., n=29) as a slow injection over 10 min. The pressor response to NA was reassessed at every hour after LPS. At 6 h, blood samples were taken and analysed for glutamate-pyruvate-transaminase (GPT) and glutamate-oxalacetate-transaminase (GOT) (liver integrity) and creatinine (Crea) and urea (renal function).

LPS caused hypotension, vascular hyporeactivity, as well as the renal and liver dysfunction ($p<0.05$, Table 1). Treatment of LPS-rats with BQ-485 aggravates the delayed hypotension, but had no effects on the vascular hyporeactivity to NA, or the rise in the serum levels of GPT, GPT, Crea and urea caused by endotoxin (Table 1). In contrast, treatment of LPS-rats with BQ-788 ameliorated the delayed hypotension and significantly enhanced the pressor responses to NA between 2h and 5h ($P<0.05$). Furthermore, BQ-788 significantly attenuated the rise in the serum levels of GPT andt GOT ($P<0.05$), but had no effects on the renal dysfunction and creatinine caused by endotoxin (Table 1).

BQ-788, a selective ET_B receptor antagonist, but not BQ-485, a selective ET_A receptor antagonist, attenuates the delayed circulatory failure (hypotension, vascular hyporeactivity), and liver failure caused by endotoxin in the anaesthetised rat. Thus, selective inhibition of ET_B receptors might be useful in endotoxaemia.

HR is a fellow of the Deutsche Forschungsgemeinschaft (Ru595/1-1).

Pittet, J.F. et al. (1991). *Ann. Surg.*, **213**, 261-264.

Table 1. Treatment	MAP (at 6 h) (mmHg)	Hyporeactivity (mmHg,3 h)	GPT (iu l^{-1})	GOT (iu l^{-1})	Crea (mM)	Urea (μM)
Sham	109±1.6	39±3	64±4	202±18	31±4	4.2±0.2
LPS + saline	83±4	27±4	510±94	860±105	79±6	16.3±0.7
LPS + BQ-485	69±6*	21±4	755±217	1001±243	89±7	19.7±0.8
LPS + BQ-788	100±6*	45±3*	261±66*	390±78*	93±10	18.1±0.8

mean ± s.e.mean. *$p<0.05$ vs. LPS + saline, unpaired Student's *t* test.

93P INFLUENCE OF $MgSO_4$ ON THE CEREBROVASCULAR EFFECTS OF ENDOTHELIN-1 IN CONSCIOUS RATS

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In conscious rats, i.v. infusion of $MgSO_4$ reverses the internal carotid vasoconstriction caused by endothelin-1 (ET-1) (Kemp *et al.*, 1993), but it is not known if this phenomenon is expressed in all tissues supplied by the internal carotid artery. Therefore, we assessed the effects of ET-1, and the influence thereupon of $MgSO_4$, on blood flow in whole brain, eyes, tongue and a sample of cranial skin in conscious rats, using the coloured microsphere technique (Kowallik *et al.*, 1991).

Under sodium methohexitone anaesthesia (40-60 mg kg^{-1}, i.p.), male, Long Evans rats (350-450 g) had a left atrial catheter implanted for injection of microspheres, and, 3-5 days later, arterial and venous catheters for reference sample withdrawal, blood pressure measurement and infusion of ET-1 and $MgSO_4$. One group (Experimental, n = 8) of conscious animals was given an i.v. infusion of $MgSO_4$ (220 µmol min^{-1} kg^{-1}) for 7 min, beginning 20 min after the onset of ET-1 infusion (12.5 pmol kg^{-1} min^{-1}). Another group (Control, n = 8) was infused with isotonic saline instead of ET-1 and $MgSO_4$. Measurements of flow were made immediately before the onset of saline or ET-1 infusion (A), and immediately before the onset of saline or $MgSO_4$ infusion (B), and during the last min of saline or $MgSO_4$ infusion (C). Flow measurements were made using 3 colours of microspheres; yellow spheres were injected immediately before the onset of saline or ET-1 infusion (A), red spheres immediately before the onset of saline or $MgSO_4$ infusion (B), and blue spheres during the last min of saline or $MgSO_4$ infusion (C). At the end of the experiment, animals were killed and tissues of interest removed and weighed. Microspheres were extracted from the tissue and reference samples, and the dye extracted from the spheres. Samples were scanned in a spectrophotometer and flows were calculated using the Dye-Trak Matrix inversion software.

Table 1. Regional blood flow (ml min^{-1} $100g^{-1}$) in control and experimental groups.

	Control A	Control B	Control C
Brain	125 ± 10	139 ± 10	132 ± 6
Eyes	165 ± 20	169 ± 14	172 ± 22
Tongue	95 ± 25	79 ± 16	85 ± 4
Skin	21 ± 3	20 ± 2	15 ± 2
	Experimental A	**Experimental B**	**Experimental C**
Brain	123 ± 9	124 ± 5	170 ± 13^{AB}
Eyes	170 ± 14	140 ±10	178 ± 6
Tongue	60 ± 8	40 ± 7^{A}	116 ± 22^{AB}
Skin	27 ± 5	12 ± 2^{A}	11 ± 2^{A}

Superscripts indicate significant differences between columns (ANOVA).

During infusion of saline there were no significant changes in tissue blood flow (Table 1). Infusion of ET-1 had no effect on brain or eye blood flow, but reduced flow through the tongue and cranial skin. $MgSO_4$ had no effect on blood flow through the eyes or cranial skin, but increased brain and tongue blood flows above baseline (Table 1). The results indicate heterogeneous effects of ET-1 and $MgSO_4$ on blood flow through regions supplied by the internal carotid artery.

This work was supported by the British Heart Foundation.

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94P ENDOTHELIN-1 IS DEGRADED BY AN ENZYME RELEASED FROM NON-ENDOTHELIAL CELLS

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ET-1, the most potent mammalian vasoconstrictor peptide known, (Yanagisawa *et al.*, 1988) is synthesised by endothelial cells and released in a principally polar direction towards the vascular smooth muscle. In the isolated perfused mesentery of the rat, ET-1 is rapidly inactivated by an enzyme released into the vasculature (Pérez-Vizcaíno *et al.*, 1995). Here, we have investigated whether this degrading enzyme is released in the absence of an endothelium.

Male Wistar albino rats (200-300 g) were sacrificed by cervical dislocation. The superior mesenteric artery was cannulated and the superior mesenteric vascular bed perfused (2 ml min^{-1}) in a closed system with warmed (37 °C) and gassed (95% O_2; 5% CO_2) Krebs' buffer. After equilibration (45 min) the endothelial layer of some preparations was removed using distilled water (Cusma-Pelogia *et al.*, 1993). Krebs' buffer (8 ml) was then recirculated for 60 min after which time 1 ml aliquots of the recirculated Krebs' were incubated for 0-60 min at 37 °C with ET-1 ($3x10^{-8}$ M) plus 1,10 phenanthroline (10^{-3} M; n=3), EGTA (10^{-3} M; n=3), phenylmethylsulfonylfluoride (PMSF, 10^{-3} M; n=4) or vehicle. In addition, recirculated Krebs' (n=3) was heated at 90 °C for 15 mins before incubation with ET-1. The ET-1 contractile activity retained within each incubate was then assayed by its ability to contract rat aortic rings denuded of endothelium. Contractions were recorded isometrically. As a control, fresh Krebs' was similarly incubated with each of the inhibitors, or was heat treated, before incubation with ET-1 and assay on aortic rings.

When ET-1 was incubated together with recirculated Krebs' from either endothelium intact or endothelium-denuded mesenteries there was a similar loss in contractile activity. For instance, at 30 min 46±13 % and 46±19 % of the original ET-1 response was retained in the two incubates, and at 60 min 33±21 % and 21±12 % was retained (n=5 for each). Using recirculated Krebs' from endothelium-denuded preparations it was found that EGTA, phenanthroline or heating of the Krebs', protected the ET-1 from degradation whereas PMSF was without effect (Table 1).

Thus, endothelial-denuded mesenteries release an ET-1 degrading enzyme similar to that released by intact preparations (Pérez-Vizcaíno *et.al.*, 1995). Clearly, this suggests that this degrading enzyme is of non-endothelial cell origin and that the activities of ET-1 within the circulation may be regulated by cell types other than endothelial.

Table 1 Contractions (% response KCl, 80 mM) induced by ET-1 incubated for 30 min with recirculated Krebs' in the presence or absence of inhibitory treatments (* $p<0.05$, paired t-test).

Treatments	+ Inhibitory Treatment	- Inhibitory Treatment
Phenanthroline	51±8	27±15*
EGTA	70±7	27±10*
PMSF	10±2	4±3
Heating	79±14	24±13*

T.D.W. holds a British Heart Foundation Lectureship (BS/95003)

Cusma-Pelogia, N., *et. al.* (1993) *J. Pharm. Tox. Methods*, 29, 157-163.
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95P ENDOTHELIN-A RECEPTOR ACTIVATION IS PARTIALLY RESPONSIBLE FOR THE ACUTE INCREASE IN HAEMATOCRIT INDUCED BY LPS

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Intravenous injection of bacterial lipopolysaccharide (LPS) causes a marked increase in haematocrit (Hct) (Smith *et al.*, 1991). As LPS can cause the release of endothelin-1 (ET-1) into the circulation (Nambi *et al.*, 1994), and as ET-1 itself can directly increase Hct (Zimmermann *et al.*, 1992), we have assessed the effects of the ET_A receptor selective antagonist, FR 139317 (see Warner, 1994), and the nonselective ET_A/ET_B receptor antagonist, PD 145065 (see Warner, 1994), on the increase in Hct caused by an i.v. injection of a bolus of LPS.

Male Wistar rats (250-350 g) were anaesthetised with sodium thiopentone (120 $mg.kg^{-1}$, i.p.) and kept warm (37 °C) by a homeothermic blanket. The jugular and femoral veins were cannulated for administration of drugs, and the carotid artery was cannulated for the measurement of mean arterial blood pressure (MAP) by a pressure transducer. Following a 30 min equilibration, LPS (30 $mg.kg^{-1}$, in 400 µl) was injected (i.v. over 30 sec). FR 139317 (100 $nmol.kg^{-1}.min^{-1}$), PD 145065 (100 $nmol.kg^{-1}.min^{-1}$) or vehicle were infused (600 $µl.h^{-1}$) for 15 min starting from 5 min before the LPS injection. Blood samples were taken for the measurement of Hct 1 min before LPS administration and up to 60 min afterwards.

1 min before LPS the Hct was 46.9 ± 0.5 % vol (n=16) and the MAP, 102.3 ± 2.7 mmHg (n=16). Administration of LPS induced an increase in Hct, and a fall in MAP. The increase in Hct was attenuated by administration of FR 139317 or PD 145065 (Table 1). The decrease in MAP was potentiated by FR 139317, but not greatly affected by PD 145065 (Table 1).

Thus, bolus injection of LPS may rapidly release ET-1 as illustrated by the effects of endothelin receptor antagonists at early time points. This indicates that ET-1 can act as a rapid responder to cardiovascular challenges. Furthermore, our results suggest that activation of ET_A receptors by endogenously produced ET-1 supports blood pressure following acute cardiovascular insult.

T.D.W. holds a British Heart Foundation Lectureship (BS/95003).

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Table 1. Effect of FR 139317 or PD 145065 on the changes in mean arterial pressure (MAP) and haematocrit (Hct) induced by LPS. * P<0.05 compared to control by a Mann Witney U test.

Time (min)	Vehicle (n=6)		+ FR 139317 (n=3-6)		+ PD 145065 (n=4)	
	Δ MAP (mmHg)	Δ Hct (% vol)	Δ MAP (mmHg)	Δ Hct (% vol)	Δ MAP (mmHg)	Δ Hct (% vol)
5	-32.8 ± 10.1	4.3 ± 2.2	-70.5 ± 6.8*	0.9 ± 0.7	-31.5 ± 14.6	0.6 ± 0.8
10	-25.6 ± 3.5	10.6 ± 1.7	-52.1 ± 5.6*	4.2 ± 0.9*	-22 ± 9.1	4.9 ± 1.8*
20	-29.3 ± 2.7	11.6 ± 0.9	-44.8 ± 6.4	7.3 ± 0.7*	-14.4 ± 6.7	7.8 ± 0.9*
30	-25.4 ± 6.5	11.9 ± 1	-37.2 ± 6.8	8.1 ± 0.6	-14.6 ± 5.3	7.9 ± 1.5
60	-29.6 ± 9.1	12.5 ± 1.9	-41.5 ± 1.5	6.3 ± 0.7*	-14.4 ± 7.8	5.1 ± 1.2*

96P RESPONSES OF GUINEA-PIG ISOLATED AORTA AND PULMONARY ARTERY TO ADENOSINE RECEPTOR AGONISTS IN NORMOXIA AND HYPOXIA

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The A_2-adenosine receptor responsible for adenosine-induced vasorelaxation in the guinea-pig aorta is thought to be mediated by the A_{2b} adenosine receptor subtype (Alexander et al., 1994). The pharmacological characterisation of the adenosine receptor mediating relaxation in the guinea-pig pulmonary artery however is less well understood (Szentmiklosi et al., 1995). The present study compares the pharmacological profile of adenosine receptor sensitivity in the guinea-pig aorta and pulmonary artery in normoxic and hypoxic conditions.

From each guinea-pig, two 3-5mm rings of endothelium-denuded thoracic aorta and pulmonary artery, were mounted under a resting tension of 1g in Krebs-bicarbonate solution at 37°C. During equilibration the tissues were bubbled in normoxic (**N**) conditions with O_2:CO_2 (95:5). For hypoxic (**H**) conditions, the gas mixture was changed to CO_2:N_2 (5:95), 20 minutes before commencing the experiment. Cumulative concentration-response curves (CCRC's) were constructed for phenylephrine (PE) in **N** and **H** conditions to determine submaximal concentrations for preconstriction. Pulmonary artery and aortic rings were precontracted with $3x10^{-6}$ and 10^{-5}M PE, respectively in, **N** or **H** conditions in the absence or presence of indomethacin (**I**, 10^{-6}M), added 30 min beforehand. When the tension had plateaued, CCRC's were constructed for the adenosine agonist. Statistical significance ($P < 0.05$) was tested using Student's t-test of paired data. EC_{50} values are given as geometric mean with 95% confidence limits.

In aortic rings, both in **N** and **H** conditions and in the absence or presence of **I**, 5'-N-ethylcarboxamidoadenosine (NECA) produced concentration-related decreases in tension. Combinations of **H** and **I** had no significant effect on the EC_{50} value(**N**, 3.7 (1.7 - 8.29)$x10^{-7}$ M, **H**, 6.7 (2.5 - 17.8)$x10^{-7}$ M, **N+I**, 6.3 (5.79 - 7.05)$x10^{-7}$ M, **H+I**, 7.4 (1.3 - 41.2)$x10^{-7}$ M. In the pulmonary artery in **N** and **H** conditions, NECA produced concentration-related *increases* in tension at low concentrations followed by concentration-dependent decreases in tension at higher concentrations. **H** had no significant effect ($P>0.05$, n=4) on the maximum tension increase (**N**, 155.9±16.4%, **H**, 141.5±19.3%). In the presence of **I** in **N** and **H** conditions, NECA relaxed the pulmonary artery in a concentration-dependent manner, and the vasoconstrictor response observed at low concentrations was abolished. The EC_{50} values in **N**,(9.6(4.44-20.9)$x10^{-7}$ M) and **H**, (1.5(0.46-4.75)$x10^{-6}$ M) were not significantly different ($P>0.05$).

The adenosine agonists, NECA, R-N^6-phenylisopropyladenosine (R-PIA), S-N^6-phenylisopropyladenosine (S-PIA), 2-p-((carboxyethyl)-phenethylamino)-5'carboxamidoadenosine (CGS 21680) and N^6-cyclopenyl-adenosine (CPA) in the presence of **I** and in **N** conditions, produced qualitively similar relaxant responses (Table 1). In both preparations, the rank order of potency was: NECA > R-PIA = CPA ≥ S-PIA > CGS21680, consistent with A_{2b} receptor involvement.

Table 1: EC_{50} values for vessel relaxation by adenosine agonists

	AORTA	PULMONARY ARTERY
NECA	9.6 (4.4 - 20)x 10^{-7} M.	6.4 (5.8 - 7.1)x 10^{-7} M.
R-PIA	1.1 (0.6 - 2.1)x 10^{-5} M.	6.5 (0.46 - 4.75)x 10^{-6} M.
S-PIA	4.3 (4.0 - 4.5)x 10^{-5} M.	4.5 (4.3 - 4.8)x 10^{-5} M.
CPA	1.9 (0.8 - 4.7)x 10^{-5} M.	6.6(2.4 - 16.7)x 10^{-6} M.
CGS21680	> 10^{-4} M.	> 10^{-4} M.

Thus, NECA contracts the pulmonary artery in **N** and **H** conditions. This is abolished by **I**, indicating involvement of cyclooxygenase products. Hypoxia does not affect the vasodilator response which appears to be mediated via A_{2b} receptors in both tissues.

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97P EFFECTS OF XR5118 ON PAI-1 AND tPA ACTIVITY AND ARTERIAL THROMBUS FORMATION IN THE ANAESTHETIZED RAT

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Plasminogen activator inhibitor type 1 (PAI-1) is the primary endogenous inhibitor of tissue plasminogen activator (tPA) and clinical evidence suggests that elevated PAI-1 activity is associated with ischaemic heart disease (Munkvad *et al.*, 1990). XR5118 [(3Z,6Z)-6-benzylidene-3-(5-(2-dimethylaminoethyl-thio)-2-thienyl)methylene-2,5-piperazinedione, hydrochloride, (X-642)] is a low molecular weight inhibitor of PAI-1 *in vitro*. In an amidolytic assay (for method see Faint *et al.*, 1995) XR5118 had an IC_{50}=3.5 μM. In the present study, XR5118 has been examined *in vivo*, on circulating tPA and PAI-1 activity, and arterial thrombus formation in the rat.

Male Wistar rats (250-350 g) were anaesthetized (Sagatal, 60 mg kg^{-1} i.p.) and XR5118 (0.1, 0.3 and 1.0 mg $kg^{-1}min^{-1}$) was infused for 60 min, with arterial blood samples taken at intervals up to 90 min, for analysis of tPA and PAI-1 activity by chromogenic assay. In separate experiments, rats were anaesthetized as above, before exposing a carotid artery and placing it over a bipolar stainless steel electrode and a flow probe. After 5 min of a 20 min infusion, the artery was electrically stimulated (6 mA for 2 min) and the time to occlusive thrombus formation (zero arterial flow) was recorded. Infusion of 0.3 and 1.0 mg $kg^{-1}min^{-1}$ XR5118 significantly increased tPA activity, relative to the vehicle (dextrose) group (figure 1). In addition, 1.0 mg $kg^{-1}min^{-1}$ XR5118 led to a significant (P<0.01) reduction in PAI-1 activity at t=60 min: 13.1±1.4, compared to 20.3±0.9 arbitrary u ml^{-1} in controls (mean±s.e. mean, n=7-10 per group). In the electrically stimulated carotid artery study, infusion of 1.0 mg $kg^{-1}min^{-1}$ XR5118 was associated with a statistically significant (P<0.01, unpaired t test) increase in the time to thrombus formation, from 21.2±2.5 min in the vehicle-treated group to 40.3±4.9 min (n=10 per group). This increase was comparable to heparin (10 iu $kg^{-1}min^{-1}$), where time to thrombus formation was 36.8±3.9 min (P<0.01).

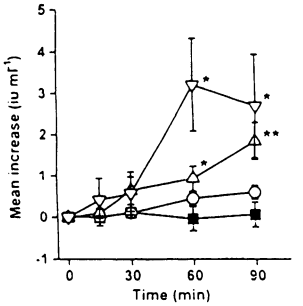

Figure 1. The effect of XR5118 on change in tPA activity. ■: vehicle, O: 0.1, Δ: 0.3 and ∇: 1.0 mg kg^{-1} min^{-1}. *, **; P<0.05, 0.01 respectively, compared to equivalent point in vehicle group, for n=4-10 per group (unpaired t test).

These results demonstrate that XR5118 is able to increase endogenous tPA activity in the rat and is capable of attenuating thrombus formation following electrical injury. We postulate that these effects of XR5118 *in vivo* are due to inhibition of PAI-1, resulting in improved fibrinolytic capacity.

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98P EFFECTS OF THE CO-POLYMER, PLURONIC F-68, ON THE RESPONSE TO PLATELET AGGREGATION AGONISTS IN HUMAN WHOLE BLOOD *IN VITRO*

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The polyol surfactant, *Pluronic*® F-68 (PF-68), has been assessed *in vivo* as a haemorrheological agent (Schaer *et al.*, 1994) and is a component of perfluorochemical emulsions, such as *Fluosol*®, which have been used clinically for myocardial oxygenation (Lowe, 1994). PF-68 can inhibit 'spontaneous' platelet aggregation (Edwards *et al.*, 1995), but the mechanism involved and its interaction with aggregation agonists remain obscure. Therefore, the effects have been studied of PF-68 on agonist-induced platelet aggregation in human blood *in vitro*.

A solution (10.0% w/v) of PF-68 (ICI, U.K.) was prepared in isotonic saline (0.9% w/v NaCl). Blood (9.0 ml) from volunteers was placed into tubes containing 50 μg ml^{-1} hirudin (*Revasc*™; Ciba, U.K.) and incubated at 37°C for 30 min. Aliquots (460 μl) of blood were placed in small plastic tubes in a water bath (37°C) and pre-incubated (2 min) with 20 μl of one of several ten-fold dilutions of PF-68. Tubes were sampled (15 μl), 20 μl of saline was added and tubes stirred continuously (1000 rpm). Sampling (15 μl) was performed at 4, 6 and 8 min and samples placed in 30 μl of a formaldehyde fixing solution. Thirty six μl of fixed sample was mixed with 9.1 ml of saline and transferred to an automatic Ultra-Flo 100 Platelet Counter for platelet counting relative to number of red cells (RBC) present. Platelet counts were expressed as % fall compared with time 0 and expressed as % aggregation. The method was modified to assess the effects of the aggregation agonists, adenosine diphosphate (ADP; 0.3-3.0 μM) or Platelet Activating Factor (PAF; 0.75-3.0 μM), by (i) replacing the addition of saline, after the sample was placed on the stirrer, with an identical volume of agonist, and (ii) reducing the sampling interval to 30 s for the first 2 min after addition, to account for the more rapid aggregation induced by such agonists. Statistical comparisons were made by the paired Wilcoxan two-tailed test.

Addition of ADP (3.0 μM) to blood induced maximum mean (± s.e. mean) platelet aggregation within 1 min [control: 88 ± 2%, n = 6; PF-68 (4 mg ml^{-1}): 83 ± 2%, n = 6]. After 4 min, for example, the mean aggregation in PF-68-treated blood (39 ± 7%) was significantly (P < 0.001) lower than control (78 ± 2%); similar results were obtained with 0.3 μM and 1.0 μM ADP. PAF (3.0 μM) similarly induced maximum aggregation within 1 min and, after 4 min, the mean aggregation in PF-68-treated blood (8 ± 3%) was also lower (P < 0.001) than control (85 ± 1%); similar results were obtained with 0.75 and 1.5 μM PAF. These results show that PF-68 can enhance the rate of platelet disaggregation following aggregation induced by ADP or PAF. Armstrong *et al.* (1995) proposed that the anti-aggregation effects of the PF-68-based *RheothRx*® involved stabilisation of RBC membranes rather than direct inhibition of ADP-induced platelet aggregation. In the present study, hirudin was used as a blood anticoagulant, rather than citrate (Armstrong *et al.*, 1995). Hirudin maintains normal concentrations of divalent cations in plasma and this may emphasise the ability of PF-68 to potentiate platelet dis-aggregation. The findings that PF-68 can inhibit RBC-induced platelet aggregation and can limit the aggregation effects of ADP and PAF may be relevant to its suggested anti-thrombotic properties.

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99P DOSE-DEPENDENT INHIBITION OF HUMAN BLOOD NEUTROPHILS BY A PERFLUOROCHEMICAL EMULSION RESPIRATORY GAS CARRIER

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Perfluorochemical (PFC) liquids can dissolve respiratory gases and their emulsions have been used as adjuncts to coronary balloon angioplasty (Lowe, 1994). Emulsified PFCs may protect against reperfusion inflammatory damage through transient alterations in blood leucocyte functions (Forman *et al.*, 1992). However, it is unclear whether such effects are caused by opsonisation of PFC emulsion droplets (typically *ca.* 0.2 μm diameter) or by direct effects of surfactant component(s) (Schaer *et al.*, 1994). Therefore, the present study has assessed the effects of a novel PFC emulsion and either the lecithin or *Pluronic®* co-polymer emulsifiers on human neutrophil function *in vitro,* as assessed by cellular chemiluminescence.

Blood was obtained from healthy volunteers and 4.5 ml aliquots placed into tubes containing 3.13% (w/v) trisodium citrate dihydrate (0.5 ml) in a water bath (37°C); samples were incubated for 30 min before use. Whole blood was used to avoid pre-activation of neutrophils by the separation procedure (Neilson *et al.*, 1992). Fifty µl of blood was added to 10 µl of either (i) a novel PFC emulsion containing 18.5% (w/v) perfluorodecalin and 1.5% (w/v) perfluorodimorpholine propane emulsified with 2.5% (w/v) lecithin (*Lipoid®* E100; Lipoid GmbH), (ii) lecithin (*Lipoid®* E100; Lipoid GmbH), (iii) commercial grade *Pluronic®* F-68 (ICI, U.K.) or (iv) *Pluronic®* PE 6800 (BASF, Germany) that had been pre-warmed to 37°C in a water bath; samples were re-incubated for a further 2 min. Fifty µl of sample was added to 1.0 ml of phosphate-buffered saline (PBS) containing *Luminol* (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich, U.K.; A8511; 100 µg ml^{-1}) and incubated for a further 5 min at 37°C. Twenty µl of phorbol 12-myristate 13-acetate (PMA; Sigma) solution (100 µg ml^{-1}) was added to each sample and the chemiluminescence recorded at 2 min intervals for up to 20 min. Statistical comparisons were made by the paired Wilcoxan two-tailed test. The mean chemiluminescence following stimulation of neutrophils with PMA in controls increased to a maximum within 10-15 min. A transient, dose-dependent, decrease in chemiluminescence, to a maximum of 54% after 12 min ($P < 0.05$), occurred when blood was pre-incubated with 10, 20 or 30 µl of the PFC emulsion compared to saline controls. For example, the mean (± s.e. mean) chemiluminescence of neutrophils incubated with 30 µl emulsion at 12 min following PMA stimulation (9.5 ± 1.3 mV, n = 6) was significantly lower ($P < 0.05$) than control (24.2 ± 2.2 mV, n = 6). In contrast, incubation of blood with lecithin up to 16 mg ml^{-1} or the *Pluronics®* up to 65 mg ml^{-1} did not alter neutrophil chemiluminescence. The present findings, showing transient inhibitory effects of a novel PFC emulsion on neutrophil function, support the use of such materials in the treatment of coronary angioplasty to facilitate improved oxygenation whilst concurrently reducing inflammatory damage caused by neutrophil-induced reperfusion injury.

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100P ARTERIAL BLOOD PRESSURE AND GROWTH PATTERNS IN SPRAGUE-DAWLEY AND SPONTANEOUSLY HYPERTENSIVE RATS FED ON CALCIUM-DEFICIENT DIETS

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The decrease of dietary calcium has been used to induce experimental models of hypertension (Togari et al., 1989). According to Metz et al. (1988), the rats on a lower calcium diet showed body weight (BW) gain. We study the influence of calcium diet deficiency on arterial blood pressure, and growth, of two different rat strains: normotensive Sprague-Dawley (SDR), and spontaneously hypertensive (SHR). After being weaned at 3 weeks, male animals of both strains were randomized with "ad libitum" intake of one of three possible diets: semi-synthetic casein with a normal-calcium content (Ca 1%) (control group), low-calcium (Ca 0.1%), and calcium-free (Ca 0.01%). The systolic (SBP) and the diastolic (DBP) arterial blood pressure were measured in 9-week-old (adult age) conscious SDR, and weekly in conscious SHR from the 6th to the 20th week of life by the tail cuff method. We took weekly measurements of animal BW up to 9 weeks in SDR, and up to 20 weeks in SHR. The data are expressed as mean values ± s.e.mean for a minimum of 6 rats. The Student's t-test for comparison of mean values was used (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

The calcium-free diet did not change the arterial blood pressure of the SDR, but the low-calcium diet increased both SBP and DBP. The SHR fed on a normal-calcium diet showed a gradual increase in SBP and DBP which reached maximum values between weeks 16-20. The calcium-free diet lessened the development of hypertension in this rat strain. On the contrary, hypertension developed most rapidily in SHR fed with the low-calcium diet, SBP and DBP reaching maximum values at 8-9 weeks. The BW of SDR fed on the calcium-deficient diets (low-calcium and calcium-free) was significantly lower than that of the animals fed on the control diet. BW gain was delayed particularly in the rats fed on the calcium-free diet. The BW gain was similar in the SHR on normal- and low-calcium diets. Nevertheless, the rat strain fed on the calcium-free diet presents a significantly reduced BW gain as compared with the animals fed on the normal-calcium diet (see table 1). We therefore concluded that dietary calcium deficiency attenuates growth in rats being the metabolism of SHR less susceptible to dietary calcium changes than that of SDR. Moreover, the results of this study suggest that calcium deficiency causes an increase in arterial blood pressure in both rat strains, but a minimum dietary calcium seems to be necessary to appreciate the vasoconstrictor effect.

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Table 1. SBP, DBP, and BW in SDR and SHR fed with diets with a different calcium content.

	% Dietary Ca		
	1	0.1	0.01
SDR (9 weeks of life)			
SBP (mm Hg)	144 ± 2	168 ± 3***	149 ± 1
DBP (mm Hg)	111 ± 2	125 ± 2***	111 ± 2
BW (g)	379 ± 6	259 ± 7***	148 ± 3***
SHR (8 weeks of life)			
SBP (mm Hg)	191 ± 5	241 ± 4***	171 ± 5**
DBP (mm Hg)	140 ± 5	189 ± 7***	126 ± 9*
SHR (20 weeks of life)			
SBP (mm Hg)	234 ± 5	225 ± 3	200 ± 2**
DBP (mm Hg)	177 ± 1	193 ± 1	133 ± 9
BW (g)	331 ± 8	328 ± 6	115 ± 6***

101P DICHLOROACETATE INCREASES THE THRESHOLD FOR CARDIAC PRECONDITIONING IN THE RAT ISOLATED PERFUSED HEART

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Recent interest has focused on the ability of short periods of ischaemia to protect the heart against prolonged ischaemia and this protective effect has been termed cardiac preconditioning (Murry *et al.*, 1986). The precise mechanisms underlying preconditioning are currently a matter of debate but it is possible that intracellular metabolism may have bearing on these events. The aim of this study was to examine the effects on preconditioning of improving the coupling between pyruvate formation and its oxidative metabolism by activating pyruvate dehydrogenase complex with dichloroacetate (DCA; Whitehouse & Randle, 1974).

Male Wistar rats (280-400g) were heparinized (1,000 U kg^{-1} i.p.) and anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.). In each case, following a thoracotomy, the heart was rapidly excised and perfused in the Langendorff mode at constant flow (20 ml min^{-1}) with oxygenated Krebs-Henseleit buffer containing 2mM pyruvate. A fluid-filled balloon catheter was inserted in to the left ventricle in order to measure developed left ventricular pressure (DLVP), from which heart rate was derived. Mechanical performance was assessed as the mathematical product of DLVP and heart rate (rate-pressure product, RPP) (Randall, 1995). After 30min equilibration cardiac preconditioning (pc) was induced by 3 cycles of either 4 or 6min ischaemia with 6 or 4min reperfusion, respectively, prior to the 30min ischaemic insult, which was followed by 60min reperfusion (15 ml min^{-1}). The non-preconditioned (non-pc) hearts were continuously perfused prior to the 30min ischaemia. Some hearts received 3mM DCA.

Addition of DCA significantly ($P<0.01$, Student's t-test) enhanced mechanical performance in that RPP=29,382± 1,625mmHg min^{-1} (mean±s.e.mean, n=21) in the presence of DCA and 22,074± 1,007mmHg min^{-1} (n=21) in its absence. DCA had no effect on coronary perfusion pressure (105±10 v. 97.1±9.3mmHg). Both the 3x4 and the 3x6min pc protocols induced significant ($P<0.05$) protection in hearts not treated with DCA in that the recoveries of RPP after 60min ($RPP_{t=60}$) of reperfusion were 8,746±1,423mmHg min^{-1} (3x4min, n=8) and 11,123±587 mmHg min^{-1} (3x6min, n=5) compared with $RPP_{t=60}$=4,041±883mmHg min^{-1} in non-pc hearts (n=8). In 6 hearts treated with DCA the 3x4min pc protocol did not provide protection ($RPP_{t=60}$=2,292±1,060mmHg min^{-1}) compared with non-pc hearts receiving DCA ($RPP_{t=60}$=3,640±1,235mmHg min^{-1}, n=6). However, in the DCA group subjected to the 3x6min protocol there was significant ($P<0.05$) cardioprotection ($RPP_{t=60}$=8,032±1,367mmHg min^{-1}, n=6).

The results of this study clearly indicate that although DCA may enhance cardiac mechanical performance, the presence of this drug increased the threshold for cardiac preconditioning in that the stimulus had to be increased by 50% to induce the same level of protection as that seen in its absence. These findings may suggest that by enhancing oxidative metabolism of pyruvate, preconditioning may be more difficult to achieve.

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102P CALCIUM-ACTIVATED AND ATP-DEPENDENT POTASSIUM CHANNELS IN HUMAN PLATELETS

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Potassium (K^+) channels are found in the membranes of virtually every human cell, and regulate a wide range of cellular functions, including electrical excitability and hormone secretion (Fine et al., 1989). Electrophysiological studies have shown the presence of calcium-activated K^+ channels in human platelets (Mahaut-Smith, 1995). Our aim here was to characterize K^+ channels in the platelets of healthy volunteers using pharmacological techniques.

We preloaded platelets with $^{86}Rb^+$ (as a tracer for K^+; Andersson & Vinge, 1991), immobilized them on an inert filter, and measured $^{86}Rb^+$ efflux using a superfusion technique (Carver et al., 1993) in the following medium (mM): NaCl (119), KCl (4.6), $CaCl_2$ (1.5), NaH_2PO_4 (1.2), $NaHCO_3$ (15), glucose (11), pH 7.4. The platelets were allowed to stabilize for 20 min at the start of each experiment and the cells were then stimulated with thrombin, superfused for 5 min.

Thrombin (0.1–0.6 IU/ml) increased $^{86}Rb^+$ efflux (Figure 1) in a concentration-dependent manner. This effect was significantly inhibited by apamin (100 nM), charybdotoxin (300 nM), and glibenclamide (20 μM) (Table 1), which on their own had no effect (not shown).

Thus, $^{86}Rb^+$ efflux from human platelets was stimulated by thrombin; the thrombin-stimulated efflux was inhibited by apamin and charybdotoxin (inhibitors of distinct calcium-activated K^+ channels) and by glibenclamide (an inhibitor of ATP-dependent K^+ channels) (Robertson & Steinberg, 1990).

These results provide evidence that thrombin-stimulated $^{86}Rb^+$ efflux from human platelets occurs via two types of calcium-activated K^+ channels (apamin-sensitive and charybdotoxin-sensitive) and via a glibenclamide-sensitive K^+ channel, which may be an ATP-dependent channel.

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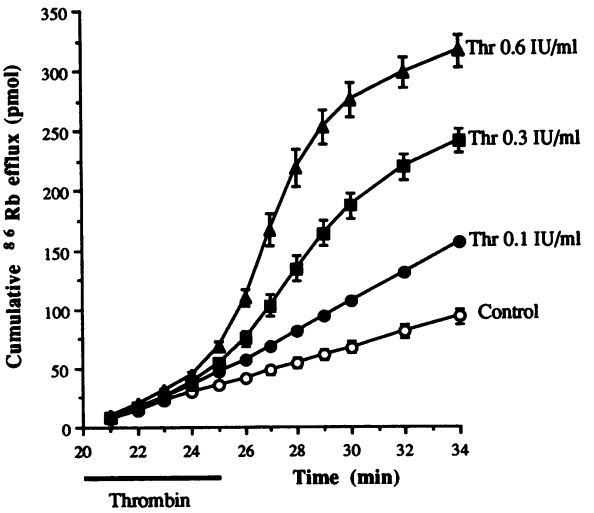

Figure 1 Cumulative ^{86}Rb efflux stimulated by thrombin (n=6; mean, s.e.mean)

Table 1 Cumulative ^{86}Rb efflux at 34 min (pmol)

	Control	Thrombin (0.3 IU/ml)	Thrombin (0.3 IU/ml) + Apamin (100 nM)	Thrombin (0.3 IU/ml) + Charybdo-toxin (300 nM)	Thrombin (0.3 IU/ml) + Gliben-clamide (20 μM)
^{86}Rb efflux	94	242	157*	196*	181*
s.e.mean	6	10	13	14	9
n	8	8	6	7	8

* Significantly different from thrombin 0.3 IU/ml ($P<0.0001$; ANOVA with repeated measures)

103P PHARMACOLOGICAL CHARACTERISATION OF $^{86}Rb^+$ FLUXES IN CHO CELLS TRANSFECTED WITH THE MOUSE BRAIN VOLTAGE-ACTIVATED K^+ CHANNEL GENE, $MK_v1.1$ (MK1)

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Voltage-activated K^+ channels play a vital role in modulating excitability in nerve and muscle cells and therefore the development of drugs which specifically target K^+ channel subtypes is thought to be an important therapeutic goal. Indeed the inherited disorders, episodic ataxia (Brown et al, 1994) and hereditary long QT syndrome (Curran et al, 1995), are associated with mutations in specific K^+ channel genes.

We have developed a high throughput $^{86}Rb^+$ flux screening procedure for detecting blockers of K_v channels (De-Allie et al, 1996). As a test system, we have used CHO cells transfected with the mouse brain voltage-activated K^+ channel gene MK-1 (Tempel et al, 1988), referred to as mKv1.1 after Chandy and Gutman (1993). These cells have previously been shown to respond to a variety of K^+ channel blockers by using patch clamp techniques (Robertson and Owen, 1993).

K^+ channel activity in mKv1.1-transfected CHO cells was studied by determining $^{86}Rb^+$ uptake in depolarized cells. Uptake into transfected cells was dependent on K^+ (external) with maximum uptake in 150mM K^+ medium. Although uptake was always a saturable process that could be fitted by a simple Langmuir isotherm, the absolute level of $^{86}Rb^+$ influx increased 354 to 860nmol $^{86}Rb^+$ mg protein^{-1}) with increasing passage number (from 8 to 20). A basal, linear flux component was always observed in non-depolarized cells (5mM external K^+). A small, saturable uptake was detected in cells transfected with the host plasmid alone, which was approximately 20% of that seen in cells transfected with mKv1.1. Approximately 25% of the $^{86}Rb^+$ uptake in depolarized CHO cells (both cells transfected with mKv1.1 and with host plasmid alone) was inhibited by ouabain, and is therefore probably associated with an active $K^+/Na^+/Cl^-$ transport process, known to be present in these cells. $^{86}Rb^+$ uptake was approximately linear for the first 30s and therefore the effect of K^+ channel blockers on $^{86}Rb^+$ uptake was determined after 30s. The ouabain-insensitive component of $^{86}Rb^+$ flux was saturably inhibited by many drugs and toxins which block voltage-activated K^+ channels, eg. TEA (IC_{50}= 0.4mM), 4-AP (IC_{50}= 5.3mM) and Toxin I (IC_{50}= 44nM). $^{86}Rb^+$ flux was comparatively insensitive to charybdotoxin (30% block at 10mM) and was not blocked by apamin (up to 50mM), a blocker of small conductance Ca^{2+}-activated channels.

These results show that this $^{86}Rb^+$ flux assay can be used for the rapid pharmacological screening of potential Kv1.1 channel blockers. By altering the particular channel introduced into CHO cells, the assay should have wide applicability to screening for specific blockers of other voltage-activated K^+ channel subtypes.

We wish to thank Dr Bruce Tempel for providing the mKv1.1 cell-line.

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104P THE BINDING OF KALIOTOXIN AND MARGATOXIN VARIANTS TO VOLTAGE-GATED POTASSIUM CHANNELS IN RAT BRAIN

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We have previously reported that the specific binding of [^{125}I]α-dendrotoxin (α-DTX) to rat brain labels the delayed rectifier class of voltage-gated potassium channels (Pryke *et al.*, 1995). In this study, we have displaced [^{125}I]α-DTX binding using modified toxins from scorpion venoms to identify the important amino acids for binding to voltage-gated potassium channels.

Kaliotoxin (KTX, 37 amino acids), 10 KTX variants with a single-amino-acid substitution, margatoxin (MgTX, 39 amino acids) and 1 MgTX variant were prepared by solid-phase peptide synthesis and used to displace 0.2nM [^{125}I]α-DTX binding to rat brain homogenates (Pryke *et al.*, 1995). Relative potencies (IC_{50}) are shown in Table1.

All amino acid substitutions resulted in a reduction in potency and, in particular, substitution of the lysine at position 27 in KTX for an asparagine resulted in the loss of binding activity. This observation is consistent with the lysine^{27} side chain projecting down into the channel pore as postulated for the block of calcium-activated potassium channels by charybdotoxin (e.g. Park & Miller, 1992). In addition, substitution of the tyrosine at position 37 in MgTX for threonine caused a 300-fold reduction in potency. Thus, maintaining a hydroxyl function in this position is not sufficient to retain potency. This is again consistent with Park & Miller (1992), where substitution with phenylalanine was tolerated. In conclusion, we have identified key amino acids for the binding of scorpion toxins to the α-DTX binding site on voltage-gated potassium channels.

Table 1. Binding potencies (IC_{50}) against [^{125}I]α-DTX binding to rat brain homogenate (mean ± s.e.mean, n=3 except *n=5).

	Original amino acid	Position	Modified amino acid	IC_{50} (nM)
KTX	*Wild type*			0.1 ± 0.04
KTX	Ser	11	Ala	1.1 ± 0.5
KTX	Ser	11	Phe	12.7 ± 3.9
KTX	Lys	27	Ala	1892 ± 112
KTX	Lys	27	Asn	> 1000
KTX	Met	29	Ala	4.09 ± 0.77
KTX	Met	29	Phe	11.5 ± 0.75
KTX	Met	29	Thr	2.98 ± 0.12
KTX	Asn	30	Gly	3.78 ± 1.09
KTX	Thr	36	Val	6.39 ± 2.7
KTX	Thr	36	Tyr	22.2 ± 0.87
MgTX	*Wild type*			0.08 ± 0.02*
MgTX	Tyr	37	Thr	21.3 ± 6.0

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105P COMPARATIVE EFFECTS OF A NON-SEDATING HISTAMINE H_1-RECEPTOR ANTAGONIST ON HUMAN Kv1.5 CHANNELS

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Recent evidence indicated that the use of terfenadine, a long-acting nonsedating histamine H_1-receptor antagonist, can be associated with prolongation of the *QTc* interval and development of polymorphic ventricular arrhythmias (*torsades de pointes*). Terfenadine is rapidly and extensively converted via cytochrome P450 CYP3A4 to its acid metabolite terfenadine carboxylate. Terfenadine-induced *QTc* prolongation has been related to the blockade of hKv1.5 channels (Yang et al., 1995). Loratadine also produces a voltage (δ = 0.18 ± 0.005, n = 6) and time-dependent inhibition of hKv1.5 current (K_D = 1.2 ± 0.1 μM) giving a value of 76.8 ± 1.8 % inhibition at 10 mV with 3 μM concentration (Delpón et al., 1995).

With a view to investigating whether or not ebastine, a newer long acting nonsedating histamine H_1 antagonist, could be a safer alternative, we have studied the effects of terfenadine and terfenadine carboxylate, ebastine and its main metabolite, carebastine, on a cloned human cardiac K^+ delayed rectifier channel (hKv1.5) stably expressed in a *Ltk*$^-$ cell line using the whole-cell configuration of the patch-clamp technique.

Terfenadine, 1 and 3 μM, induced 42 ± 6% (n = 5, P < 0.01) and 69 ± 4% (n = 5, P < 0.01) inhibition of hKv1.5 current measured at the end of 500 ms depolarizing pulses from -80 mV to +60 mV. Block induced by terfenadine was voltage-dependent and it was described by an electrical distance (δ) of 0.18 ± 0.02 (n = 5). Terfenadine-induced block was also time-dependent, so that it did not modify the activation time course, but induced a fast decline of the current during depolarizing steps to +60 mV (τ = 32 ± 4 ms. n = 5). Terfenadine carboxylate, 3 μM, did not modify the amplitude of the hKv1.5 current (0.03 ± 0.01%. n = 4, P > 0.05).

In contrast to the high potency of terfenadine and loratadine to inhibit hKv1.5 current, ebastine (1 and 3 μM) decreased the amplitude of hKv1.5 current by only 8 ± 2% (n = 6, P < 0.05) and 13 ± 2% (n = 5, P < 0.05), respectively. Moreover, ebastine did not modify the kinetics of the current at either concentration. Carebastine did not modify the magnitude or the kinetics of the hKv1.5 current. Thus, the inhibition produced by 3 μM carebastine averaged a mean value of 5 ± 1% (n = 5, P > 0.05).

These results confirmed those previously reported for terfenadine and terfenadine carboxylate (Rampe *et al.*, 1993; Yang *et al.*, 1995) and suggested that because of their lower potency to inhibit hKv1.5 channels, both ebastine and carebastine may represent safer alternatives in clinical practice.

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106P NITRIC OXIDE ACTIVATES LARGE CONDUCTANCE POTASSIUM CHANNELS IN RAT CEREBRAL ARTERY SMOOTH MUSCLE CELLS

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The relaxation induced in smooth muscle by the endogenous vasodilator molecule nitric oxide (NO) has been attributed in certain blood vessels and species to the opening of K^+ channels. The type of K^+ channel activated by NO appears to differ between blood vessels. Robertson et al. (1993) and Archer et al. (1994) both identified the large conductance calcium-activated K^+ (BK_{Ca}) channel as the target for NO in cerebral and pulmonary artery preparations respectively. More recently, Murphy and Brayden (1995) identified the ATP-dependent K^+ (K_{ATP}) channel as the target for NO-induced hyperpolarisation in a mesenteric preparation.

In this study we have attempted to identify the K^+ channels activated by NO in single cells isolated from the rat basilar artery. Unlike the majority of other patch clamp studies of this type we have recorded steady state whole cell currents at membrane potentials approximating to the physiological resting potential of these cells. NO was applied in the form of the NO donor compound SIN-1A. Smooth muscle cells were enzymatically isolated from rat basilar artery and whole cell currents recorded using the conventional configuration of the patch clamp technique. Voltage ramps were run to activate whole cell currents using appropriate compensation for capacitance and series resistance. All experiments were conducted at room temperature.

Approximately 50% of cells held at -60 or -40 mV in symmetrical 140mM K^+ responded to the application of SIN-1A (50-100uM). In cells that responded an inward current developed within 7 minutes of beginning SIN-1A superfusion(-700 +/- 450 pA n=7). The SIN-1A induced current was identified as being due to the activation of a K^+ conductance on the basis of whole cell ramp data, which demonstrated that the reversal potential of the current activated by NO was dependent on the $[K^+]_o$. Once activated, currents persisted following washout of SIN-1A showing no reduction even after washing for 6 minutes. The current was unaffected by 5uM glibenclamide and 100 nM apamin but was rapidly inhibited by 100 nM iberiotoxin (IbTX), a selective inhibitor of BK_{Ca} channels.

The currents activated by NO were significantly increased above control levels at all potentials($p<0.05$ Students paired t-test). For example currents were increased from -28 ± 14 pA to -87.5 ± 20 pA at -50 mV (n=3) and from 80 ± 20 pA to 2850 ± 964 pA at +50 mV (n=3) and significantly reduced following the addition of 100 nM IbTX. This NO-induced current increased as the membrane potential was driven in a depolarising direction with the result that at +50 mV current levels were increased 35 fold with respect to control values, as opposed to a 3 fold increase at -50 mV. The conductance of the channel activated by NO was estimated to be 255 ± 20 pS (n=7) in symmetrical 140 mM K^+, a value characteristic of BK_{Ca} in smooth muscle.

This study provides further evidence that NO modulates K^+ channels in arterial smooth muscle cells. We have identified the channel activated by NO as BK_{Ca} which agrees with some recent reports. This activation occurred in approximately 50% of cells studied and we found no evidence for the activation of K_{ATP} or an apamin-sensitive K^+ channel by NO in cells from the basilar artery.

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107P RUBIDIUM ANTAGONISES THE RELAXANT EFFECT OF THE POTASSIUM CHANNEL OPENER SDZ PCO400 IN THE MOUSE ILEUM

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Yeung *et al.* (1995) have demonstrated in the mouse ileum that increasing concentrations of rubidium (Rb) caused progressive reduction in relaxant potency of pinacidil (PIN). PIN is reported to have potassium channel opening and other actions (Steinberg *et al.*, 1988). In the present study, a more selective potassium channel opener (KCO), SDZ PCO400 (SDZ) is used to compare the influence of Rb on actions of this KCO and to investigate antagonism by the potassium (K) channel blocker, glibenclamide (GBC), in the presence of Rb.

Mucosa-free preparations from the distal ileum of male BKW mice (28-40g) were placed under 0.5g tension (37℃, 95% O_2/5% CO_2) in normal Krebs solution (KS) containing 5.95mM K (Rb=0) or in isosmotic KS in which K was replaced by increasing concentrations of Rb (1.19-4.75mM). Isometric contractions were elicited by electrical field stimulation (0.2Hz, 30V, 1ms pulse width). When twitch height was constant, a cumulative concentration-response curve to SDZ (0.1-200μM) or vehicle was performed. This procedure was repeated following 20min incubation with a single concentration of GBC (0.1-1μM) or vehicle. Relaxant potency was expressed as geometric mean EC_{50} (the concentration required to reduce twitch height by 50%) with 95% confidence limits. The initial twitch response was expressed as tension (g) ± s.e.mean and the effect of GBC was expressed in terms of SDZ dose ratio ± s.e.mean. Statistical analysis was performed using Student's unpaired t-test.

The initial twitch heights were not affected by exposure of preparations to KS containing increasing concentrations of Rb, with values of 0.63±0.04g (normal KS, n=12) and 0.59±0.03g (4.75mM Rb, n=12). The relaxant potency of SDZ was progressively attenuated as the concentration of Rb increased (Table 1) with 160-fold reduction at 4.75mM Rb. GBC antagonised in a concentration-dependent manner the relaxant effect of SDZ. The antagonism was attenuated by increasing concentrations of Rb: no antagonism was apparent at 4.75mM Rb (Table 1). No vehicle effect was observed (n≥4).

Table 1 Effect of increasing concentrations of Rb on responses to SDZ alone (n=24 in all cases) and in the presence of GBC (n=4 in all cases) in the mouse ileum.

Rb (mM)	SDZ EC_{50} (μM) (mean, 95%CL)	SDZ+GBC (μM) (Dose Ratio ± s.e.mean) 0.1 GBC	0.3 GBC	1.0 GBC
0	0.5 (0.5-0.7)	2.8±0.4	4.4±0.4	28.0±2.2
1.19	1.2 (1.0-1.6)***	2.3±0.3	5.2±0.6	18.8±1.5*
2.38	1.9 (1.5-2.5)***	4.7±1.3	8.0±0.8**	20.2±2.9
3.56	34.4 (26.8-49.5)***	2.5±0.7	3.0±0.6	2.8±0.5***
4.75	79.5 (70.2-91.7)***	1.2±0.1*	1.1±0.1***	1.1±0.1***

*P<0.05, **P<0.01, ***P<0.001 (0 *vs* 1.19-4.75mM Rb)

The reduction in both relaxant potency of SDZ and its antagonism by GBC in increasing concentrations of Rb is consistent with the suggestion that Rb blocks K channels. These reductions were much greater for SDZ than previously reported for PIN (Yeung *et al.*, 1995). This may be associated with the selectivity of SDZ for K_{ATP} channels, indicated by the greater antagonism of SDZ by GBC: PIN has been reported to have additional sites of action (Steinberg *et al.*, 1988). Thus differences in relaxant potency of KCOs in KS containing Rb could be indicative of their selectivity for K channels.

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108P DOES HALOTHANE AFFECT FENTANYL INHIBITION OF ADENYLYL CYCLASE IN SH-SY5Y CELLS

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Opioids and volatile anaesthetic agents are well known to interact in the production of the anaesthetised state in humans and animals, but the site of this interaction is undetermined. We have previously shown that there is no interaction between halothane and opioids at either the level of the opioid receptor (Campbell *et al* 1995a) or at opioid receptor-G-protein coupling (Campbell *et al* 1995b). In this study we sought to determine whether halothane affects fentanyl induced inhibition of forskolin stimulated cAMP formation in SH-SY5Y cells.

Undifferentiated SH-SY5Y cells were cultured in minimum essential medium. Whole cell suspensions in Krebs/HEPES buffer (pH 7.4) were incubated (total assay volume 300 μl) with IBMX (1mM), forskolin (10 μM) and fentanyl (up to 10 μM). Cells were incubated for 20 min at 37°C, in the presence of either humidified air or humidified air containing halothane (2.0 %), following a 15 min pre-incubation to allow equilibration of halothane between gaseous and aqueous phases. The reaction was terminated by the addition of 20 μl HCl (10 M). The cAMP concentration of the supernatant was determined by a specific radioreceptor mass assay (Hirst & Lambert, 1995). Halothane was delivered by a calibrated Fluotec 3 vaporiser, and the concentration checked regularly with an anaesthetic agent monitor (Capnomac). Buffer concentrations were measured by gas chromatography, using a modification of the technique of Rutledge *et al.* (1963). A vaporiser dial setting of 2.0 % resulted in an aqueous halothane concentration of 660 ± 40 μM. Results are mean ± s.e.mean (n = 9 or 10).

Basal and forskolin stimulated cAMP levels were 65 ± 7 and 1260 ± 100 pmol/mg protein, respectively, and represented a 19-fold increase over basal. Halothane had no significant effect on these data; the corresponding results in the presence of halothane 2.0 % were 68 ± 5 and 1310 ± 150 pmol/mg protein (also a 19-fold increase).

Fentanyl dose dependently inhibited forskolin stimulated cAMP formation; maximal inhibition with 10 μM fentanyl was 20 %. There was no significant difference in cAMP formation at any fentanyl concentration between the cells incubated in the presence of halothane 2.0 % and those incubated in air.

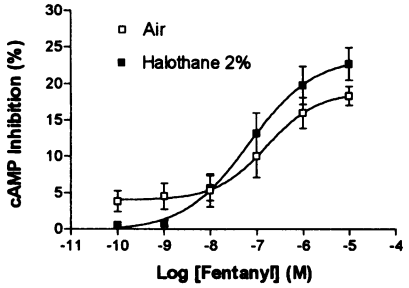

Figure 1. Effect of halothane 2% on the dose related inhibition of forskolin stimulated cAMP formation.

These data suggest that at least in this cell system, halothane does not interact with the μ opioid induced inhibition of cAMP formation. A cellular basis for the interaction of opioids and volatile anaesthetics is not yet excluded, as such an interaction may occur at the level of phospholipase C or at calcium channels.

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109P MODULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE BY ANTI-OXIDANTS IN THE MACROPHAGE CELL LINE J774

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The induction of nitric oxide synthase (iNOS) in smooth muscle cells and macrophages by cytokines present in atherosclerotic lesions suggests a potential role for NO. It has been shown (Verbeuren *et al.*, 1993) that iNOS is induced in aortae from cholesterol fed rabbits. In addition to their critical role in the modification of lipoproteins, reactive oxygen species are also of interest in atherosclerosis as modulators of an nuclear factor κB-like (NFκB) transcription factor (Schreck *et al.*, 1991). NFκB controls the expression of a number of genes involved in atherosclerosis (Collins, 1993) including iNOS (Lowenstein *et al.*, 1993). The beneficial effects of anti-oxidants in atherosclerosis could be the result, at least in part, of the inhibition of NFκB. To this end we have investigated how the production of NO from the murine macrophage cell line J774 stimulated with lipopolysaccharide (LPS), is affected by different anti-oxidants.

J774 cells were grown to confluence in DMEM with 10% foetal calf serum. Cells were incubated for 18h with 10μg/ml LPS in the presence of the anti-oxidants: trolox C; butylated hydroxy toluene (BHT); vitamin C; pyrrolidine dithiocarbamate (PDTC) or N-acetyl cysteine (NAC). Nitrite accumulation was measured using the Griess reaction, before cells were harvested and lysed; cell viability was assessed using the MTT assay; NOS activity in lysate preparations was determined by measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline (Yang *et al.*, 1994).

Incubation of J774 cells with 10μg/ml LPS resulted in 23.2±2.7μM nitrite (mean ± s.e.mean). Nitrite from non-induced cells was below levels of detection (n=16). Vitamin C and NAC had no effect on LPS-induced nitrite production (n=3). BHT, Trolox C and PDTC produced a dose-dependent decrease in nitrite production; IC_{50}'s were 80μM, 80μM and 7.9μM respectively (n=3). Anti-oxidants had no effect on cell viability at the concentrations used. Lysate prepared from LPS-induced J774 cells produced 109±19 pmole/mg protein citrulline compared to 3.9±0.25 pmol/mg protein from non-induced cells (n=6). Incubation of lysate with the NOS inhibitor L-NAME (10μM) resulted in complete inhibition of citrulline production (n=6). Removal of calcium from the incubation medium had no effect on activity (n=6). Levels of iNOS activity in LPS-induced cells, as measured by the citrulline assay, were unaffected by coincubation with BHT, Trolox C or vitamin C (n=3). NAC (1mM) and PDTC (32μM) abolished iNOS activity.

Our results demonstrate that the inhibition of NO production in J774 cells can be facilitated, not only by blocking enzyme induction, but also by destruction of NO. It is therefore likely that a general reduction in cellular oxidative potential is not sufficient to block iNOS activation. Both thiol containing anti-oxidants were able to abolish induction suggesting that by reducing thiol depletion these reagents may inhibit iNOS activation possibly, in part, by blocking NFκB-like transcriptional activation.

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110P REACTIVE OXYGEN SPECIES IN TNFα-MEDIATED EXPRESSION OF ADHESION MOLECULES IN EA.hy 926 CELLS

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An early event in atherosclerosis is a change in the functional integrity of the endothelium resulting in increased adherence of leukocytes (Faruqi *et al.*, 1993). Factors found in atherogenic lesions, such as the TNFα and IL-1 are able to induce the synthesis of reactive oxygen species (ROS). Concurrently, TNFα and IL-1 can induce the expression of adhesion molecules VCAM-1, ICAM-1 & E-Selectin on the endothelial cell surface. Transcriptional activation leading to increased monocyte adhesion may involve redox signalling pathways. We have therefore investigated the regulation of TNFα-induced expression of cell adhesion molecules in the human endothelial cell line EA.hy 926 (ECs) using inhibitors of various redox signals. We measured monocyte binding to ECs, using a myeloperoxidase based 96 well plate assay, and cell adhesion molecule expression with an ELISA.

Expression of ICAM-1 by TNFα was increased 10-14 fold after 4h (n=3) and remained constant for 24h. VCAM-1 expression increased 10-12 fold after 24h (n=3). E-Selectin expression increased 10% over basal after 2-4h (n=3) and then gradually decreased back to basal levels in the following 24h. Monocyte adhesion also increased after 6h ($1.71x10^5±5.74x10^3$ cells/well) and 24h ($1.42x10^6±4.93x10^3$ cells/well) incubation with TNFα (10ng/ml). Pyrrolidine dithiocarbamate (PDTC), a putative NFkB inhibitor, (Mauri *et al.*, 1993) acetovanillone (NADPH Oxidase inhibitor) and rotenone (electron transport chain inhibitor) inhibited VCAM-1 expression, after 6 and 24h. None of the inhibitors in Table 1 had any effect on ICAM-1 expression. Aspirin (1mM) and allopurinol (500μM) had no effect on ICAM-1 and VCAM-1 expression suggesting that ROS generation via xanthine oxidase and cyclooxygenase is not involved. The inhibitors used had no effect on cell viability, as measured by MTT assay.

Inhibitor	Time (h)	%Inhibition of VCAM (±sem)
PDTC (100μM)	6	88.6±7.2
	24	54.6±6.0
Rotenone (10μM)	6	61.6±0.7
	24	98.4±0.4
Acetovanillone	6	62.9±7.4
(100μg/ml)	24	83.8±3.0

Table 1. Effect of ROS inhibition on VCAM-1 expression.

The above data indicate that leakage of $O_2^{\cdot-}$ from the electron transport chain, and $O_2^{\cdot-}$ synthesis from an NADPH Oxidase-like activity, are possible sources of signalling ROS in VCAM-1 expression. TNFα-mediated expression of ICAM-1 and VCAM-1 is differentially regulated in ECs. Transcriptional regulation via a redox sensitive mechanism appear to play a role in VCAM-1, but not ICAM-1 expression. The nature of this radical signalling mechanism is not known.

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111P FORMATION OF 7-HYDROXYMETHOTREXATE FROM METHOTREXATE WITH HUMAN AND GUINEA-PIG ALDEHYDE OXIDASE

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7-Hydroxymethotrexate (7-OHMTX) is the major metabolite of methotrexate (MTX) after high-dose therapy (Ertmann *et al.*, 1985). Although the oxidation of MTX to 7-OHMTX is rapidly catalysed by rabbit liver aldehyde oxidase (AO) (Fabre *et al.*, 1986), human liver is reported to have negligible MTX-oxidising activity (Johns, 1967). In this study, incubations of MTX with human and guinea pig liver AO have been analysed by HPLC and the interaction of AO inhibitors with 7-OHMTX formation has been investigated.

(+) MTX was incubated with partially purified human and guinea pig liver AO in 0.067 M phosphate buffer pH 7.0 at 37°C as described previously (Beedham *et al.*, 1995). HPLC analysis of the clarified supernatant at 305 nm from human and guinea pig incubations indicated the formation of a single metabolite which was identical to that found with rabbit AO.

Table 1 shows 7-OHMTX concentrations in MTX (300 μM or 50 μM) incubations after 240 or 180 min with human and guinea pig AO respectively. With both enzymes, oxidation of MTX to 7-OHMTX was significantly reduced by menadione and chlorpromazine, potent AO inhibitors. Allopurinol, a selective inhibitor of xanthine oxidase had little effect on 7-OHMTX formation. Neither famciclovir or 6-deoxypenciclovir, substrates of human liver AO, or their metabolite, penciclovir, caused significant reduction of 7-OHMTX production.

Table 1. Inhibition of 7-OHMTX formation from (+) MTX catalysed by human and guinea pig liver AO

Inhibitor (100μM)	7-OHMTX Concentration (μM) Human Liver AO (after 240 min)	Guinea Pig AO (after 180 min)
Control	0.24 ± 0.04	1.87 ± 0.34
Menadione	0	0.30 ± 0.08*
Chlorpromazine	0	0
Allopurinol	0.25 ± 0.07	1.98 ± 0.27
Famciclovir	0.15 ± 0.08	1.84 ± 0.26
6-Deoxypenciclovir	0.17 ± 0.06	1.79 ± 0.27
Penciclovir	ND	1.86 ± 0.24

Data are presented as mean ± s.e.mean (n = 4)
ND not determined * $p < 0.01$ using an unpaired Student's t-test

In conclusion these results show that, *in vitro*, human and guinea pig liver AO catalyse the oxidation of (+) MTX to 7-OHMTX and thus it is likely that this enzyme is responsible for 7-OHMTX production during MTX therapy.

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112P STUDIES ON THE CHEMOSENSITIVITY OF HUMAN CULTURED MCF-7, MCF-7 ADR AND MT-1 BREAST TUMOURS AND K562 LEUKAEMIA CELL LINES TO NOVEL STEROIDAL ANTITUMOUR PRODRUGS

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The specificity with which oestrogens bind to nuclear oestrogen receptors (ER) may be exploited by synthesising prodrugs which incorporate both oestrogenic and anticancer moieties. Doxorubicin (DOX) an anthracycline antibiotic drug used in the chemotherapy of advanced breast cancer is effective but relatively nonselective against hormone-dependent tumours, hence could be targeted to breast tumours if incorporated within oestrogenic prodrugs. In these studies three series of steroidal prodrugs were synthesised. In the first series, hydrocarbon spacer groups (6C and 12C) were covalently linked to oestrone (E) at the 3-position. In the second series, similar spacer groups (2C and 12C) were linked to E at the 17-position. In the third series, carboxylic acid derivatives of E (first and second series) were amide-linked to DOX via daunosamine amino group.

Prodrugs were biologically evaluated against cultured human cell lines including ER positive (>10 fmol receptor/mg cytosolic protein, Wittliff, 1984) MCF-7, ER negative MT-1 and MCF-7ADR breast tumour, and K562 leukaemia cells in RPMI 1640 medium. Percentage cell survival was determined after 96 h exposure, using drugs in the range 0.001 μM to 100 μM, via the colorimetric MTT assay (Jabbar *et al.*, 1989) using a Labsystems Multiskan Plus ELISA reader, set at 550 nm.

The IC_{50} values of tested compounds are shown in Table 1. CCRL 1026 (17-carboxymethyl oxime of E), CCRL 1020 and 1022 (3-ester of E with C6 and C12 side chains respectively), all aliphatic carboxylic acid derivatives, were inactive against all cell lines tested. CCRL 1023 (DOX-ester with C12 side chain in amide linkage to daunosamine amino group) was unselective, and was less cytotoxic than DOX against all cell lines. CCRL 1035 (DOX amide-linked to CCRL 1026) was inactive against all breast tumour cell lines. CCRL 1036 and 1024 (DOX amide-linked to CCRL 1020 and 1022, respectively) were relatively inactive and unselective against all cell lines. However CCRL 1033 (DOX amide-linked to 17-position of E via C12 spacer group) was selectively cytotoxic against MCF-7 (ER positive) breast tumour cells and was similar in potency to DOX.

Table 1: IC_{50} values (μM ± SD) for antitumour agents, doxorubicin, its ester analogue, oestrone derivatives and its novel prodrugs (n=6).

Compound	MCF-7	MT-1	MCF-7ADR	K562
DOX	0.4 ± 0.1	0.5 ± 0.2	8.0 ± 0.5	0.2 ± 0.1
CCRL 1020	> 100.0	> 100.0	> 100.0	> 100.0
CCRL 1022	> 100.0	> 100.0	> 100.0	40.0 ± 4.6
CCRL 1024	10.5 ± 5.2	10.5 ± 2.3	9.0 ± 4.0	3.0 ± 4.4
CCRL 1036	11.0 ± 0.1	12.0 ± 1.2	11.5 ± 2.6	11.0 ± 2.3
CCRL 1023	9.0 ± 2.4	5.0 ± 1.2	12.0 ± 4.3	2.8 ± 0.8
CCRL 1026	> 100.0	> 100.0	> 100.0	> 100.0
CCRL 1033	0.7 ± 0.3	10.4 ± 2.1	10.5 ± 5.1	10.5 ± 4.4
CCRL 1035	> 100.0	> 100.0	> 100.0	11.7 ± 4.1

These results support the hypothesis that steroidal antitumour prodrugs require side chains greater than 2C in length plus either a 3- or 17-OH group in the steroid moiety, for ER selectivity. Hence CCRL 1033 is a potential lead compound for the development of further novel oestrogenic/antioestrogenic antitumour prodrugs.

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113P FURTHER CHARACTERISATION OF THE MOUSE δ-OPIOID RECEPTOR IN NG108-15 CELLS

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The concept of heterogeneity of δ-opioid receptors has been demonstrated in *in vivo* experiments, but there is only limited evidence from biochemical studies (Traynor & Elliott, 1993). Heterogeneity of the δ-opioid receptor population may arise as a result of different protein entities, differential post-translation processing or different intracellular coupling systems. We have further characterised a mouse δ-opioid receptor endogenously expressed in NG108-15 cells using ligand binding assays together with [^{35}S]GTPγS binding assays as a measure of receptor-G protein coupling (Traynor & Nahorski, 1995).

NG108-15 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% foetal calf serum and HAT (hypoxanthine, 13.6mg.ml^{-1}; aminopterin, 0.18mg.ml^{-1}; thymidine, 3.9mg.ml^{-1}) at 37°C in a 5% CO_2 atmosphere. Cells were harvested in Hepes buffered saline, membranes prepared in Tris HCl buffer (50mM, pH7.4; for ligand binding assays) or Hepes buffer (20 mM, pH 7.4, containing 100mM NaCl and 10mM $MgCl_2$; for [^{35}S]GTPγS assays) and assays were performed as described earlier (Traynor & Nahorski, 1995).

The opioid antagonist [^{3}H]diprenorphine afforded a Bmax value of 559 ± 61 fmols. mg^{-1} protein similar to the δ-agonist [^{3}H]DPDPE (501 ± 94 fmols. mg^{-1} protein). Competition for [^{3}H]diprenorphine binding by the putative δ_1 antagonist BNTX (benzylidene naltrexone) and the putative δ_2 antagonist NTB (naltriben) afforded shallow displacement curves with Hill coefficients of 0.45 ± 0.02 and 0.51 ± 0.05 respectively (n = 3). These were resolved into high affinity (BNTX 90 ± 27 pM; NTB 1.2 ± 0.9 pM) and lower affinity (BNTX 9.8 ± 2.1 nM; NTB 0.47 ± 0.05 nM) components, each representing approximately 50% of the displacement curve.

DPDPE (EC_{50} 31 ± 5 nM, n = 3) stimulated [^{35}S]GTPγS binding in NG108-15 cell membranes to 78.0 ± 2.0 fmols. mg^{-1} protein from a basal value of 36.4 ± 1.4 fmols.mg^{-1} protein. Maximal stimulation of [^{35}S]GTPγS binding decreased in the order DSLET>DPDPE>deltorphin II>etorphine>levorphanol=diprenorphine>nalorphine. BNTX had a weak partial agonist effect, Naltriben had no effect and ICI 174864 acted as an inverse agonist, causing a decrease of 17.0 ± 9.3 % in basal [^{35}S]GTPγS binding with an EC_{50} of 34.3 ± 7.6nM (n = 3). The effect of DPDPE was antagonised by naloxone (Ke 25nM) and by both the δ_1 preferring BNTX and the δ_2 preferring NTB with affinities (Ke 1.5 ± 0.3nM and 71 ± 24pM respectively) approximately midway between their high and low affinities defined by binding assay.

The results demonstrate that [^{35}S]GTPγS binding is a useful measure of opioid efficacy at δ-receptors. The ligand-binding assays suggest some form of δ-receptor heterogeneity, whilst the [^{35}S]GTPγS data are suggestive of a single, possibly δ_2, opioid receptor population in NG108-15 cells.

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114P THE RELATIVE DISTRIBUTION OF Cu/Zn AND Mn SUPEROXIDE DISMUTASE IN ADULT RAT BRAIN

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Superoxide dismutase (SOD) exists in a Cu/Zn-dependent cytosolic form (Cu/ZnSOD) and a Mn-dependent mitochondrial form (MnSOD). In rodents, the repeated administration of antiparkinsonian drugs such as deprenyl and pergolide increase SOD activity in tissue homogenates (Carrillo et al., 1992; Clow et al., 1993), but the regional specificity of this effect has not been studied in tissue slices. Indeed, the distribution of SOD protein and mRNA in the rat brain is unknown. We now report on the distribution of Cu/Zn- and MnSOD mRNA in the adult rat brain.

Male Wistar rats (200-250g; n=6) were killed by terminal anaesthesia, the brains were removed and snap frozen at -40°C in isopentane and coronal sections (12μm) were cut at -20°C. The gene expression of both Cu/Zn- and MnSOD was determined using *in situ* hybridization histochemistry with Cu/Zn- and MnSOD oligodeoxynucleotide cDNA probes with the sequences 5'-CC-AGT-CTT-TGT-ACT-TTC-TTC-ATT-TCC-ACC-TTT-GCC-CAA-GTC-ATC-3' and 5'-TGA-TCT-GCG-CGT-TAA-TGT-GCG-GCT-CCA-GCG-CGC-CAT-AGT-C-3' respectively.

The distribution was assessed by autoradiography and image analysis. Results were analyzed using paired Student's t-test for significance.

There was a wide distribution of mRNA for both Cu/Zn- and MnSOD in all areas of basal ganglia (Table 1). In addition, high levels of gene expression were detected in other areas, namely the hippocampus, dentate gyrus and both the parietal and piriform cortex. In all cases, there was a significantly higher expression of Cu/ZnSOD mRNA compared to MnSOD mRNA ($p<0.05$ using paired Student's t-test).

The results show a wide distribution of both Cu/Zn- and MnSOD in the rat brain. The ability of deprenyl and pergolide to elevate the activity of SOD may be important to their proposed neuroprotective effects, particularly as SOD levels are also increased in Parkinson's disease (Saggu et al., 1989).

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Table 1. The relative distribution of Cu/Zn- and MnSOD mRNA (pCi/mg) in the basal ganglia of adult rat brain. Mean ±SEM for n=6 for all areas.

	Nucleus Accumbens	Striatum	Globus Pallidus	Substantia Nigra pars compacta	Substantia Nigra pars reticulata
Mean Cu/ZnSOD	62.8±2.3	60.6±1.9	59.4±1.9	72.4±5.8	54.4±1.3
Mean MnSOD	16.4±0.9	16.0±0.7	18.9±2.3	24.3±2.3	18.3±1.4

$p<0.05$ in all cases, comparing levels of Cu/ZnSOD mRNA to MnSOD; paired Student's t-test.

115P EFFECTS OF PHOSPHODIESTERASE INHIBITORS ON HUMAN LUNG MAST CELL AND BASOPHIL FUNCTION

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Recent studies suggest that cyclic nucleotide phosphodiesterase (PDE) exists as multiple molecular forms (Beavo *et al.*, 1990; 1991; Barnes 1995). In the present study we have attempted to establish the role of PDE isozymes in the regulation of human basophil and human lung mast cell (HLMC) function by examining the effects of non-selective and isozyme-selective inhibitors of PDE on the anti-IgE-induced release of histamine and the generation of both sulphopeptidoleukotrienes (sLT) and prostaglandin D_2 (PGD_2).

The non-selective PDE inhibitors theophylline and 3-isobutyl-1-methylxanthine (IBMX) inhibited anti-IgE-induced histamine release from both basophils (n=5) and HLMC (n=6) in a dose-dependent fashion. IBMX was more potent (IC_{50}s; 0.05mM, basophils; 0.85mM, HLMC) than theophylline (IC_{50}s; 0.2mM, basophils; 1.7mM in HLMC) as an inhibitor of anti-IgE-mediated histamine release.The PDE IV inhibitor rolipram (10μM) attenuated the release of both histamine (40±8% inhibition, mean±s.e.m.; $p<0.05$) and the generation of sLT (44±16% inhibition; $p<0.05$) from basophils (n=4) but was ineffective at inhibiting histamine release, sLT generation and PGD_2 generation in HLMC (n=4). Alternative PDE IV inhibitors, nitraquazone (10μM) and RP 73401 (10μM), were found to be effective inhibitors of histamine release (44±7% and 29±5% inhibition, respectively; $p<0.05$) and sLT generation (54±19% and 35±13% inhibition, respectively; $p<0.05$) in basophils, but less effective ($p>0.05$) at inhibiting histamine release and the generation of both sLT and PGD_2 from HLMC (n=4). Alternative isozyme-selective inhibitors (10μM) including 8-methoxymethyl-IBMX (PDE I inhibitor), siguazodan (PDE III inhibitor), and zaprinast (PDE V inhibitor) did not inhibit mediator release to a significant ($p>0.05$) extent from either basophils or HLMC. In basophils, a combination of rolipram (PDE IV inhibitor) and SK&F 95654 (PDE III inhibitor) was more effective at inhibiting histamine release than when the two drugs were used individually (n=4). Rolipram (10μM) inhibited histamine release from basophils by 29±5%, SK&F 95654 (10μM) by 0±2% and a combination of both drugs inhibited histamine release by 46±6%. In HLMC, a combination of rolipram and SK&F 95654 was ineffective (n=5). In basophils (n=4), rolipram (10μM) acted to potentiate the inhibitory effects on histamine release of forskolin (10μM), a direct activator of adenylate cyclase (inhibition by forskolin, 10±2%; by rolipram, 39±6%; by forskolin and rolipram combined, 70±2%). In HLMC (n=6), rolipram was less effective at potentiating the inhibitory effects of forskolin (inhibition by forskolin, 18±5%; by rolipram, 6±5%; by forskolin and rolipram combined, 29±6%).

These results suggest that modulation of the PDE IV isoform can regulate basophil responses, whereas an association of the PDE IV isoform with the regulation of HLMC function remains uncertain.

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116P MODULATION OF NEUROTRANSMISSION IN THE GUINEA-PIG VAS DEFERENS BY SOMATOSTATIN AND NEUROTENSIN

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Stimulation of the sympathetic nerve supply to the guinea-pig vas deferens causes frequency-dependent contractions of the smooth muscle through the co-release of noradrenaline (NA) and adenosine triphosphate (ATP) (Burnstock, 1985). Somatostatin nerves have also been found in the vas deferens (Pekary *et al.*, 1984) but their role in the function of this tissue is not yet clear. In order to examine the possible influence of these peptidergic nerves on the vas deferens, male guinea-pigs (250-300 g body weight) were killed and their vasa deferentia removed. The prostatic part of each vas was mounted in physiological saline solution (modified Krebs-Henseleit solution) at 37°C, aerated with 95% O_2 and 5% CO_2 in an organ bath. Field stimulation could be applied and isometric contractions recorded. The effects of somatostatin (SOM) and neurotensin (NT) on the contractions produced by NA, ATP or field stimulation were recorded. Differences between mean responses in the presence of either SOM or NT and their respective controls was compared for significance by Student's t-test). Field stimulation (8 Hz, 0.3 ms duration at submaximal voltage, either 80 or 160 pulses) at 8 min intervals produced contractions with an initial "twitch" component, mediated by ATP, followed by a second "tonic" component, which is partly nonadrenergic-noncholinergic and partly due to NA release (Stjarne & Astrand, 1985). Both components disappeared in the presence of TTX (1 μM) suggesting their neuronal origin. SOM (1 μM) reduced the amplitude of both components. However, SOM (1 μM) had a greater effect on the initial twitch response mediated by ATP (a reduction of 62.3 ± 6.4%, $n = 6$, $p < 0.001$, compared with control; the reduction in the "tonic" component was 33.4 ± 5.4% ($n = 6$; $p < 0.01$, compared with control). This difference in the effect of SOM on "twitch" and "tonic" components was significant with 1 μM of SOM ($p < 0.01$). SOM (1 μM) influenced neither the spontaneous nor the ATP (up to 100 μM) and NA (up to 10 μM)-induced contractions of the vasa, suggesting a lack of interaction by SOM with ATP and NA receptors on the smooth muscle.

Conversely, when NT (1 μM) was applied 4 min before field stimulation, an increase in both components of the isometric contraction was seen but with a more pronounced effect on the tonic, NA-mediated response. NT (1 μM) caused an increase of the "twitch" responses by 38.5 ± 0.5% ($P < 0.01$, $n = 6$) and of the "tonic" responses by 67.1 ± 1.5% ($P < 0.001$, $n = 6$). NT (1 μM) also potentiated by 49.8 ± 1.3% ($P > 0.05$, $n = 6$) the NA (10 μM)-induced maximum contractions of the vasa. The data suggest the involvement of pre- and postjunctional adrenergic pathways in the contractile effect of NT on guinea-pig vasa deferentia.

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117P STUDIES ON THE BINDING OF [^{14}C]THIOPENTONE TO RAT CEREBROCORTICAL MEMBRANES

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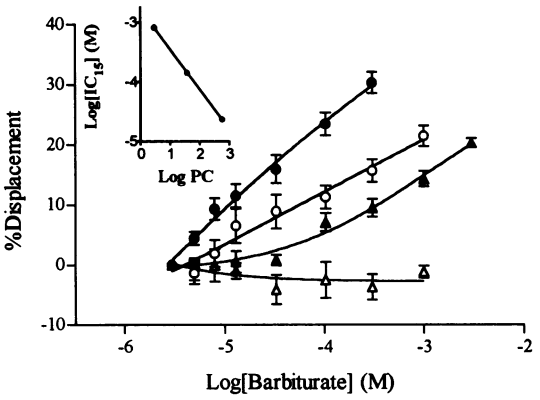
The relationship between anaesthetic potency and lipid solubility suggests a unitary mechanism of general anaesthetic action (Koblin, 1994) yet there are reports showing interaction with $GABA_A$ receptors, voltage sensitive Ca^{2+} channels (VSCCs) and other sites (Lynch & Pancrazio, 1994). In this study we have examined the binding of a radiolabelled barbiturate [^{14}C]thiopentone to determine whether a specific anaesthetic binding site exists in the brain.

Cerebrocortical membranes were prepared from female rats (250-300g) in 50 mM Tris.HCl, pH 7.4 by homogenization and centrifugation. Binding assays were performed in 1ml of Tris.HCl, pH 7.4 for 90 mins at room temperature using approximately 1 mg of protein and 3 μM [^{14}C]thiopentone. Bound and free radioactivity were separated by vacuum filtration. Attempts to define non-specific binding with excess thiopentone failed due to low solubility, hence B_{max} and K_d could not be determined. Data are presented as displacement curves for % displacement of total binding. Displacement studies were performed with thiopentone (10^{-6}-3×10^{-4}M), pentobarbitone (10^{-6}-10^{-3}M), phenobarbitone (10^{-6}-3×10^{-2}M) barbituric acid (10^{-6}-10^{-3}M), propofol (10^{-5}-10^{-3}M), ketamine (10^{-5}-10^{-3}M), alphaxalone (3×10^{-6}-3×10^{-4}M) etomidate, (3×10^{-6}-3×10^{-4}M) and midazolam (10^{-9}-10^{-4}M). All data are expressed as mean±s.e.mean (n=4-7).

The binding of [^{14}C]thiopentone was time dependent reaching apparent equilibrium at 90mins (1069±6 DPM/mg protein). Binding was enhanced (23±4%, EC_{50}=6±1 mM) by Mg^{2+}. [^{14}C]thiopentone binding was displaced dose dependently by three barbiturates with a rank order potency of thiopentone > pentobarbitone > phenobarbitone. The non-anaesthetic barbiturate, barbituric acid was ineffective, Figure 1. With the exception of barbituric acid there was a significant correlation between the concentration of barbiturate that produced 15% displacement and lipid-water partition coefficient (PC) (Ferko, 1990) (r^2=1.0). With the exception of propofol (at high concentrations, 18±2% displacement at 1mM) all non-barbiturate anaesthetic agents tested were ineffective in displacing [^{14}C]thiopentone.

Figure 1. The effect of thiopentone (●), pentobarbitone (○), phenobarbitone (▲) and barbituric acid (Δ) on [^{14}C]thiopentone binding to rat cerebrocortical membranes.



These data suggest that a single anaesthetic binding site is unlikely to exist in the brain. Further studies are underway to determine the identity of the barbiturate binding site.

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118P A MUSCLE-TYPE NICOTINIC ACETYLCHOLINE RECEPTOR BINDING ASSAY, USING WHOLE TE 671 CELLS, FOR RAPID SCREENING OF NEUROMUSCULAR BLOCKING AGENTS

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Neuromuscular blocking agents (NMBAs) are used in clinical anaesthesia to induce muscle relaxation. They exert their effect by antagonising the action of the transmitter acetylcholine on nicotinic receptors at the motor endplate of the neuromuscular junction. This study describes the measurement of the inhibition of [^{125}I]α-bungarotoxin ([^{125}I]α-BGT) binding by a range of nicotinic acetylcholine receptor (nAChR) agonists and antagonists, using the human cell line TE 671 as a source of muscle type nAChRs (Schoepfer *et al.*, 1988). The aim of this study was to develop a radioligand binding assay to determine the affinity of compounds for the nAChR.

TE 671 cells (passage 3-20), were routinely cultured in Dulbecco's Modified Eagle Medium and seeded into 24 well culture plates ($5x10^5$ cells/well) 24 hours prior to use. Following washing (x2) with phosphate buffered saline, cells were incubated (30 min, 37°C, 95%O_2/5%CO_2) with either 0.1mM tubocurarine (to evaluate non-specific binding) or appropriate concentrations of test compound in the presence of excess Hepes-Krebs buffer (NaCl 118mM, $NaHCO_3$ 30mM, KCl 5mM, KH_2PO_4 1mM, $MgSO_4$ 1mM, Glucose 11mM Hepes 20mM $CaCl_2$ 2.5mM) pH 7.4. Following addition of [^{125}I] α-BGT (0.1-10nM for saturation experiments, 0.1nM for inhibition studies) cells were incubated under the Reactions were terminated by removal of the incubation mixture and after washing, samples were solubilised (1ml of 0.1M NaOH) and the radioactivity counted.

From saturation experiments, the mean±s.e. mean (n=3) pK_D value for [^{125}I] α-BGT was 9.15±0.06 (K_D=0.7nM Bmax=24fmol/mg). Of the five aminosteroidal antagonists tested, pancuronium and vecuronium were more potent inhibitors of [^{125}I]α-BGT binding (pIC_{50}=7.11±0.09 (n=12) and 7.39±0.18 (n=6) respectively) than Org 9487(1-[(2β,3α,5α,16β,17β)-3-acetyloxy-17-(1-oxopropoxy)-2-(1-piperidinyl-16-yl]-1-(2-propenyl) piperidinium bromide), pipecuronium and rocuronium. The mean±s.e.m. pIC_{50} value for these compounds (6.91±0.29, 6.72±0.12 and 6.64±0.08 respectively) indicated a 4-5 fold decrease in activity compared to vecuronium. Comparisons of the pIC_{50} values for the 3 agonists tested showed that acetylcholine (6.91±0.04) had a greater affinity for the nAChR than either carbachol (5.95±0.05) or nicotine(5.75±0.09).

The results show that this assay provides a rapid, reproducible system which can be used to determine the affinity of compounds for the nAChR. The TE 671 whole cell binding assay is now routinely used in our laboratory as a primary screen to evaluate the binding activity of potential new NMBAs.

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119P DEPRESSION OF THE RAT HAMSTRING FLEXOR REFLEX BY (±)-BACLOFEN AND MUSCIMOL

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The spinal GABAergic system is known to modulate sensory input. Chronic noxious stimulation of the rat hindpaw by the injection of Freund's complete adjuvant or carageenan, results in an increase in the levels of GABA and GAD, an augmentation of the GABA immunoreactive cells and a reduction in the $GABA_B$ receptor binding in the superficial dorsal horn receiving the afferent input from the inflamed region. These effects are abolished by peripheral neurectomy or by destruction of the primary afferent C-fibres with capsaicin (Castro-Lopes et al. 1992; Castro-Lopes et al. 1994; Castro-Lopes et al. 1995).

The purpose of the present series of experiments was to investigate the effects of the $GABA_A$ and $GABA_B$ receptor agonists (muscimol and baclofen respectively) on an electrophysiological model of nociception; the hamstring flexion reflex, in the rat.

Female Wistar rats (200-350 g) were anaesthetised using 1-2% halothane in O_2/N_2O mixture and the left jugular vein was cannulated for the administration of drugs. EMG activity was recorded via a pair of stainless steel needle electrodes inserted into the biceps femoris muscle. Electrical stimuli (1 ms duration, 10 mA, at 0.16 Hz) were applied by another pair of needle electrodes inserted into the lateral two digits of the foot. At this stimulation strength both A and C-fibre mediated EMG responses were activated. The spikes of the EMG response were counted and recorded on a computer for subsequent analysis. All values are mean ± SEM.

(±) Baclofen was applied cumulatively at doses over the range of 0.5 - 20 mg /kg i.v. to 8 animals. Baclofen had little effect on the reflex activity at doses lower that 2.5 mg/kg and inhibited both the A and C-fibre mediated responses at higher doses, with an estimated ED_{50} on the C-fibre response of 5.3 ± 0.4 mg/kg. The $GABA_B$ antagonist CGP 35348 (Olpe et al. 1990), had no effect on its own, but significantly reversed the inhibitory effect of 20 mg/kg baclofen from 5.0±4.6% to 59.5 ± 12.9% (at 100 mg/kg, $p<0.05$ t-test) and 108.4 ± 22.7% (at 200 mg/kg, $p<0.01$) of the control responses.

Muscimol given at doses of 0.5 - 6 mg/kg i.v. was applied to 5 preparations, resulted in a 47.9%± 25.7 reduction of the reflex response at 6 mg/kg, but considerable variability was observed between preparations and therefore statistical significance was not reached. At doses higher than 6 mg/kg animals died due to respiratory arrest. The $GABA_A$ antagonist bicuculline given at a dose of 0.25 mg/kg caused a transient reversal (approximately 10 minutes) of the inhibitory effect of muscimol.

Baclofen is a muscle relaxant at doses greater than 4 mg/kg but inhibits mono- and poly-synaptic reflex activity at lower doses (Bowery 1982). It is concluded that $GABA_B$ receptors inhibit the hamstring flexion reflex and may therefore contribute to the muscle relaxant properties of baclofen.

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120P RS 16566: A NOVEL, HIGH AFFINITY LIGAND FOR THE (R) ZACOPRIDE BINDING SITE

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Racemic zacopride displays high affinity $5\text{-}HT_3$ receptor antagonism and $5\text{-}HT_4$ receptor agonism (Smith *et al.*, 1988; Craig & Clarke, 1990). At these receptors, the binding of the zacopride enantiomers is (S) > (R) (Pinkus et al., 1990). High affinity binding sites for [3H] (R) zacopride, present in rat brain, ileum and NG 108-15 cells, have been identified that exhibit the opposite enantiomeric selectivity (Kidd *et al.*, 1992). This site has been termed the (R) zacopride site. The pharmacology and CNS distribution of the binding site is inconsistent with other receptors or ion channels, including the $5\text{-}HT_3$ and $5\text{-}HT_4$ receptors (Kidd *et al.*, 1993; see Eglen, 1995, for review). We now report the identification of a novel high affinity ligand, RS 16566, for the (R) zacopride binding site.

Methods. Radioligand binding studies were conducted in membranes prepared from rat cerebral cortex ($5\text{-}HT_3$), guinea-pig striatum ($5\text{-}HT_4$) and NG 108-15 cells ((R) zacopride site) according to methods described elsewhere (Eglen *et al.*, 1994).

Results. RS 33800 (*endo*-3-(6-chloro-1-isopropylbenzimidazole-4-caboxamido)-tropane) acted as a high affinity ligand for the (R) zacopride site, with an affinity approaching that of (R) zacopride itself. RS 33800 thus provided an achiral lead for further studies. The effect was studied of substituting the (R)-3-quinuclidinyl and (S)-3-quinuclidinyl moieties for the tropane ring system to yield the enantiomeric amides, RS 16566 ((R)-3-(6-chloro-1-isopropylbenzimidazole-4-carboxamido)quinuclidine) and RS 16456 ((S)-3-(6-chloro-1-isopropylbenzimidazole-4-carboxamido)quinuclidine)), respectively. RS 16566 exhibited a 30 fold higher affinity at the (R) zacopride binding site than (R) zacopride itself,. RS 16456, in contrast, was 150 less potent than (R) zacopride at this site. These structures can be compared to the other aminoquinuclidines, enantiomers, (R) and (S) RS 56532, (R) and (S) RS 42358 (table 1; Eglen *et al.*, 1994; Wong *et al.*, 1994). Of these, RS 16566 exhibited the highest affinity at this site, although it also possessed high affinity for the $5\text{-}HT_3$ receptor.

Table 1. Affinities (pKi) of various ligands at $5\text{-}HT_3$, $5\text{-}HT_4$ and (R) zacopride binding sites.

Ligand	$5\text{-}HT_3$	$5\text{-}HT_4$	(R) zacopride
(R) zacopride	8.4 ± 0.08	5.6 ± 0.13	8.3 ± 0.22
(S) zacopride	9.7 ± 0.03	6.4 ± 0.11	5.3 ± 0.31
RS 33800	9.0 ± 0.10	6.8 ± 0.07	7.7 ± 0.10
(R) RS 16566	9.3 ± 0.03	7.6 ± 0.01	9.8 ± 0.42
(S) RS 16456	9.9 ± 0.08	6.8 ± 0.21	6.2 ± 0.30
(R) RS 56532	9.1 ± 0.06	6.5 ± 0.06	6.1 ± 0.21
(S) RS 56532	8.0 ± 0.10	7.6 ± 0.12	< 5.0
(R) RS 42358	7.6 ± 0.06	< 5.0	7.5 ± 0.34
(S) RS 42358	9.8 ± 0.04	< 5.0	< 5.0

Values are mean ± s.e. mean, n=4. All Hill coefficients were not significantly different to unity.

Conclusion. RS 16566 represents a novel high affinity ligand to label the (R) zacopride binding site. However, additional studies are required to identify compounds selective for the (R) zacopride site *per se*. Nonetheless, under appropriate conditions of $5\text{-}HT_3$ blockade, RS 16566 may provide a useful probe to explore the nature of the (R) zacopride binding site.

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121P AGONIST-DEPENDENT DESENSITISATION OF m1-MUSCARINIC RECEPTOR-MEDIATED INOSITOL POLYPHOSPHATE RESPONSES IN CHINESE HAMSTER OVARY CELLS

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The m1-muscarinic receptor, in common with the m3- and m5-muscarinic receptor subtypes, couples preferentially to phosphoinositidase C (PIC) via G-proteins of the $G_{q/11}$ class. This study concentrates on the regulation of agonist-sensitive inositol (1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) generation in Chinese hamster ovary cells transfected with m1-muscarinic receptor cDNA (CHO-m1 cells). The m1-muscarinic receptor expression level was ~1.9pmol [^{3}H]-n-methylscopolamine /mg protein. Data are quoted as mean ± S.E.M. for n =3-4 determinations. Data were analysed using the two-tailed student t-test and significance was accepted when $p \leq 0.05$.

Stimulation of CHO-m1 cells with methacholine (1mM) resulted in a biphasic pattern of $Ins(1,4,5)P_3$ production . Addition of agonist resulted in $Ins(1,4,5)P_3$ increasing from a basal level of 30.7 ± 4 pmol/mg protein to a peak level of 345.4 ± 34.4 pmol/mg protein within 10s. By 60s $Ins(1,4,5)P_3$ generation was typically 111. 3 ± 14.2 pmol/mg protein indicating that there had been a diminution of the peak response. Over the next 9min there followed a sustained but slowly increasing phase of agonist-stimulated $Ins(1,.4,5)P_3$ accumulation. The EC_{50} for the methacholine stimulated peak was 1.47 ± 0.1μM compared with 0.2μM for the 5min time point , suggesting that the sustained phase was more sensitive to agonist than the peak phase.

Agonist-dependent desensitisaton of the initial methacholine-evoked burst of $Ins(1,4,5)P_3$ production was then investigated . Prestimulation with methacholine (1mM) for 5min. resulted in a rightwards shift in the dose response curve for the methacholine-stimulated peak without any reduction in maximal responsiveness. The EC_{50} for the control 10s response was 2.36 ± 0.8μM compared with 8.58 ± 1.7μm for prechallenged cells. These data suggested the presence of a large receptor reserve and that desensitisation resulted in a partial loss of functional receptors. Desensitisation was rapid requiring only 1min preincubation with agonist and the EC_{50} for methacholine -induced desensitisation was 8.2 ± 2.2μM.

We decided to examine the relationship between agonist-efficacy and desensitisation by employing a partial agonist Experiments comparing the dose- dependencies of methacholine- and arecoline-stimulated $Ins(1,4,5)P_3$ accumulation at 10s confirmed that arecoline was a partial agonist . The EC_{50} for arecoline (11.2 ± 5.1μM) was not significantly different from that obtained with methacholine (4.8 ± 2.3 μM) . However, the maximum response obtainable with arecoline was only 58 ± 10% of that observed with methacholine. Prestimulation with methacholine (1mM) resulted in a 34.2 ± 9.2% desensitisation of the 10s arecoline (1mM) response. This is consistent with desensitisation resulting in a partial loss of functional receptors since such an effect should result in desensitisation of the arecoline response which probably does not possess a large receptor reserve.

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122P 5-HYDROXYTRYPTAMINE-STIMULATED PHOSPHOLIPASE D ACTIVITY IN THE RABBIT ISOLATED MESENTERIC ARTERY

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In the rabbit mesenteric artery the contractile response to 5-hydroxytryptamine (5HT) is usually only apparent if the tissue is partially depolarised with elevated extracellular KCl (Plane *et al.*, 1995) or co-stimulated with an alternative contractile agonist (Choppin and O'Connor, 1995). Such a functional response has been attributed to the $5HT_1$-like receptor (Choppin and O'Connor, 1995) however the second messenger pathways coupled to receptor stimulation are as yet undefined. In view of evidence suggesting that in the presence of elevated, extracellular [KCl], 5HT fails to elicit an increase in phospholipase C activity (Seager *et al.*, 1994) we aimed to establish whether phospholipase D (PLD) is involved in the 5HT-induced response in this tissue.

Female New Zealand White rabbits (2-3 kg) were anaesthetised (sodium pentabarbitone, 60mg/kg), killed by rapid exsanguination and the mesenteric bed removed. Third order mesenteric arteries were labelled for 20 h at 37°C in phosphate-free Krebs-Henseleit Buffer (KHB) containing [^{33}P] orthophosphate (5 μCi/ml, specific activity 3000Ci/mmol). Individual arteries were subsequently incubated for 30 min in standard KHB in a shaking water bath at 37°C and 100mM butanol added 5 min prior to stimulation. Reactions were terminated after 3 min with 10% perchloric acid. Lipids were extracted and [^{33}P]phosphatidyl butanol (PBut) separated by thin layer chromatography, identified and quantified according to the method of Purkiss and Boarder (1993). The generation of PBut in the presence of butanol is considered unique to PLD and therefore reflects PLD activity.

Neither KCl (45mM) nor 5HT (1μM) elicited an increase in PBut relative to the high basal levels (26240 ± 1642 dpm/mg protein, n=3, mean ± s.e.mean). However, in the presence of 45 mM KCl, an increase in the accumulation of PBut was observed with 5HT-stimulation. This effect was concentration-dependent, with a maximal, 84%, increase (48390 ± 4742 dpm/mg protein, $P<0.01$, Student's t-test, n=5) observed with 3μM 5HT, EC_{50} = 0.19 ± 0.07μM. 5HT (3μM)-elevated PBut levels (40001 ± 567 and 35971 ± 3751 dpm/mg protein) were significantly inhibited ($P<0.01$) to approximately basal values (6499 ± 104 and 7526 ± 656 dpm/mg protein, n=3) in either calcium-free KHB or after 20 min prior incubation with the PKC inhibitor, Ro 31 8220 (10μM), (Davis *et al.*, 1989), respectively. Both basal and 5HT (3μM)-stimulated PBut levels (5180 ±858 and 8971 ± 366 dpm/mg protein) were attenuated to near zero after labelling in the presence of pertussis-toxin (PTX), (100ng/ml, n=4). Although this suggested a role for a PTX-sensitive G-protein in the signal transduction response, control experiments demonstrated that PTX treatment during labelling significantly reduced the incorporation of [^{33}P] orthophosphate into the lipid pool by 90% ($P<0.01$), an effect which clearly complicates the interpretation of these data.

In summary, 5HT stimulated a concentration-dependent increase in PLD activity in the rabbit mesenteric artery. This effect was only observed under depolarising conditions and therefore correlates with equivalent contractile studies. The response was dependent upon the presence of extracellular calcium and was inhibited by the protein kinase C inhibitor, Ro 31 8220.

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123P EFFECT OF THE SPIN TRAP REAGENT PBN ON DEGENERATION OF 5-HT NEURONES INDUCED BY P-CHLOROAMPHETAMINE OR FENFLURAMINE ADMINISTRATION

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We recently demonstrated that administration of the spin trap reagent α-phenyl N-tert butyl nitrone (PBN) concurrently with 3,4-methylene-dioxymethamphetamine (MDMA or ecstasy) prevented the degeneration of 5-HT neurones in cerebral cortex and hippocampus which normally occurs after MDMA injection (Colado & Green, 1995). We have now examined whether this free radical scavenger also protects against the degeneration induced by injection of two other 5-HT neurotoxins, namely p-chloroamphetamine (PCA) and fenfluramine.

Male DA strain rats were injected with either PBN (150mg kg^{-1} i.p.) or saline. Ten min later they were injected with either PCA (2.5mg kg^{-1} i.p.), fenfluramine (15 mg kg^{-1} i.p.) or saline. After a further 120 min PBN pretreated animals received a further dose (150mg kg^{-1} i.p.) of this compound. Seven days later all groups were killed and [^{3}H]-paroxetine binding performed in cortical tissue (Hewitt & Green, 1994). This ligand can be used as a marker of intact 5-HT nerve terminals and has thus been used as an index of neurodegeneration (see Hewitt & Green, 1994).

Both PCA and fenfluramine produced a substantial loss in [^{3}H]-paroxetine binding (Table). PBN alone did not alter [^{3}H]-paroxetine binding but markedly attenuated the loss which followed PCA administration without significantly altering the loss produced by fenfluramine (Table). These effects cannot be attributed to changes in body temperature since neither neurotoxin produced significant hyperthermia, nor did PBN produce hypothermia in neurotoxin-treated rats.

We conclude that PCA, like ecstasy, probably induces neurodegeneration because of oxidative stress produced by free radical formation while fenfluramine-induced damage involves a different mechanism.

Table Effect of PCA, fenfluramine and PBN on [^{3}H]-paroxetine binding (1nM) in rat cerebral cortex

	[^{3}H]-paroxetine binding (fmol mg^{-1} protein)	
	Saline	***PBN***
Saline	53.1 ± 4.6 (11)	57.4 ± 4.5 (11)
PCA	24.5 ± 1.8 (12)†	42.7 ± 3.5 (13)*
Fenfluramine	28.7 ± 3.6 (8)	36.9 ± 3.4 (8)

†Different from saline $p<0.001$; *Different from PCA treated $p<0.001$. Data was analysed by one-way ANOVA followed by post-hoc 2 tailed t-tests.

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124P 5-HT_{2B} RECEPTOR mRNA IN GUINEA-PIG SUPERIOR CERVICAL GANGLION

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In addition to containing 5-HT_3 and 5-HT_{2A} receptors, the guinea pig superior cervical ganglion (SCG) contains a putative 5-HT receptor pharmacologically most similar to, but not identical with, other members of the 5-HT_2 receptor family (Watkins and Newberry, 1996). We are currently trying to determine the relative abundance of the mRNAs for the 5-HT_2 receptor family in this preparation.

Adult male Dunkin-Hartley guinea pigs and Sprague-Dawley rats were anaesthetised with pentobarbitone, perfused transcardially with saline and total RNA prepared from guinea pig SCG and rat brain (with Trizol). RNAs were DNase-treated, then used in reverse transcriptase (RT) reactions with a reverse primer for the rat 5-HT_{2A} (Watts et al., 1994) or 5-HT_{2B} (Foguet et al., 1992) receptor mRNA sequence. RT products were used as templates in polymerase chain reactions (PCR), containing reverse and forward primers for these sequences. For the 5-HT_{2A} receptor mRNA sequence, the reverse and forward primers were complementary to and homologous with bases 514-534 and 232-258, respectively. For 5-HT_{2B}, the forward primer matched bases 1255-1279 but the reverse primer for the RT (1631-1653) and PCR (1568-1589) differed. To control for specificity in the PCR reactions duplicates were performed using RT products obtained using a reverse primer for β-actin mRNA. PCR products were analysed by UV illumination, following migration on ethidium bromide-agarose gels. Southern blots of PCR products derived from presumed 5-HT_{2B} mRNA were probed with a ^{32}P-radiolabelled oligonucleotide homologous to bases 1323-1344 of the 5-HT_{2B} receptor mRNA sequence (an internal portion of the predicted PCR product).

After 35 PCR cycles, specific bands were obtained from rat brain RNA, consistent in size with those predicted from the rat 5-HT_{2A} and 5-HT_{2B} receptor mRNA sequences (303 & 335, respectively, n=6 rats) (Flanigan et al., 1995). Similar bands were also obtained with guinea pig SCG RNA, following 35 (5-HT_{2A}) and 40 (5-HT_{2B}) PCR cycles (n=4). The 5-HT_{2B} receptor products were confirmed by Southern blot analysis. Furthermore, the use of DNase-treated RNA suggests that the source of these signals is RNA, rather than genomic DNA.

Our data confirm the expected presence of the 5-HT_{2A} receptor mRNA in guinea pig SCG and also provide strong evidence for the expression of 5-HT_{2B} receptor mRNA in this tissue, albeit at lower levels.

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125P β_3-ADRENOCEPTORS IN ADIPOSE TISSUE AND GUT OF GENETICALLY OBESE C57BL/6J (ob/ob) AND LEAN C57BL/6J (+/+) MICE

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White adipose tissue (WAT) from genetically obese C57BL/6J (ob/ob) mice displays reduced adenylate cyclase activity in response to stimulation with adrenaline and the β_3-adrenoceptor (ar) selective agonist BRL 37344 compared with lean C57BL/6J (+/+) controls. WAT and brown adipose tissue (BAT) from obese mice contains markedly reduced levels of β_3-ar mRNA compared to their lean counterparts (Collins *et al.*, 1994). In rat ileum there is abundant evidence from functional (see Arch & Kaumann, 1993), receptor binding (Roberts *et al.*, 1995) and molecular studies for the presence of β_3-ar's mediating relaxation. This study was carried out to determine if β_3-ar's are expressed in mouse ileum and whether their expression and function in this tissue is reduced in obese mice in the same way as has been reported for adipose tissue.

Mouse terminal ileum was mounted in an organ bath in modified Kreb's solution (37°C, pH 7.4) under a resting tension of 5mN. Relaxation responses were recorded isotonically in tissues pre-contracted with carbachol (10μM). Ileum taken from either obese or lean mice relaxed in a concentration-dependent manner to addition of the selective β_3-ar agonist CL 316,243 (pEC_{50} 7.90 ± 0.13, and 7.77 ± 0.19 respectively, n=7). In both mouse strains the β_1-, β_2-ar selective antagonist (-) propranolol (1μM) shifted the concentration-response curve for CL 316,243 to the right in a parallel manner with pK_B values of 6.31 ± 0.22 (n=7; obese) and 6.40 ± 0.08 (n=6; lean). The β_3-ar selective antagonist SR 58894A (3-(2-allylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol hydrochloride; Manara *et al.*, 1995) caused similar rightward shifts but with pK_B values of 8.22 ± 0.06 (n=6; obese) and 8.27 ± 0.12 (n=5; lean). These results demonstrate that functional β_3-ar's mediating relaxation are present in mouse ileum from both obese and lean mice.

In molecular studies, RNA was extracted by the acid/guanidinium/phenol/chloroform method, DNase treated and checked for integrity. RNA (1μg) was reverse transcribed and PCR (23-30 cycles) conducted on 1/10 the cDNA using ^{33}P labelled primers. Product was gel electrophoresed, blotted onto Hybond N+ and detected by phosphorimaging (Molecular Dynamics SI). WAT and BAT from obese mice had markedly lower levels of β_3-ar mRNA than their lean counterparts whereas ileum from both strains showed similar levels of β_3-ar mRNA. These studies confirm the findings of Collins *et al.*, in WAT and BAT but clearly show that β_3-ar expression in ileum is controlled by different mechanisms.

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126P IMPAIRED CYCLIC AMP SIGNALLING IN HEARTS FROM RATS CHRONICALLY INFUSED WITH ISOPRENALINE

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In studies of cardiac β-adrenoceptor desensitization, attention has focussed on the number and function of β-adrenoceptors (Muntz *et al.*, 1994) and G proteins (Eschenhagen *et al.*, 1992). This study investigated if β-adrenoceptor desensitization in rat heart is associated with changes in the adenylate cyclase-cAMP-dependent protein kinase (cAMPdPK) signalling pathway. Male Sprague-Dawley rats (240-260 g) were infused with vehicle (1 mM HCl) or isoprenaline (400 μg/kg/hr) for 14 days via osmotic minipumps (Alzet 2002) subcutaneously implanted at the back of the neck. Inotropic responses were examined in electrically stimulated left and right ventricular papillary muscles (1 Hz, 5 msec). Cumulative concentration-response curves were obtained for isoprenaline, the adenylate cyclase activator forskolin, the cell-permeable cAMP analogue dibutyryl cAMP and calcium chloride. One concentration response curve was performed in each tissue. Following chronic infusion of rats with isoprenaline, the inotropic responses of left and right papillary muscles to isoprenaline were abolished (Table 1), indicating marked β-adrenoceptor desensitization. Reduced inotropic responses were also observed when adenylate cyclase was directly stimulated with forskolin and when a cAMP analogue was used as the agonist, however, responses to calcium were unchanged. These findings indicate a post-receptor impairment in the signalling pathway, possibly at the level of cAMPdPK, the functional integrity of the contractile apparatus remaining intact.

To further investigate the impaired cAMP signalling, [^{3}H]cAMP binding was carried out using slide-mounted sections (10 μm) of left ventricle. Sections were preincubated in Krebs-HEPES buffer (Gundlach & Urosevic, 1989) for 10 min at 37°C to remove endogenous cAMP, incubated for 30 min in buffer at 4°C containing 1 mM 3-isobutyl-1-methylxanthine and 5 nM [^{3}H]cAMP, (non-specific binding defined using 10 μM cAMP), washed for two periods of 1 min in buffer at 4°C, wiped from the slide and counted. In left ventricle from isoprenaline-treated rats [^{3}H]cAMP binding was increased by 30% (vehicle 45.6±4.1 fmol/mg protein n=6; isoprenaline 59.0±3.1 fmol/mg protein n=6, $p<0.05$), which may indicate increased inhibitory (R) subunits of cAMPdPK which form inactive tetramers (R_2C_2) with catalytic (C) subunits and upon binding cAMP liberate active C subunits. These findings indicate that cardiac β-adrenoceptor desensitization involves not only changes to β-adrenoceptor function but also to post-receptor signalling which may be associated with alterations to cAMPdPK.

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Table 1. Mean pD_2 values (± s.e. mean) for agonists in cardiac tissues from vehicle- and isoprenaline-treated rats (* $p<0.05$, ** $p<0.01$)

		Isoprenaline	Forskolin	Dibutyryl cAMP	$CaCl_2$
Left Papillary	- Vehicle	7.44±0.31 (n=4)	5.80±0.07 (n=10)	4.07±0.06 (n=8)	2.52±0.01 (n=12)
	- Isoprenaline	No response (n=6)	5.07±0.07 (n=9)**	3.62±0.07 (n=8)**	2.55±0.01 (n=12)
Right Papillary	- Vehicle	7.53±0.12 (n=5)	6.02±0.08 (n=6)	4.07±0.06 (n=7)	2.46±0.04 (n=7)
	- Isoprenaline	No response (n=6)	5.25±0.06 (n=6)**	3.74±0.06 (n=6)**	2.58±0.04 (n=6)

127P KINETIC ANALYSES OF INTERNALIZATION AND RECYCLING OF THE HUMAN β_2-ADRENOCEPTOR

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Heptahelical G protein-coupled receptors desensitize upon occupancy by agonist. Significant uncoupling from down-stream effectors occurs during desensitization, constituting an autoregulatory mechanism whereby cells can limit the degree to which they are stimulated. The human β_2-adrenoceptor (β_2AR) offers a well-described model system in which to study receptor desensitization. β_2ARs internalize rapidly upon binding of agonist, resulting in re-distribution of receptors away from the plasma membrane and into endocytic vesicles (von Zastrow and Kobilka, 1994). Experiments were performed to determine if internalized β_2ARs represent a static endosomal pool, or rather, form a dynamic population of receptors that cycle between the plasma membrane and endosomal compartments.

Transfected HEK293 cells that express epitope-tagged β_2ARs at high levels (12β6 line; 320,000 receptors per cell) were used to quantify the kinetics of ligand-induced receptor trafficking. Receptor internalization and recycling were measured by flow cytometry which quantified cell surface levels of the hemagglutinin epitope on the receptor ectodomain by binding of the specific monoclonal antibody 12CA5. Agonist binding is not limiting under these conditions. Receptor trafficking data were fitted to a one-phase exponential kinetic model and yielded a value K, which was equal to the combined rate constant term ($k_e + k_r$), where k_e and k_r are the rate constants for endocytosis and recycling, respectively (units: min^{-1}).

Isoprenaline (ISO;5μM) caused internalization of ß$_2$ARs at 37°C which followed first order kinetics. Cell surface receptors stabilized at 22.2±4.5% of that in control cells after 15 minutes exposure to agonist (n=6). Prior treatment of cells with ISO (5μM;15 minutes), in order to internalize ß$_2$ARs, accelerated the kinetics of subsequent ISO-induced ß$_2$AR internalization (naive cells, mean ± s.e.mean, $k_e + k_r$ 0.31±0.03, n=6; ISO-treated cells, $k_e + k_r$ 0.43±0.03, n=7; $P<0.05$). Recycling of internalized ß$_2$ARs to the cell surface, after removal of ISO, followed first order kinetics (k_r 0.08±0.02, n=3). This value was applied to this mathematical model for receptor trafficking in order to calculate the k_e value for ßARs: calculated k_e value, 0.21±0.03.

These data demonstrate that the sequestered ß$_2$AR represents a dynamic pool of trafficking receptors with steady-state distribution between the cell surface and internal compartments. Further, the results suggest that receptor internalization may be influenced by down-stream signal transduction mechanisms.

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128P DIVERGENT EFFECTS OF AGENTS THAT INCREASE INTRACELLULAR Ca^{2+} ON HUMAN EOSINOPHIL AND NEUTROPHIL APOPTOSIS

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Eosinophils and neutrophils undergo constitutive cell death by apoptosis, however the underlying intracellular regulatory mechanisms are unknown. The glucocorticoid, dexamethasone, has opposing effects on the rate of apoptosis in these cells, promoting eosinophil, while inhibiting neutrophil apoptosis (Meagher *et al.*, 1995), suggesting that apoptosis is regulated differently in these closely related cell types. We also reported that elevation of the intracellular Ca^{2+} ($[Ca^{2+}]_i$) in neutrophils by calcium ionophores, caused an inhibition of apoptosis (Whyte *et al.*, 1993).

In this study we examined the effects of agents which elevate $[Ca^{2+}]_i$ upon the rate of granulocyte apoptosis using (a) thapsigargin, which increases $[Ca^{2+}]_i$ by a mechanism involving the mobilization of intracellular Ca^{2+} stores (Takemura *et al.*, 1989), and (b) A23187, a calcium ionophore.

Human eosinophils and neutrophils were isolated (Stern et al., 1992) and incubated (2×10^6 ml^{-1}) in serum supplemented Iscove's at 37°C for 20 h, in the presence or absence of thapsigargin (2 μM) or A23187 (0.1 μM). After 20 h, apoptosis (% mean ± s.e.mean) was determined by morphological criteria. Apoptosis occurred without loss of cells or necrosis, as assessed by trypan blue exclusion.

Thapsigargin and A23187 caused an inhibition in the rate of neutrophil apoptosis, whereas eosinophil apoptosis was promoted. Hence, basal neutrophil apoptosis after 20 h was 71.2 ± 6.4, whereas thapsigargin and A23187 reduced apoptosis to 20.2 ± 4.0 ($p<0.05$) and 41.0 ± 8.1 ($p<0.05$) respectively (n=3-7). In contrast, basal eosinophil apoptosis increased from 9.7 ± 1.7 to 62.8 ± 21.0 and 23.1 ± 4.1 in the presence of thapsigargin and A23187 respectively (n=3-6).

Thus, elevation of $[Ca^{2+}]_i$ mimicks the effect of dexamethasone, promoting eosinophil, while inhibiting neutrophil apoptosis. The effects of thapsigargin indicate that mobilisation of intracellular calcium stores may be an important factor in the regulation of granulocyte apoptosis. Eosinophil and neutrophil apoptosis appears to be regulated differently, an observation that may be important in the understanding of allergic inflammation.

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129P INHIBITION OF HUMAN NEUTROPHIL APOPTOSIS BY HYPOXIA IS MEDIATED BY A FERRO-PROTEIN SENSOR

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Neutrophil apoptosis represents a major mechanism involved in the resolution of inflammation. In contrast to the ability of hypoxia to induce apoptosis in several cell lines we have recently shown that hypoxia causes a dramatic decrease in the rate of neutrophil apoptosis (Hannah *et al.*, 1995). This effect was not mimicked by antioxidant treatment or heat shock and was not associated with an increase of bcl-2 expression. We have now examined whether hypoxic inhibition of neutrophil apoptosis involves a ferro-protein oxygen sensing mechanism as demonstrated for hypoxic regulation of erythropoietin gene expression (Eckardt *et al.*. 1993).

Human neutrophils were isolated from peripheral venous blood (Haslett *et al.*, 1985) and cultured in Iscove's DMEM at 37°C either in a normoxic (21% O_2) or hypoxic environment (0.1% O_2). Cells were also exposed under normoxic conditions to the mitochondrial inhibitors: sodium azide (1 mM); potassium cyanide (1 mM) and rotenone (0.1 $\mu g/ml^{-1}$) or to the iron chelators desferrioxamine mesylate, DFO, (1-1000 μM) and 1,2-diethyl-3-hydroxypyridine-4-one, CP94, (1-1000 μM). After 20 hours cytocentrifuge samples were prepared and apoptosis assessed morphologically. (Data are expressed as mean ± s.e. mean, n=3).

Neutrophil apoptosis was reduced from 62.5 ± 3.7% in 21% O_2 to 19.5 ± 0.1% under hypoxic conditions. The iron chelators DFO and CP94 both mimicked the effect of hypoxia in a concentration-dependent manner. For example, in the presence of 300 μM DFO and 300 μM CP94 the % apoptosis was reduced from 62.5 ± 3.7% to 36.7 ± 3.4% and 30.5 ± 1.7% respectively. This effect could be overcome by the addition of a molar excess of Fe^{2+} ions in the form of $FeCl_2$. Neither potassium cyanide, sodium azide nor rotenone significantly affected the rate of neutrophil apoptosis. This indicates that the sensing system is specific for hypoxia and not responsive to the metabolic consequences of interrupting mitochondrial electron transport.

These results demonstrate that chelatable iron is closely involved in the oxygen sensing mechanism involved in the hypoxic inhibition of neutrophil apoptosis. This leads to the proposal that the control mechanism may involve the interaction of oxygen with a ferro-protein sensor.

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130P A SINGLE VOLTAGE SENSITIVE CALCIUM CHANNEL TYPE UNDERLIES THE INWARD CURRENT IN THE SKELETAL MUSCLE FIBRES OF PRE-PUPAL HOUSEFLY LARVAE

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In insect skeletal muscle, voltage clamp studies have shown that calcium is the major carrier of inward current at the non-synaptic membrane(Washio 1972). Recent studies of single channel and whole cell calcium currents, particularly in insect neurons (Leung and Byerly 1991, Pearson *et. al.* 1993) suggest the existence of multiple calcium channel sub-types with unique properties. We have used two electrode voltage clamping to investigate inward barium currents in body wall muscle fibres of larval houseflies *Musca domestica* and their sensitivity to organic and inorganic calcium channel blockers.

Pre-pupal 3rd instar housefly larvae (Cooper strain) were dissected to expose the musculature of the abdominal body walls, The preparation was perfused with housefly saline (mM: NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 0.75; $NaHCO_3$, 2; and HEPES, 5; pH 7.2) for 30 - 45 minutes at room temperature. The perfusion medium was switched to saline containing barium (mM: NaCl, 35; KCl, 5; tetraethylammonium chloride, 100; $NaHCO_3$, 2; $MgCl_2$, 1; $BaCl_2$, 5; pH 7.2) for 10 minutes before recording barium currents. Ventrolateral muscle fibres were impaled with two electrodes (3 - 10 MΩ, containing 3M KCl). After observation of a steady resting membrane potential the fibres were clamped at a holding potential of -70mV using an Axoclamp-2A amplifier. Currents were elicited by stepping the membrane potential in the range -60mV to +30mV for 200-600ms.

Voltage-sensitive inward barium currents (up to 1.5μA) were activated at potentials positive to -50mV, and had an apparent reversal potential of 20mV. The inward current was completely abolished by 10mM Co^{2+} and exhibited differing sensitivities to Cd^{2+} (IC_{50} 5μM) and Ni^{2+} (IC_{50} 150μM). High concentrations (>1μM) of the organic L-type calcium channel antagonists nifedipine (IC_{50} 33μM), verapamil (IC_{50} 40μM) and diltiazem (IC_{50} 142μM) resulted in partially reversible block of the inward current. The inward current was reduced in the presence of tetrandrine (6,6,7,12tetramethoxy-2,2-dimethylberbaman) (1.6μM), The T-type calcium channel antagonist amiloride (250μM and 500μM) and the peptide toxins ω-conotoxin GVIA (1μM) and ω-aga IVA (100nM and 500nM) were ineffective. For all dose/response relationships n=4-6 preparations for each data point.

The pharmacological and electrophysiological profile constructed suggests a single calcium channel type underlies the inward current in these muscle fibres. These data also indicate that this calcium channel has different properties from the presently defined vertebrate calcium sub-types and also to the described calcium channels in other invertebrate preparations.

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131P CONTRACTILE EFFECTS OF ENDOTHELIN-1 AND ENDOTHELIN-3, MEDIATED BY ET_A AND ET_B RECEPTOR SUBTYPES, IN VENTRICULAR CARDIOMYOCYTES ISOLATED FROM RABBIT MYOCARDIUM

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Two distinct subtypes of endothelin (ET) receptors, ET_A and ET_B, have been distinguished pharmacologically by the different potencies displayed by ET isopeptides; ET-1 has a higher affinity than ET-3 for the ET_A receptor subtype, whereas the isopeptides have almost equal affinity for the ET_B receptor subtype (Warner, 1995). ET-1 is a positive inotropic agent in mammalian myocardium, but the receptor subtypes present in cardiomyocytes have not been ascertained. The aim of this study was to investigate the contractile effects of ET-1 and ET-3, and to use the ET_A receptor selective antagonists, BQ-123 and PD155080 (Doherty *et al.*, 1995), and the ET_B receptor selective antagonists, BQ-788 and RES-701, to determine the receptor subtype(s) coupled to responses in isolated ventricular cardiomyocytes. Cells were isolated from adult, male, New Zealand White rabbits (2.5-3kg), using Langendorff perfusion with collagenase, and equilibrated with ET-1 or ET-3, in Tyrode's solution (37°C), for 10 min following a 5 min pre-incubation in the absence or presence of the various antagonists (1μM). Contractile function, as measured by mechanical cell shortening (dL) and expressed as a percentage of diastolic length, was monitored using video microscopy. Concentration-response relationships (non-cumulative) were established for ET-1 and ET-3 (10^{-11} to 10^{-7}M) in the absence and presence of the various antagonists, and potency values (pD_2) determined. E_{max} values were expressed as % maximal responses to Ca^{2+} (12mM). Data were expressed as mean±s.e.mean, and comparisons were made using analysis of variance followed by a multiple range test (Duncan's); values of $P<0.05$ were taken as significant.

The contractile amplitude of cells which were maximally activated with Ca^{2+} (12mM) was 16.4±0.35%dL (n=16). ET-1 was more potent than ET-3, and the mean EC_{50} values were 10pM and 3.3nM, respectively. The ET_A receptor-selective antagonists, PD155080 and BQ-123, produced a significant shift in the ET-1 concentration-response relationship; the ET_B receptor-selective antagonist, BQ-788, had less effect, while RES-701 had no effect on the contractile response to ET-1 (Table 1). Because of the lower potency of ET-3, the antagonists, PD155080, BQ-123 and BQ-788, at a concentration of 1μM, produced such large shifts in the ET-3 concentration-response curves that dose ratios could not be quantified. There was no apparent effects of the antagonists on E_{max} values indicating, in all cases, the competitive nature of the antagonism at these receptors.

Table 1. Effects of ET receptor antagonists on ET-1 and ET-3; *$p<0.05$ with respect to ET-1

	pD_2	Dose ratio	n	E_{max}(%)
ET-1	11.0±0.06	-	14	93±2.3
+PD155080	8.58±0.13*	284	6	86±3.6
+BQ-788	9.92±0.13*	13	5	89±2.3
+BQ-123	8.34±0.27*	487	6	84±3.4
+RES-701	10.8±0.19	1.7	5	85±4.8
ET-3	8.48±0.27	-	16	84±2.8
+RES-701	8.45±0.15	1.2	5	87±2.5

In conclusion, the order of potency of the endothelin agonists to increase contractile activity, and its antagonism by BQ-123 and PD155080, indicate that this response is mediated by ET_A receptors. It would appear, however, that the contractile effect of the endothelins can be influenced, to some extent, by a subtype of ET_B receptor which is sensitive to BQ-788, but not to RES-701.

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132P STUDIES ON P_2 PURINOCEPTORS, HISTAMINE RECEPTORS AND ENDOTHELIN RECEPTORS ON PRIMARY AND PASSAGED RAT BRAIN ENDOTHELIAL CELLS

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The nature of receptors on brain capillary endothelial cells is important for the understanding of cerebral function and improved treatment of certain brain disorders. These receptors include those for endothelin and histamine (Purkiss et al., 1994; Vigne et al, 1990) and the P_2-purinoceptors for ATP and ADP (Nobles et al, 1995). The study of these receptors has been made more complex by the variety of cell culture preparations (e.g. primary, highly passaged, and immortalised). In the present study, we have compared the responses of unpassaged primary rat brain capillary endothelial cells with those in early passage numbers.

Rat brain endothelial cells (RBECs) were isolated from adult Wistar rats essentially as described by Abbott et al (1992). Cells were used at a preconfluent stage, either as unpassaged primary cultures (day 6-28) or after passages 1-3 (day 1 - 14 from final passage) Cells were loaded with 2 μM Fura-2 AM (30 min at 36°C). Imaging of fluorescence at 340 and 380 nm and analysis of the fluorescent ratios (an indication of Ca^{2+}_i, i.e. cytosolic Ca^{2+}) was by an ImproVision microspectrofluorimetric system. Drugs were applied directly to the cells on cover slips using a multibarrel perfusion system.

Increases in Ca^{2+}_i in primary cells were seen in essentially all primary cells in response to 100 μM histamine in 92% (192 out of 209) cells, to 100 μM ATP in 99% (1037 out of 1051) cells, but not to 100 nM or 1 μM endothelin-1 (2 out of 182 cells). The change in ratio for histamine was 0.76 ± 0.03 (n = 125) while for ATP it was 1.15 ± 0.04 (n = 203). Ca^{2+}_i was also raised in 96% (130 out of 135) of cells in response to 300 μM UTP and 86% (196 out of 227) of cells exposed to 30 μM 2-methylthio ATP and 82% (80 out of 98) of cells exposed to 100 μM ADP. The size of the ratio change, expressed as % of the size of a subsequent response to 100 μM ATP, was: UTP, 166 ± 21 %, n = 99; 2-methylthio ATP, 122 ± 5, n = 99, and ADP, 99 ± 5, n = 68. None of these responses were significantly different ($P > 0.05$) from that for the subsequent addition of ATP. The responses tp purinergic agonists did not differ between passaged and unpassaged cells except in the case of α,β-methylene ATP (100 or 200 μM), to which a response was absent in unpassaged cells but was present in 96% (87 out of 91) passaged cells. In passaged the response to α,β-methylene ATP was smaller than that to a subsequent addition of ATP (52 ± 0.3 % of the ATP response, n = 87, $P < 0.05$).

These results show that primary rat brain microvascular endothelial cells contain Ca^{2+}_i coupled receptors for histamine but not endothelin. They also contain receptors with characteristics expected for P_{2Y}-purinoceptors and either P_{2U}- or pyrimidinoceptors. The unpassaged cells contain no α,β-methylene ATP responsive P_{2X}-purinoceptors, but responses to α,β-methylene ATP are apparent after one or more passages, suggesting the recruitment of $P2X_1$ or $P2X_3$ purinoceptors.

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133P ENDOTHELIAL P_{2Y}- AND P_{2U}-PURINOCEPTORS PHOSPHORYLATE AND ACTIVATE MITOGEN-ACTIVATED PROTEIN KINASES: STUDIES WITH A NOVEL KINASE ASSAY AND IMMUNOBLOT

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Bovine aortic endothelial cells contain co-existing phospholipase C coupled P_{2Y}- and P_{2U}- purinoceptors (Wilkinson et al, 1993). These receptors also stimulate tyrosine phosphorylation, a requirement for agonist stimulated prostacyclin production (Bowden et al, 1995). Here we use a modified assay and immunoblot procedure to show that these two P_2-purinoceptors stimulate tyrosine phosphorylation and activate enzyme activity of both p42 and p44 forms of mitogen activated protein kinase (MAPK) in cultured endothelial cells.

Bovine aortic endothelial cells were cultured as described previously (Wilkinson et al, 1993) and used as monolayers in 175 or 80 cm^2 flasks afer 24h serum free. Western blots used an antiserum specific for the tyrosine phosphorylated form of MAPK, with quantification using laser densitometry. A peptide kinase assay used the substrate peptide APRTPGGRR , chosen as an efficient substrate for MAPK activity. Extracts were fractionated on Resource Q columns with a NaCl gradient.

Under conditions which gave little detectable basal MAPK phosphorylation we found that 30 μM 2MeSATP, 300 μM UTP (selective P_{2Y}- and P_{2U}- purinoceptor stimulation respectively) and 300 μM ATP (a non-sective P_2-purinoceptor agonist) each gave an increase in phosphorylation of both p42 and p44 MAPK bands, using an antiserum specific for tyrosine-phospho-MAPK. For example, densitometry results for the p42 form: control, 0.10 ± 0.02; 2MeSATP, 1.11 ± 0.23 ($P < 0.05$); UTP, 1.33 ± 0.34 ($P < 0.02$); ATP, 1.13 ± 0.06 ($P < 0.002$); data are mean ± s.e.mean, n = 3, of arbitrary density units, with significance of difference from controls by ANOVA an Dunnet's multiple range tests. Using immunoblots with an antiserum which was for the unphosphorylated forms of MAPK, there was no difference between the density of bands for the stimulated and control samples, but there was a slightly reduced mobility of immunoreactive bands from the stimulated samples. On Resource Q chromatography two peaks of phospho-MAPK immunoreactivty were resolved, the first mainly p42, and the second mainly p44. Each of these immunoreactive peaks corresponded to peaks of peptide kinase activity.

These results show that the P_{2Y}- and the P_{2U}- purinoceptors on cultured bovine aortic endothelial cells each stimulate the tyrosine phosphorylation, and activate the enzymic activity of, both p42 and p44 forms of MAPK. Elsewhere we present evidence that this is involved in the control of prostacylin production by these two receptors (Boarder et al, 1996).

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134P STRUCTURE AND FUNCTION OF A NOVEL VOLTAGE-GATED, TETRODOTOXIN-RESISTANT SODIUM CHANNEL SPECIFIC TO SENSORY NEURONS

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Voltage-gated sodium channels play a fundamental role in the regulation of neuronal excitability. These channels can be distinguished based on their primary structure, kinetics and their sensitivity towards the neurotoxin, tetrodotoxin (TTX) (Catterall, 1992). In the present study, we describe the structure of a novel voltage-gated sodium channel, PN3 (Peripheral Sodium Channel 3), its cellular localisation in the dorsal root ganglia (DRG) and its functional expression in Xenopus oocytes.

Structure of PN3. Sequence analysis of a full-length cDNA clone, 7.3, isolated from a rat DRG cDNA library revealed that it has a 5868-base open reading frame, coding for a 1956 amino acid protein. The structural features of the α-subunit of a voltage-gated sodium channel are conserved in PN3. It contains four domains (I-IV), each consisting of 6 membrane-spanning segments (S1-S6); positively charged residues in the voltage sensor, S4; the inactivation gate between IIIS6 and IVS1; sites for cAMP-dependent phosphorylation and N-linked glycosylation. Among all known sodium channels, PN3 sequence is most homologous to the cardiac channel.

Expression of PN3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses of total RNA isolated from a variety of tissues indicated that PN3 mRNA is expressed only in regions containing sensory neurons within the peripheral nervous system. A strong positive signal was obtained in DRG and nodose ganglia. No signal was observed in brain, spinal cord, superior cervical ganglia, heart or skeletal muscle. *In situ* hybridisation using PN3-specific probes (oligonucleotides synthesised from the 3'-untranslated region) indicated that, within the DRG, PN3 mRNA is expressed in 76% of the small neurons (which primarily give rise to C-fibres) and 33% of the large neurons (which primarily give rise to Aβ fibres).

Functional analysis of PN3. Expression of PN3 in *Xenopus* oocytes produced an inward current with slow inactivation kinetics. The current was voltage-dependent and was carried by sodium ions, since reduction of extracellular sodium ions from 90mM to 20mM resulted in a hyperpolarising shift in the reversal potential from +46±2.1 mV to -7.4±9.08 mV, (mean ±SE, n=3). PN3 exhibited little or no activation at -10 mV, whereas most cloned sodium channels begin to activate between -60 and -30 mV in this expression system (Cribbs et al., 1990; Patton and Goldin, 1991; Trimmer et al., 1989). PN3 sodium current is resistant to high concentrations of TTX (IC_{50} ≥100μM), unlike the brain-type and skeletal muscle channels that are sensitive to TTX (IC_{50}=5-18nM) and the TTX-insensitive cardiac channels (IC_{50} ~1-6μM) (White et al., 1993). The amino acid critical for TTX-sensitivity, Phe/Tyr in TTX-sensitive channels and Cys in the TTX-insensitive cardiac channel (IS5-IS6) (Kallen et al., 1994) is replaced by Ser in PN3.

The biophysical and pharmacological properties of this novel sodium channel, PN3, correlate well with those described for a TTX-resistant sodium current in the small neurons of DRG. PN3 may play a role in primary afferent hyperexcitability and sensitisation.

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135P COMPARISON OF RELAXANT PROPERTIES OF OESTROGENS AND BISINDOLYLMALEIMIDE, A SELECTIVE INHIBITOR OF PROTEIN KINASE C, IN ISOLATED RAT AORTA

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We have demonstrated oestrogen-induced relaxation of rat aorta which appeared to be independent of the endothelium and extracellular calcium; relaxation was unaffected by indomethacin, L-NAME or methylene blue (Babaei *et al.* 1995). Since increased oestrogen production in ovine ovarian cycles and pregnancy was reported to be associated with increased blood flow and decreased protein kinase C (PKC) activity in uterine arteries (Magness *et al.* 1991) and activation of PKC was shown to increase vascular muscle tone in vitro (Rasmussen *et al.* 1984), we investigated the possibility that oestrogen-induced aortic relaxation may be associated with inhibition of PKC.

Rings of aorta 3-5 mm wide from male Hooded Lister rats (250-400g, Bradford strain) were placed in Krebs' solution containing 10μM indomethacin under 2g tension (37°C; 95% O_2, 5% CO_2). Rings were contracted to a similar extent by $PGF_{2\alpha}$ (10μM, an approximately EC_{80} concentration) or a direct activator of PKC, phorbol 12,13-dibutyrate (PDB), which caused concentration-dependent contraction. When contraction was stable 17β–oestradiol (β-EST), 17α–oestradiol (α-EST), oestrone (ESTN), diethylstilboestrol (DESB), (5-20μM) nifedipine (NIF) a calcium channel blocker or the selective inhibitor of PKC, bisindolylmaleimide (BIM) was applied for 40 mins. No vehicle effects occurred in this concentration range; higher oestrogen concentrations elicited vehicle effects. Statistical analysis on original data was performed using Student's unpaired t-test.

Oestrogens caused concentration-dependent gradual relaxation of contraction induced by both $PGF_{2\alpha}$ (10 μM) and PDB (2 nM) in all tissues. BIM relaxed contractions produced by PDB in a concentration-dependent manner but was relatively ineffective in relaxing $PGF_{2\alpha}$-induced contraction. Comparison of the relaxation (expressed as % reversal of contraction) produced by the oestrogens (20μM), NIF (0.1μM) and BIM (1-2μM) is shown in Table 1.

Table 1. Relaxation of contraction produced by PDB or $PGF_{2\alpha}$ expressed as % reversal of contraction. * indicates significant difference in extent of relaxation between groups, $p<0.01$; n=4-9 for each agent.

Relaxant Agent	PDB (2 nM)	$PGF_{2\alpha}$ (10μM)
β-EST (20 μM)	100	57.4 ± 5.5 *
α-EST (20 μM)	22.83 ± 3.3	15.5 ± 1.7
ESTN (20 μM)	30.75 ± 5.0	12.9 ± 2.1 *
DESB (20 μM)	100	100
BIM (1 μM)	81.20 ± 5.0	10.0 ± 2.5 *
BIM (2 μM)	100	12.5 ± 1.0 *
NIF (0.1 μM)	100	48.6 ± 3.14 *

The lack of effect of BIM on contraction elicited by $PGF_{2\alpha}$ and the different extent of relaxation of $PGF_{2\alpha}$- and PDB-induced contraction produced by various oestrogens and NIF suggests that while PDB mimics responses stimulated by physiological agonists, it activates contraction by pathway(s) different from those utilised by $PGF_{2\alpha}$. If oestrogen-induced relaxation primarily involved PKC inhibition, BIM would be expected to elicit relaxation of $PGF_{2\alpha}$ contraction comparable to that of the oestrogens; this was not observed.

These results do not exclude the possibility that oestrogens act on an isoform of PKC not affected by BIM but it appears unlikely that oestrogens relax vascular muscle by simple PKC inhibition.

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136P EFFECTS OF HALOPERIDOL ON EXCITATORY AND INHIBITORY AMINO ACID RECEPTORS EXPRESSED IN *XENOPUS* OOCYTES INJECTED WITH RAT BRAIN RNA

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Glutamate and γ-aminobutyric acid (GABA) receptors are potential targets for neuroleptics (Sherman et al., 1991). We have used two-electrode voltage clamp to investigate the effects of haloperidol (HAL) on glutamate and GABA receptors, in *Xenopus* oocytes injected with rat brain RNA.

Whole brain RNA (12μg/μl) from male CD rats (175-200g) was injected (50nl) into mature oocytes (Brackley et al., 1990). Responses to N-methyl-D-aspartate (NMDA), kainate and GABA were tested from day 5 post-injection.

HAL (10^{-9}M - 10^{-3}M) failed to evoke responses (n=50). Dose-inhibition curves for HAL on responses to 10^{-4}M NMDA, kainate or GABA suggested that it preferentially inhibits NMDA responses. HAL's IC_{50}s (±S.D.) were ~$1.8\times10^{-6}\pm9.0\times10^{-7}$M (NMDA;n=6), >$10^{-3}$M (kainate; n=6) and ~$3.6\times10^{-4}\pm1.9\times10^{-5}$M (GABA;n=4). Although, HAL (10^{-9}M - 10^{-3}M) dose-dependently inhibited all 3 responses, complete inhibition was not achieved. HAL's antagonism of kainate and GABA responses was non-competitive (maximal response reduced by ~35%, 5×10^{-4}M HAL (n=4) and ~18%, 10^{-5}M (n=2)) and voltage-independent (n=7). Washing for 10-30 min (2 ml/min) with frog Ringer saline fully reversed the kainate response inhibition. Antagonism of the GABA response was only partially reversed. NMDA responses were potentiated (>100%) by HAL in some oocytes (n=7) and antagonised in others (n=14). The latter was voltage-dependent in 7/8 oocytes (Fig. 1) and only partially reversible by washing (30 min; 2 ml/min) with frog Ringer saline.

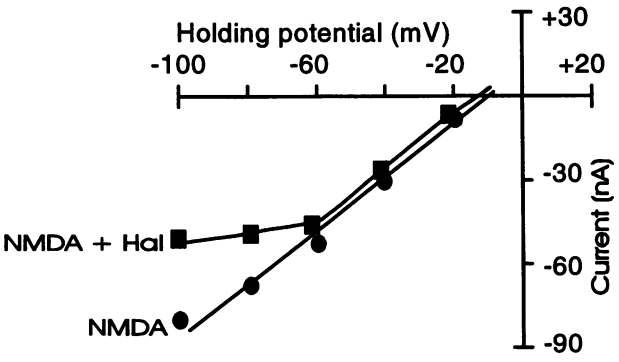

Fig 1 Voltage-dependent inhibition of NMDA responses by HAL

The finding that HAL potentiates or inhibits NMDA responses agrees with Fletcher and Macdonald (1993). These authors found the antagonism of hippocampal NMDA receptors in the mouse to be voltage-independent and suggest the potentiation is due to HAL acting as a partial agonist at the strychinine-insensitive glycine site on NMDA receptors. In the rat, however, the voltage-dependent inhibitory action of HAL indicates a binding site in the ion-channel of the NMDA receptor.

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137P PERTUSSIS TOXIN ENHANCES PHOSPHOINOSITIDE SIGNALLING IN BABY HAMSTER KIDNEY (BHK) CELLS EXPRESSING RECOMBINANT TYPE 1α METABOTROPIC GLUTAMATE RECEPTORS

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Metabotropic glutamate receptors (mGluR) constitute a family of G-protein coupled receptors that are activated by L-glutamate (Glu), the principal excitatory neurotransmitter in the brain. Of the eight mGluR subtypes cloned to date, mGluR1 and mGluR5 preferentially couple to phosphoinositide-specific phospholipase C (PLC). In the present study, baby hamster kidney (BHK) cells stably transfected with mGluR1α were used to study phosphoinositide turnover stimulated by this receptor subtype. A reported characteristic of the mGluR-mediated phosphoinositide response is its partial inhibition by pertussis toxin (PTX) in BHK and CHO cells expressing mGluR1α (Aramori and Nakanishi.,1992; Thomsen *et al.*,1993). Here, we have investigated further the effects of PTX on mGluR1α-mediated phosphoinositide signalling in BHK cells.

BHK-mGluR1α cells (passage 3-40) were maintained in DMEM, 5% dialysed foetal calf serum, 2 mM glutamine, 50 μg ml^{-1} gentamicin, 0.5 mg ml^{-1} G418 and 1 μM methotrexate. For [^{3}H]-$InsP_1$ determinations cells were labelled with [^{3}H]-inositol (1 μCi ml^{-1}) for 48 h. For [^{3}H]-$InsP_1$ and $InsP_3$ mass measurements, cells were washed in oxygenated Krebs-Henseleit buffer (KHB) and preincubated with KHB supplemented with 10 mM LiCl for 15 min. Test compounds were added and cells incubated at 37°C for a further 30 min and experiments terminated with trichloroacetic acid. [^{3}H]-$InsP_1$ and $InsP_3$ were measured in neutral cell extracts (Challiss *et al.*, 1994). Where indicated, cell cultures were pre-incubated with PTX for 22-24 h.

Glu, quisqualate (Quis) and 1**S**,3**R**-ACPD induced dose-dependent increases in [^{3}H]-$InsP_1$ accumulation (basal, 365 ± 19; +Glu, 2873 ± 224 d.p.m./mg protein). Respective EC_{50} values were 19 μM, 1.8 μM and 256 μM, with Glu (1 mM) and Quis (100 μM) evoking similar maximal responses (749 ± 58 (n=5); 734 ± 22 (n=5) % over basal accumulation), whilst 1**S**,3**R**-ACPD (1 mM) appeared to be a partial agonist (369 ± 22 (n=3) % over basal). In all cases, the agonist-stimulated response was dramatically attenuated in the absence of extracellular Ca^{2+}. Glu (300 μM; EC_{50}, 17 μM) and Quis (30 μM; EC_{50}, 0.7 μM) each caused a rapid increase in $InsP_3$ accumulation which was about 5 fold over basal values (basal, 43 ± 4; +Glu, 201 ± 8 pmol/mg protein) 30 s after addition, whilst 1**S**,3**R**-ACPD (1 mM) did not significantly increase $InsP_3$ accumulation. Pre-treatment of BHK-mGluR1α cells with PTX (1-100 ng ml^{-1}) resulted in a dose-dependent increase in basal [^{3}H]-$InsP_1$ accumulation which was 15.5 ± 1.4 fold-over-control levels at 100 ng ml^{-1} PTX. This effect of PTX was not observed in untransfected BHK cells. In PTX-treated BHK-mGluR1α cells, Glu evoked a modest increase in [^{3}H]-$InsP_1$ over this elevated basal accumulation (PTX/basal, 6032 ± 1284; PTX/Glu, 9270 ± 1278 d.p.m./mg protein). The PTX effect on the phosphoinositide cycle under basal conditions was also observed at the level of $InsP_3$ accumulation (-PTX, 66.4 ± 6.2; +PTX, 130.2 ± 23.7 pmol/mg protein (n=4)).

Thus, basal [^{3}H]-$InsP_1$ and $InsP_3$ levels were increased in PTX-pretreated BHK-mGluR1α cells suggesting that PTX treatment can dramatically enhance basal phosphoinositide turnover. The mechanism(s) underlying the unmasking of this 'constitutive activity' is currently being investigated.

We thank the Wellcome Trust for the award of a Toxicology studentship to A.M. Carruthers and Christian Thomsen (Novo Nordisk, Denmark) for providing the BHK-mGluR1α cells.

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138P GLUTAMATERGIC AND GLYCINERGIC POSTYNAPTIC POTENTIALS IN PRIMARY CULTURES OF RAT MOTONEURONES

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Primary cultures of neurones derived from rat embryo ventral horn display membrane properties characteristic of motoneurones (Elliott *et al.*, 1995). Considerable synaptic activity is seen in these long-term cultures. We report here experiments to investigate the nature of this synaptic input and its possible modulation by presynaptic receptor activation (Evans, 1989).

Enriched motoneurone cultures were prepared from 14 day embryonic rat ventral horn by density gradient centrifugation. These were maintained in 35mm Petri dishes containing a monolayer of spinal cord glial cells, in the presence of ciliary neurotrophic factor for 11-66 days. About 85% of the presumptive motoneurones showed intense choline acetyltransferase immuno-reactivity.

Electrophysiological studies of the neurones were obtained using whole cell patch clamp recording. In current clamp mode neurones had a mean R.M.P. of -67±7mV (±S.D., n=76), Rin 105±65MΩ and action potential amplitudes of 96±12mV. Most of the cultures demonstrated marked spontaneous synaptic activity, which was completely blocked by Cd^{2+} (0.5mM, n=4). Fast EPSPs were blocked by 10μM CNQX (n=61) and IPSPs blocked by 10μM strychnine (n=20). D-AP5 (20-50μM) depressed synaptic activity in 4/5 experiments. A number of cholinergic antagonists were tested but had no clear inhibitory actions except for dihydro-ß-erythroidine (10-100μM, n=4).

The spontaneous activity was inhibited by 2 min superfusion of 5μM baclofen (n=14), 10nM 5-carboxamidotryptamine (5-CT, n=9), adenosine (1-10μM, n=3) and L-AP4 (50-250μM, n=5), but not by 1S, 3R ACPD (100μM, n=2). Neither baclofen nor 5-CT had significant ($P>0.05$, paired t-test) effects on R.M.P. (control -63.9±2.8mV / 5-CT -63.6±2.8mV, n=7: control -65.6 ±3.8mV / baclofen -65.3 ±3.7mV, n=4) or Rin (control 113±26MΩ / 5-CT 117±27MΩ: control 222±92MΩ / baclofen 216±85MΩ).

In voltage-clamp (+TTX) fast inward currents were recorded following applications of AMPA (0.3-100μM) with an EC_{50} of 2.8±0.8μM (±s.e.mean, n=3). AMPA-induced currents were antagonized by 3μM CNQX with a pA_2 of 6.41. 5-CT (10nM, 4min, n=3) had no significant effect on the inward currents recorded in response to repeated applications of 5μM AMPA (ANOVA). NMDA (100μM, n=3) evoked no inward current in normal recording solution; a small response was seen in Mg-free solutions, which were markedly potentiated by 3μM glycine. Concentration-dependent inward currents were also evoked by glycine (10-300μM) with an EC_{50} of 57±10μM and antagonized by strychnine (0.1-0.5μM, $pA_2 \approx 6.8$)

It is concluded that glutamatergic and glycinergic interneurones are present in these cultures and make synaptic connections with the presumptive motoneurones. The presence of cholinergic neurotransmission needs confirmation. The spontaneous synaptic transmission appears to be susceptible to presynaptic inhibition.
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139P DEXAMETHASONE INDUCES RENAL CYTOCHROME P450-DEPENDENT NITRIC OXIDE AND EICOSANOID FORMATION IN THE RABBIT

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Dexamethasone (DEX) treatment of rabbits enhances the cyclooxygenase (COX)-independent component of the vasodilator response to arachidonic acid (AA) by 5-fold in the isolated, perfused kidney (Sessa *et. al.*, 1990). In the rat, DEX induces cytochrome P_{450} (P_{450}) 4A and 3A, isozymes which can generate AA metabolites (Lin *et. al.*, 1994) and nitric oxide (NO), the latter from L-hydroxy-arginine (Boucher *et. al.*, 1993). We, therefore, studied the mechanism by which glucocorticoid treatment enhances the renovascular response to AA.

Male NZW rabbits (2.3-2.5 kg) were treated with DEX acetate (2.5 mg/kg; s.c.) or corn oil vehicle (2ml) for 6 days. After anaesthesia, kidneys were excised and perfused with oxygenated Krebs-Henseleit buffer at a constant flow of 1ml/g/min at 37^{0}C. In the presence of indomethacin (2.8μM), kidneys were constricted with phenylephrine (1-3μM) to raise perfusion pressure from *ca* 40 to 100mmHg (Carroll *et. al.*, 1992). The vasodilator responses to AA (10μg) were tested before and after inhibition of P450 activity with 12, 12-dibromododec-11-enoic acid (DBDD; 10μM) and NO synthesis with L-nitroarginine (LNA;50μM). In control kidneys the residual AA response after indomethacin treatment (-8±4 mmHg) was unaffected by DBDD (n=6) or LNA (n=6), whereas in DEX treated kidneys DBDD (n=6) reduced the AA response from -25±9 to -10±6 mmHg and LNA (n=6) treatment inhibited the response to AA from -27±8 to -2±1 mmHg ($P<0.05$). Thus, after DEX treatment the COX-independent response to AA may involve a NO component.

Renal cortical microsomes were prepared from DEX treated (n=6) and control (n=6) rabbits to measure P450 activity. The NADPH (1mM) dependent conversion of ^{14}C-AA (7μM; 300μg protein) was increased from 20.5±0.6% in controls to 28.6±1.6% after DEX treatment ($P<0.05$). NO synthesis, measured fluorometrically, by renal cortical microsomes (200μg) incubated with 100μM L-hydroxy-arginine and NADPH (1mM) was increased from 30±12 to 176±40 pmol NO_2^-/30 min (control vs DEX treatment; $P<0.05$).

In summary, DEX-treatment induces P450 activity resulting in enhanced AA metabolism and NO synthesis. Each of these P450-dependent pathways may contribute to the renal COX-independent vasoactivity of AA.

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140P POSSIBLE BIOCHEMICAL MECHANISMS INVOLVED IN CARDIOTOXICITY: A COMPARISON BETWEEN BBR 2778, A NOVEL NON-CARDIOTOXIC COMPOUND, MITOXANTRONE AND DOXORUBACIN

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BBR 2778, (6,9-bis{[(2-amino)ethyl]amino}benzo[g]isoquinoline-5,10-dione dimaleate), is a new azaanthracenedione derivative with antitumor activity in *"in vitro"* and *"in vivo"* models (Pezzoni *et al.*, 1993) and devoid of cardiotoxicity in rodents (Cavalletti *et al.*,1993). The antineoplastic drugs doxorubicin (Dx) (Young *et al.*,1981) and mitoxantrone (MTX) (Faulds,1991) show clinical cardiotoxicity. A number of data suggest that cardiotoxicity could be related to the reduction of the quinone moiety to semiquinone radicals by flavoproteins, such as NADH dehydrogenase (NADH dehyd.) and NADPH cytochrome P_{450} reductase (NADPH CYP red.), with generation of superoxide anion (O_2^-) as a consequence of the semiquinone reoxidation (Tarasiuk *et al.*,1992; Kharasch *et al.*,1981).

We compared the ability of BBR 2778, Dx and MTX (100 μM) to oxidize NADH and NADPH and their ability to generate O_2^- after enzyme reduction. Moreover, we determined the substrate properties of the compounds for NADH dehyd. by calculating the IC_{10} (compound's concentration at which total cytochrome c (cyt. c) reduction is decreased by 10 %). The rates of NADH (μmol/min) and NADPH oxidation (nmol/min/mg protein) were determined spectrophotometrically at 340 nm by using an extinction coefficient (ε) of 6.22 $mM^{-1}cm^{-1}$. NADH dehyd. (0.15 - 0.75 U/ml), liver microsomal NADPH CYP red. (3 - 6 mU/ml) were used (Tarasiuk *et al.*, 1992; Kharasch *et al.*, 1981). The amount of O_2^- production (μmol/min) was determined spectrophotometrically by the rate of superoxide dismutase inhibitable cyt. c reduction at 550 nm and at an ε value of 19.6 $mM^{-1}cm^{-1}$. The IC_{10} (μM) was determined using compound concentrations up to 200 μM,and a large excess of both cyt. c and compounds with respect to NADH concentration (Tarasiuk *et al.*,1992). The results with Dx (see Table) are in agreement with those reported in literature (Mimnaugh *et al.*,1982; Stefanska,1993) and confirm that the affinity of Dx towards flavoprotein enzymes and the consequent oxygen free radicals production could be one of the biochemical mechanisms responsible of Dx cardiotoxicity. On the contrary, the results obtained with BBR 2778 and MTX do not correlate with the lack of cardiotoxicity of the former and the significant cardiotoxic effects of the latter. We are now investigating other possible mechanisms (such as iron dependent and NADPH dependent liver microsomal lipoperoxidation, cardiac cell energy state and cardiac mitochondrial calcium flux) that are known to play a role in the cardiotoxicity, in order to explain the opposite effects of MTX and BBR 2778.

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Table: NADH and NADPH oxidation rate and O_2^- production by 100 μM Dx, MTX and BBR 2778 catalyzed by NADH dehyd. and NADPH CYP red. IC_{10} values are determined by NADH dehyd. cytochrome c reduction. Results are expressed as mean (SD) of 6 replications.

COMPOUNDS	NADH ox. (μmol/min)	NADPH ox. (nmol/min/mg prot.)	O_2^- prod. (μmol/min)	IC_{10} (μM)
Doxorubicin	27.18 (1.5)	25.24 (3.29)	25.21 (1.06)	3.01 (0.70)
Mitoxantrone	4.53 (1.0)	6.76 (3.18)	0.47 (0.37)	> 200 —
BBR 2778	20.49 (0.8)	11.75 (2.61)	8.45 (0.25)	11.83 (0.90)

141P CHARACTERISATION OF A cAMP-RESPONSIVE REPORTER CHO CELL LINE USING ENDOGENOUS RECEPTORS

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A cAMP-responsive reporter cell line has been established through the stable transfection of Chinese hamster ovary (CHO) cells with a luciferase reporter plasmid (donated by Himmler *et al.*, 1993). Reporter cells show dose-dependent luciferase expression when incubated with forskolin to directly stimulate adenylyl cyclase (AC), owing to the presence of six cAMP response elements in the promoter region of the plasmid. In order to characterise receptor mediated changes in luciferase expression, reporter cells were screened for a G-protein coupled receptor (GPCR) subtype which would stimulate AC and one which would inhibit forskolin-stimulated AC. A stimulatory response was observed following incubation of cells with calcitonin gene-related peptide (CGRP), indicating the presence of CGRP or calcitonin receptors. An inhibitory response was observed following incubation of cells with 5-HT, consistent with the presence of members of the 5-HT_1 receptor family.

Calcitonin was also found to stimulate luciferase expression (pEC_{50} = 10.2 ± s.e.mean 0.04) and was approximately 10,000 times more potent than CGRP (6.6 ± 0.05) indicating that this response is mediated by calcitonin receptors. This classification is confirmed by the observation that the selective CGRP antagonist, CGRP-(8-37), was unable to attenuate the CGRP response in the reporter cell line, (Chiba *et al.*, 1989).

Additional 5-HT receptor agonists also inhibited forskolin stimulated luciferase expression in the rank order 5-CT(8.21 ± 0.11) >5-HT(7.59 ± 0.06) >CP-93,129(7.22 ± 0.10) >sumatriptan(5.42 ± 0.06) >8-OHDPAT(<5), consistent with the presence of 5-HT_{1B}-like receptors (Giles *et al.*, 1994). Inhibition by 5-HT was reversed by co-incubation with pindolol (5.54 ± 0.06) or cyanopindolol (6.82 ± 0.06), but not by the 5-HT_2 antagonist ketanserin, demonstrating the specificity of the response. Co-incubation with pindolol caused a parallel rightward shift of the 5-HT dose-response curve as expected for a competitive antagonist (pKb = 7.05 ± 0.18).

The reporter gene system showed the expected pharmacology for the endogenous receptors present in CHO cells and generated EC_{50} values which were comparable with those obtained by other groups using cAMP accumulation assays (Giles *et al.*, 1994; Chiba *et al.*, 1989). This characterisation thus confirms the value of the reporter gene system as a functional assay for both positively and negatively coupled GPCRs.
(Unless otherwise stated, values quoted are the mean pEC_{50} ± s.e.mean and n ≥ 3 in all cases.)

This work was supported by the BBSRC.

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142P STABLE EXPRESSION OF G-PROTEIN COUPLED RECEPTOR KINASE 2 DOMINANT NEGATIVE MUTANT (GRK2 DNM) IN NG108-15 CELLS

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A family of G-protein coupled receptor kinases (GRKs) is thought to play a major role in the phosphorylation and hence homologous desensitization of G-protein coupled receptors (Premont *et al* 1994). Although the phosphorylation of purified receptors by purified GRKs has been well documented, the role of GRKs in mediating desensitization in intact cells has not been widely studied. In a recent study (Kong *et al* 1995), human bronchial epithelial cells stably expressing GRK2 DNM (Lys^{220} in the catalytic domain mutated to Arg) displayed reduced desensitization of β-adrenoceptor stimulated adenylyl cyclase following agonist exposure. In the present study we attempted to stably transfect NG108-15 neuroblastoma x glioma hybrid cells with this GRK2 DNM construct.

Initial Western blotting of NG108-15 total cell protein with specific antibodies (a monoclonal antibody that recognises amino acids 500-531 of bovine GRK2 and a polyclonal antibody that recognises amino acids 648-671 of bovine GRK3) revealed that these cells express significant quantities of GRK2 (around 75ng/mg cell protein) and much lower levels of GRK3 (less than 5ng/mg cell protein). Purified recombinant GRK2 and GRK3 was employed for quantification. NG108-15 cells were then transfected with either GRK2 DNM (GRK2 with Lys^{220} mutated to Arg in the expression vector pCMVneo) or with the expression vector (pCMVneo) alone using lipofectamine (GIBCO BRL) according to the manufacturer's instructions. Geneticin (400μg/ml medium) resistant NG108-15 cell clones were isolated and expanded for Western blotting analysis.

Of 29 geneticin resistant clones transfected with GRK2 DNM, 18 showed enhanced immunoreactivity to the GRK2 antibody in Western blotting experiments (GRK2 wild type and GRK2 DNM proteins have similar electrophoretic mobility and are equally recognised by the GRK2 monoclonal antibody). Of these, two were selected for further study, and titration experiments revealed that each expressed the GRK2 DNM protein in at least a 20-fold excess over endogenous wild type GRK2. These clones did not display altered levels of GRK3 immunoreactivity as compared to non-transfected cells. A number of clones were also isolated that had been transfected with the pCMVneo vector alone. These did not display altered expression of GRK2 or GRK3 when compared to non-transfected cells. Initial experiments indicate that plasmid transfected and GRK2 DNM transfected cells show no difference in cyclic AMP accumulation following acute (8min) incubation with the IP prostanoid receptor agonist iloprost (1μM) or the direct activator of adenylyl cyclase, forskolin (10μM). These cells therefore represent an interesting model system with which to study the functional significance of GRK2 in the desensitization of G-protein coupled receptors.

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143P THE RESPONSE OF INTACT AND DAMAGED FASCIAE TO POTASSIUM AND CALCIUM IONS

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In 1963 Hadfield suggested that the subcutaneous fascia was pivotal to the process of wound contraction. In fascia the major cell is the fibroblast/ myofibroblast and its contraction, by unknown agents, reduces wound size. Using rabbit fascial fibroblasts , Yen Chow et al., (1984) showed these cells to have unusual resting membrane potentials and responses to potassium and sodium ions.This study investigated potassium and calcium ions on rat fascial fibroblasts.

Rat subcutaneous fascia (RF, n=8) from unwounded animals and granulation tissue (GT) from around subcutaneously placed silicone rods (either 1 (n=8) and 2 weeks (n=8), Malata et al., (1993), were obtained from pentobarbitone killed animals. Tissue strips (20 x 10 mm) for RF and GT were placed under a tension of 2g, superfused at 37°C, 1ml/min using Krebs solution and equilibrated for > 1 hr. Isometric responses were measured (Grass FTO3C) to bolus additions of calcium (0.75 to $3x10^{-4}$ moles) and potassium chloride (1 to $4x10^{-4}$ moles).

Both RF and GT gave dose dependent, reversible, repeatable relaxatory responses to potassium chloride and contractile responses to calcium chloride (figure 1a and b). All the responses had dose dependent durations of effect but none lasted more than 5 minutes. The overall degree of reactivity of the RF was greater than that of the GT. At the doses tested, for both calcium and potassium, the magnitude of the relaxatory and contractile responses were significant less in the granulation tissue. For potassium the reduction was; $P<0.01$ for both 1 and 2 week capsules. Similarly, for calcium; the 1 and 2 week capsules were significantly (Fig 1b) less reactive than nonwounded fascia.

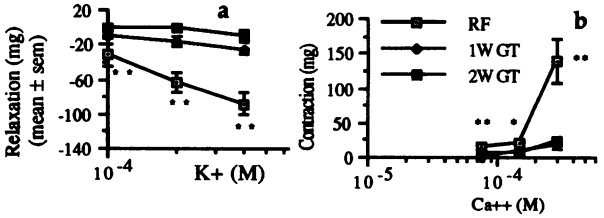

Figure 1a) Relaxatory responses in rat fascia (RF) and 1 and 2 week old granulation tissue (GT) to potassium chloride and b) Contractile response in RF and GT to calcium chloride, * $P<0.05$, ** $P< 0.01$ [unpaired Student's t-test].

Since RF and GT respond in a similar way to both the ions this suggests at least two of the properties of the fibroblast/ myofibroblast population in both the undamaged fascia and silicone induced tissue are similar in their contractile/relaxatory nature. The finding that GT is less reactive than RF is unexpected since it would contain more myofibroblasts and therefore a greater contractile ability. This suggests that fascial fibroblast transformation into myofibroblasts changes its phenotype, losing its reactivty to calcium and potassium ions. Further studies to determine if myofibroblasts accquire other sensitivities to compensate for this apparent loss are currently in progress.

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Malata, C.M.,et al., (1993) *Br. J .Pharmacol.*, **110**, 73P
Yen Chow, et al., (1984) *In Vitro*, **20**, 677-685.

144P *IN VIVO* GUT SELECTIVITY OF THE NOVEL MUSCARINIC ANTAGONIST, DARIFENACIN, IN THE CONSCIOUS DOG

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Darifenacin ((S)-2- {1-[2- (2, 3-dihydrobenzofuran-5-yl) ethyl] - 3 pyrrolidinyl}-2, 2 -diphenylacetamide) is a competitive muscarinic antagonist which *in vitro* has been shown to have good selectivity for the M_3 subtype over both neuronal M_1 (25 fold) and cardiac M_2 (100 fold) receptors (Wallis *et al*, 1995). We have conducted experiments in the conscious dog to ascertain the *in vivo* functional efficacy and selectivity of darifenacin for the gut over other parameters.

All experiments were conducted in fed male Beagle dogs (n=4). Each had previously been prepared with a chronically implanted miniature pressure transducer (Konigsberg Inc.) in the jejunum to measure digestive (food-induced) intestinal motility. Heart rate was determined from the Lead II ECG measured from subcutaneous titanium electrodes. In addition, pupil diameter was quantified under fixed illumination with the aid of an ophthalmoscope and dryness of the mouth, an index of basal salivation, was determined as the change in weight of a swab when placed in the cheek jowl for 1 minute. All parameters were recorded for 1 hour pre-dose and 4 hours post-dose.

Oral (0.03-3 $mgkg^{-1}$) administration of darifenacin induced a dose related inhibition (up to 90%, $P<0.01$) of digestive motility with a calculated ED_{50} value of 0.1 $mgkg^{-1}$. Peak inhibition was seen within 1 hour of dosing with activity demonstrable for at least 4 hours at doses above 0.03 $mgkg^{-1}$. Importantly, darifenacin caused no tachycardia at the doses tested and mydriasis only occurred at 3 $mgkg^{-1}$ ($P<0.01$). Darifenacin (0.1-3$mgkg^{-1}$) evoked a dose related dryness of the mouth, indicative of an inhibition of salivation, with an ED_{50} of 0.3 $mgkg^{-1}$. In previous experiments (McRitchie *et al*, 1993) atropine had no selectivity for the gut over salivation with ED_{50} values of 0.04 and 0.06 $mgkg^{-1}$ respectively. In addition, atropine caused significant tachycardia ($P<0.05$) at dose levels above 0.1 $mgkg^{-1}$ p.o. and mydriasis after 0.3 $mgkg^{-1}$.

In separate experiments (n=4), the chronic efficacy of darifenacin was assessed following repeated oral administration at 0.1 $mgkg^{-1}$, once daily for 10 days. The peak inhibition of digestive motility on days 4,7 and 10 was similar to that observed on both day 1 and in the dose response study. The effect on whole gut transit was also assessed on days 1,4,7 and 10 by measuring the recovery of radio-opaque markers from the faeces over a 24 hour period after food. An increased number of markers expelled (i.e. decreased transit time) was observed on day 1 (90% pellets recovered in darifenacin treated animals vs. 49% control; $P<0.01$) but on subsequent days the transit time was not significantly different from control. No effects on stool frequency or consistency were observed on any day. Thus, the efficacy of darifenacin was maintained on repeated oral administration without propensity to induce constipation.

In conclusion, darifenacin is a novel M_3 selective muscarinic receptor antagonist which caused reproducible inhibition of dog intestinal motility at doses that have minimal anticholinergic effects on heart rate (>30 fold selectivity) or pupil size (30 fold selectivity), and a reduced effect on salivation.

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Wallis, R.M, Burges, R.A, Cross, P.E. *et al* (1995). Pharmacol. Res. 31S 54.

145P ROLE OF M_2 AND M_3 MUSCARINIC RECEPTORS IN MEDIATING REFLEX BLADDER CONTRACTIONS IN THE ANAESTHETISED RAT

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The parasympathetic nervous system plays a key role during micturition by mediating reflex bladder contractions (De Groat *et al.*, 1993), although the role of muscarinic receptor subtypes in this process has not been clearly defined. In the present study, we investigated the effects of several subtype-selective and non-selective muscarinic antagonists on volume-induced bladder contractions (VIBC) in the anaesthetised rat. The inhibitory potencies of these compounds *in vivo* were compared to their affinity estimates (pA_2's) for antagonizing (+)-cis-dioxolane-induced contractions of the rat isolated bladder.

In vitro experiments: Strips of rat bladder were mounted in organ baths containing oxygenated Krebs solution (35°C, pH 7.4) for measurement of isometric tension. Two consecutive cumulative concentration-effect curves to (+)-cis-dioxolane were constructed in each tissue. Antagonists were equilibrated with the tissue for 90 minutes prior to the construction of the second curve. All antagonists produced concentration-dependent dextral displacement of the curves to (+)-cis-dioxolane. The rank order of pA_2 estimates (95% confidence interval in parenthesis): atropine, 9.1 (9.0 - 9.2) > 4-DAMP (4-diphenylacetoxy-N-methylpiperdine methiodide), 8.9 (8.7 - 9.2) > zamifenacin, 8.3 (7.9 - 8.6) = darifenacin, 8.3 (8.1 - 8.5) > p-F-HHSiD (para-fluoro-hexahydrosiladifenidol), 7.4 (7.3 - 7.5) > pirenzepine, 6.8 (6.7 - 6.9) > methoctramine, 5.9 (5.7 - 6.1) was consistent with the involvement of M_3 receptors.

In vivo experiments: Female Sprague-Dawley rats were anaesthetised with urethane and instrumented for the measurement of blood pressure, heart rate and intra-bladder pressure. Drugs were administered intravenously. Rapid infusion of saline into the bladder (200 $\mu l.min^{-1}$ for ~ 5 min), followed by a maintainence infusion of 5 $\mu l.min^{-1}$, evoked rhythmic VIBC (amplitude ~ 25 mm Hg) that could be partially inhibited (~ 55 %) by atropine (0.3 mg/kg; iv). The effect of atropine was mimicked by other muscarinic antagonists with the following rank order of potency ($ID_{35\%}$-dose ($nmol.kg^{-1}$; iv) required to produce 35% inhibition of the amplitude of VIBC; 95% confidence interval in parenthesis): 4-DAMP, 8.1 (2.6 - 24.9) > atropine, 20.7 (12.8 - 36.2) > methoctramine, 119.9 (34.3 - 205.2) > zamifenacin, 264 (165.6 - 415.2) ≅ darifenacin, 283.3 (127.9 - 618.9) > pirenzepine, 369.1 (56.8 - 2242.8) > p-F-HHSiD, 1053.8 (398.7 - 2705.6). Atropine, 4-DAMP and methoctramine produced dose-dependent increases in heart rate, presumably through M_2 receptor antagonism, with ED_{50}'s ($nmol.kg^{-1}$; iv) of 16.9 (10.6 - 27.3), 789.3 (133.7 - 4659.1) and 68.6 (51.4 - 102.8), respectively, whereas darifenacin, zamifenacin, pirenzepine and p-F-HHSiD had no significant effect on heart rate. The $ID_{35\%}$ (inhibition of VIBC) and ED_{50} (tachycardia) of methoctramine did not differ significantly from each other. Pre-treatment with propranolol (1 $mg.kg^{-1}$; iv) increased the $ID_{35\%}$ ($nmol.kg^{-1}$; iv) of methoctramine from 95.9 (70.3 - 126.9) to 404.5 (269.1 - 610.2), but had no significant effect on its tachycardiac potency.

The data suggest that cholinergic nerves mediate VIBC in anaesthetised rats via activation of both M_2 and M_3 receptors. Furthermore, it is proposed that M_3 receptor activation causes direct detrusor contraction whereas M_2 receptor activation causes contraction, indirectly, by reversing sympathetically (β-adrenoceptor)- mediated relaxation.

De Groat, W. C., Booth, A. M. & Yoshimura, N. (1993) in *Nervous control of Urogenital Tract*, (Maggi, C. A., ed). pp 227-290. Harwood Academic Publisher.

146P THE EFFECT OF PLATELET ACTIVATING FACTOR (PAF) ON THE RESPONSIVENESS OF RAT AND GUINEA-PIG ISOLATED TRACHEAL STRIPS TO ACETYLCHOLINE AND PROSTAGLANDIN $F_2\alpha$

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Platelet activating factor (PAF) has been implicated in asthma causing hyperresponsiveness of airway smooth muscle (Cuss *et al.*, 1984). Webber *et al.*, (1992) showed that PAF increased methacholine induced contractions of ferret tracheal smooth muscle. The aim of this study was to determine the effect of PAF on rat and guinea-pig tracheal smooth muscle and the effect of PAF on tracheal responses to acetylcholine (ACh) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$).

Male Dunkin-Hartley guinea-pigs (500-600g) or Sprague-Dawley rats (300-350g) were killed by stunning and bleeding. Four strips from guinea-pig trachea or a complete rat trachea cut in a spiral preparation was suspended under 0.5g tension in Greenberg-Bohr buffer containing bovine serum albumin, 0.25% w/v (GBB-BSA). Responses were recorded with an isotonic transducer and oscillograph.

Rat trachea produced a 0.85 ± 0.02 mm contraction with 0.1μM $PGF_2\alpha$ and 1.15 ± 0.04 mm in the presence of 1μM PAF (n=4). In each case despite the continued presence of agonist tracheae relaxed to the original resting tension within 90 sec. Pre-incubation with PAF (1μM) for 3 min prior to addition of $PGF_2\alpha$ (0.1μM) produced a 54.4±2% increase in $PGF_2\alpha$-induced contraction ($P \leq 0.05$, n=4) to the $PGF_2\alpha$ alone or vehicle matched control responses. Guinea-pig trachea produced contractions of 1.65 ± 0.08 mm, 2.25 ± 0.10 mm, and a 47.1 ± 2.2% increase respectively ($P \leq 0.05$, n=4). Also investigated were the effects of PAF and $PGF_2\alpha$ on cumulative ACh (1nM-300μM) induced contractions of trachea. Incubation (15 min) with PAF (1μM), $PGF_2\alpha$ (0.1μM) or PAF (1μM) and $PGF_2\alpha$ (0.1μM) increased the sensitivity and maximum response to ACh (see Table 1).

This study indicates that both PAF and $PGF_2\alpha$ induce hyperresponsiveness of both rat and guinea-pig tracheae to ACh. Further, the increased responsiveness of tracheae to ACh in the presence of $PGF_2\alpha$ is enhanced by PAF. This enhancement suggests their roles in the hyperreseponsiveness observed in asthma.

Cuss, F. M., Dixon, C.M.S & Barnes, P.J. (1984). *Lancet.* II,189-192.
Webber, S.E., Morikawa, T. & Widdicombe, J.G. (1992). *Br. J. Pharmacol.* 105, 230-237.

Table 1. Effects of PAF (1μM) and $PGF_2\alpha$ (0.1μM) on ACh induced contractions of rat and guinea-pig trachea. Mean EC50 and maximum response values are shown, s.e.m. values did not vary from means by >5%. Data with PAF or PGF2α were significantly different ($P \leq 0.05$) from ACh control data. Data in presence of both PAF and PGF2α differed significantly ($P \leq 0.05$) from when either agent was added singularly.

	RAT			GUINEA-PIG		
	n	EC50 (μM)	max (mm)	n	EC50 (μM)	max (mm)
ACh + GBB-BSA	9	9.00	2.18	4	10.18	4.98
ACh + PAF	4	4.95	3.01	4	6.60	6.05
ACh + PGF2α	4	5.10	2.40	4	2.60	5.65
ACh +PAF+PGF2α	4	3.80	3.10	4	1.90	6.75

147P RENAL A_1 ADENOSINE RECEPTOR mRNA LEVELS DURING THE DEVELOPMENT OF ACUTE RENAL FAILURE

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Renal vasoconstriction produced by adenosine is mediated by A_1 adenosine receptors. Gould *et al.* (1995a) have previously demonstrated, in the rat, a selective increase in the renal vasoconstrictor response to adenosine in acute renal failure (ARF) induced by glycerol, but not mercuric chloride ($HgCl_2$). Furthermore, the enhanced response to adenosine was accompanied by an increase in the density of renal A_1 adenosine receptors in glycerol-induced ARF (Gould *et al.*, 1995b). The aim of this study was to determine whether this increase in receptor density in glycerol-induced ARF is associated with enhanced levels of A_1 adenosine receptor mRNA.

ARF was induced in male Wistar rats (200 - 250g) either by injection of 50% v/v glycerol in saline (10 ml/kg i.m.) or $HgCl_2$ (2 mg/kg s.c.). Control animals were given equivalent volumes of saline. At 0.5, 16 and 48 h after injection, both kidneys were removed and total RNA extracted. β-Actin and A_1 adenosine receptor mRNA levels were quantitated by the reverse transcriptase polymerase chain reaction.

Renal A_1 adenosine receptor mRNA levels were significantly ($P<0.05$) elevated at 0.5, 16 and 48 h following induction of glycerol-induced ARF (Table 1). Levels of A_1 adenosine receptor mRNA increased during the development of ARF such that at 48 h there was a 3-fold increase relative to saline-injected rats. By contrast to saline-injected rats, no statistically significant increase in A_1 adenosine receptor mRNA levels was detected 48 h following injection of $HgCl_2$. The level of mRNA for β-actin in the kidney 48 h following injection of saline was $2.7 \pm 0.8 \times 10^9$ (n=5) copies/μg total RNA, and there was no significant difference in the levels of β-actin mRNA in kidneys of rats with either glycerol or $HgCl_2$-induced ARF compared to their respective time-matched controls.

The present findings suggest that the increased A_1 adenosine receptor density noted in glycerol-induced ARF is associated with elevated levels of A_1 adenosine receptor mRNA, implying increased transcription of the gene for this receptor. The increase in A_1 adenosine receptor mRNA found here at 48 h is similar to the increase in Bmax for renal A_1 adenosine receptors (2.5-fold) in rats with glycerol-induced ARF noted by Gould *et al.* (1995b). The absence of any statistically significant change in levels of mRNA for A_1 adenosine receptor in rats with $HgCl_2$-induced ARF, together with the lack of change in mRNA for β-actin, in either form of ARF, suggests that the increase in A_1 adenosine receptor mRNA noted in glycerol-induced ARF is not a general response to renal dysfunction.

This work was supported by the Wellcome Trust.

Gould, J., Bowmer, C.J. & Yates, M.S. (1995a) *Nephron.* 71, 184-189
Gould, J., Bowmer, C.J. & Yates, M.S. (1995b) *Br.J.Pharmacol.* 115, 5P.

Table 1 Renal A_1 adenosine receptor mRNA levels and plasma urea concentrations in rats with ARF.

Group	A_1 mRNA (copies/μg total RNA x10^6)	Urea (mg/100ml)	Group	A_1 mRNA (copies/μg total RNA x10^6)	Urea (mg/100 ml)
Saline (i.m.) 0.5 h	7.6 ± 1.0	52 ± 5	Glycerol (i.m.) 0.5 h	12.3 ± 0.7*	64 ± 13
Saline (i.m.) 16 h	7.1 ± 0.6	51 ± 4	Glycerol (i.m.) 16 h	19.4 ± 0.5**	216 ± 30
Saline (i.m.) 48 h	7.0 ± 0.4	41 ± 2	Glycerol (i.m.) 48 h	23.4 ± 1.8*	299 ± 89
Saline (s.c.) 48 h	12.1 ± 2.2	37 ± 2	$HgCl_2$ (s.c.) 48 h	8.0 ± 5.8	377 ± 23

Values are mean ± s.e.mean (n=5). * $P<0.05$; ** $P<0.001$ relative to saline-injected rats (Student's t-test)

148P THE EFFECT OF COOLING ON OVINE TRACHEAL AND BRONCHIOLAR SMOOTH MUSCLE

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Inhalation of cold air and exercise are recognized causes of bronchoconstriction in asthmatics. It was suggested that this results from cooling of the airway wall (Souhrada and Souhrada 1981). The aim of this study was to determine the direct effect of cooling on smooth muscle of ovine airways.

Tracheal strips and bronchiolar rings were prepared from male Merino sheep as described previously (Thulesius and Mustafa, 1994). They were suspended in Krebs' solution at 37°C, pretensioned at 2g and 1g respectively, gassed (95% O_2, 5% CO_2) and isometric tension recorded. Tissue responses were examined at 5°C intervals over the range 30°C - 5°C.

Cooling-induced contraction (CIC) was quick in onset and sustained in both tracheal and bronchiolar preparations. Increases in tension were temperature-dependent (Table 1). Maximal contraction in tracheal muscle was seen at 5°C and at 15°C in bronchiolar rings. On readjustment to 37°C the tone returned rapidly to basal levels in both preparations. Removal of tracheal and bronchiolar epithelium did not affect CIC. Incubation with tetrodotoxin (1μM) had no effect on CIC in either preparation, nor was the response to cooling significantly inhibited by pretreatment with indomethacin (1μM; 10μM), nordihydroguaiaretic acid (1μM), atropine (1μM), mepyramine (1μM) and phentolamine (1μM). However, bathing both tissues in Ca^{2+} free Krebs' solution containing 1mM EGTA reduced CIC at all temperatures. Incubation for 45 min reduced CIC at 20°C by 79.23 ± 1.98% in tracheal (n=6) and 32.7 ± 6.4% in bronchiolar (n=4) muscle, while incubation for 90 min resulted in reductions of 92.84 ± 2.9% and 58.3 ± 16.0% respectively ($P<0.05$).

Table 1. Effect of cooling on tracheal and bronchiolar tension (mg/mg tissue wt)

Temp °C	Trachea (n=32) +epi	Bronchiole (n=22) +epi
30	8.16 ± 1.04	8.33 ± 1.07
25	13.85 ± 1.72	14.55 ± 1.73
20	20.64 ± 2.77	22.45 ± 3.27
15	30.82 ± 4.12	25.77 ± 3.52
10	43.16 ± 4.51	22.74 ± 3.36
5	63.4 ± 5.1	

Data are expressed as increases, mean ± s.e.m., above basal tone at 37°C (8.20 ± 0.74 for trachea and 3.58 ± 0.50 for bronchiole). Responses were compared with basal tone using Student's paired t-test. $p<0.001$ in all cases.

In conclusion, cooling ovine tracheal and bronchiolar preparations rapidly induces contractile responses inversely proportional to temperature, which are not dependent on the presence of respiratory epithelium. The contractile effects do not appear to involve arachidonic acid metabolites nor a neurogenic process. However, extracellular Ca^{2+} is necessary.

Souhrada, M. and Souhrada, J.F. (1981) Respir. Physiol. 44, 311-323.
Thulesius, O. and Mustafa, S. (1994) Clinical Physiol. 14, 135-143.

149P INHIBITION OF INTERSTITIAL COLLAGENASE AND GELATINASE DOES NOT AFFECT INTIMAL THICKENING IN THE BALLOON CATHETER-INJURED RAT CAROTID ARTERY

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In the rat, neo-intima is barely existent in healthy arteries, but develops greatly after injury. Migration of smooth muscle cells is an important event in the formation of a neo-intima after arterial injury in the rat. There is ample evidence which suggests that matrix metalloproteinases (MMPs) degrade the basement membrane in which vascular smooth muscle cells reside, allowing migration, and that inhibition of these enzymes could reduce intimal thickening following intra-vascular injury. This series of experiments was undertaken to determine the role of MMPs in intimal formation after balloon-catheter injury in the rat.

UK-181,587 ({4-(N-hydroxyamino)-2(R)-[3-phenylpropyl]succinyl} -L-beta-cyclohexylalanine-N-[(2-{4-aminosulphonylphenyl})ethyl] amide) is a potent inhibitor of rat gelatinase (IC_{50} 20nM) and of rat collagenase (IC_{50} 30nM). Rats were anaesthetised and implanted with an indwelling intravenous infusion catheter accessing the left jugular vein, then allowed to recover. The distal part of the catheter was attached to an infusion pump. The rats were infused at $0.5ml.h^{-1}$ with either UK-181,587 ($80\mu g.kg^{-1}.min^{-1}$) prepared in hydroxypropyl-β-cyclodextrin (OHP-β-CD) (final concentration 5% w/v) or OHP-β-CD vehicle. This infusion rate of UK-181,587 was sufficient that a ten-fold dilution of blood samples reduced the gelatinolytic activity of exogenously added gelatinase (MMP-2) by 88 ± 5% (n=3).

Further rats were continuously infused intravenously with either UK-181,587 ($80\mu g.kg^{-1}.min^{-1}$) or vehicle and subjected to balloon over-stretch injury of the left carotid artery 2 days after the infusion commenced. Four days after injury, the number of smooth muscle cells present on the lumenal side of the internal elastic lamina in the UK-181,587-treated group was reduced to 2.9 ± 1.6 from 42.3 ± 0.3 (vehicle), (mean ± s.e. mean, n=3), confirming that migration had been inhibited.

To determine the effect of UK-181,587 on intimal formation, rats were prepared for continuous intravenous infusion for 16 days. One group of rats were infused with UK-181,587 ($80\mu g.kg^{-1}.min^{-1}$) in OHP-β-CD as above, the other group were infused with vehicle. Two days after beginning the infusion, all rats were subjected to a balloon over-stretch injury of the left carotid artery. The rats were allowed to recover and were killed 14 days later. The carotid arteries were pressure fixed with formaldehyde and glutaraldehyde, then sectioned transversely, stained and the areas of the vessel measured by computer assisted morphometry (Table 1).

Table 1.
Mean cross-sectional areas ± s.e.mean (sq mm).

	Vehicle (n=8)	UK-181,587 (n=7)
Intima	0.220 ± 0.025	0.216 ± 0.012
Media	0.120 ± 0.008	0.130 ± 0.010
Lumen	0.142 ± 0.034	0.135 ± 0.027

There were no significant differences ($p<0.05$, Student's t-test) between UK-181,587 treatment and vehicle treatment in terms of intima, media or lumen area.

We conclude that, although UK-181,587 has an inhibitory effect on gelatinase activity and on the migration of smooth muscle cells in the rat *in vivo*, it does not affect the formation of a neo-intima 14 days after injury in this model.

150P (S)-N^5-(1-IMINOETHYL)-ORNITHINE AND AMINOGUANIDINE EFFECTS ON ELECTRICAL FIELD-INDUCED NON-ADRENERGIC, NON-CHOLINERGIC RESPONSES OF GUINEA-PIG ISOLATED TAENIA CAECI

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Immunohistochemical studies have shown nitric oxide synthase (NOS) to be present in myenteric plexus neurones from the guinea pig taenia caeci (Saffrey *et al*, 1992). Non-adrenergic, non-cholinergic (NANC) electrical field stimulation of this tissue *in vitro* produces relaxations which are inhibited by the nitric oxide synthesis inhibitors N^{ω}-nitro-L-arginine (NOARG) and N^{ω}-nitro-L-arginine methyl ester (NAME), and reversed by L-arginine, suggesting a role for nitric oxide (NO) in these responses (Piotrowski *et al*, 1993, Piotrowski *et al*, 1994). 7-nitroindazole, a NOS inhibitor which displays selectivity for the neuronal (nNOS) and endothelial (eNOS) isoforms (Babbedge *et al*, 1994), is without effect in this tissue (Piotrowski *et al*, 1994).

To investigate further the nitrergic mechanisms in the taenia caeci, 2cm long segments of this tissue were removed from freshly killed guinea pigs (310-590g) and set up in 10ml isolated tissue baths maintained at 37°C and supplied with Kreb's solution containing atropine (10μM) and guanethidine (10μM) and bubbled with 95%O_2/5%CO_2. Supramaximal electrical field stimulation, using 1ms rectangular pulses, was applied in 20Hz trains lasting 10s and repeated every 90s. Mechanical activity of the preparations was recorded isotonically using a resting tension of 0.5g. The NANC relaxations obtained were expressed as means±sem of control relaxations.

Under these conditions, the NOS inhibitors aminoguanidine (100μM) or (S)-N^5-(1-Iminoethyl)ornithine (NIO) (10μM) appeared to have little or no effect on the electrically-evoked NANC relaxations (98.6±4.1%, n=10 n.s. and 101.9±1.4%, n=6 n.s. respectively). In tissues treated with either of these two drugs, NOARG (10μM) was still able to cause a reduction of the NANC relaxations (85.6±2.2% * and 87.0±5.1% * respectively), whilst subsequent application of L-arginine (1mM), produced a recovery of these responses (100.6±2.4% n.s. and 95.6±6.9% n.s. respectively).

These data provide further evidence that the nitrergic system in the guinea pig taenia caeci differs from that described in other tissues. One possible explanation, consistent with our present observations and with those previously reported (Piotrowski *et al*, 1993, Piotrowski *et al*, 1994), is that a novel isoform of nNOS is present in the taenia.

(*=$p<0.05$, n.s.=not significant, compared to pre-drug controls, Student's t-test)

This work was supported by NESCOT.

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151P DEXAMETHASONE ENHANCES GR205171 INHIBITION OF CYTOTOXIC-INDUCED PLASMA PROTEIN EXTRAVASATION IN FERRETS

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Tachykinin NK_1 antagonists have anti-emetic properties in the ferret (Bountra *et al.*, 1993), and are able to reduce plasma protein extravasation (PPE) caused by cyclophosphamide (CYP) in the rat bladder (Ahluwalia *et al.*, 1994), and by CYP or X-irradiation (Rx) in the ferret bladder and intestine (Alfieri & Gardner, 1995). Glucocorticosteroids are commonly used to augment anti-emetic therapy, and have been shown to inhibit capsaicin- or substance P-induced PPE in the rat urinary bladder (Bacci *et al.*, 1993).

This study was undertaken to determine whether the efficacy of GR205171, a tachykinin NK_1 receptor antagonist (Gardner et al, 1996), in inhibiting PPE induced by CYP or Rx can be improved by prior administration of dexamethasone (DEX). Adult male ferrets (0.8-1.5kg) were dosed subcutaneously with either saline (1ml kg^{-1}), GR205171 (0.3mg kg^{-1}), DEX (1mg kg^{-1}) or the combination of these doses of GR205171 and DEX, before administration of either CYP or Rx at previously determined ED_{100} emetogenic doses. DEX was administered 2h before the antineoplastic agent, and saline or GR205171, 5min before. Anaesthesia was induced with pentobarbitone sodium (60mg kg^{-1}), and Evans blue dye (50mg kg^{-1}) was administered intravenously 15min before the blood was flushed out with saline (time of sacrifice, see Table 1). The bladder, portions of duodenum and jejunum, a kidney and a lobe of lung were removed and the dye was extracted with formamide for 24h at 60°C, and quantified by spectrophotometry at 620nm. Pre-treatment with GR205171 or DEX significantly inhibited the CYP-induced PPE in the bladder, and this inhibition was increased when these drugs were co-administered (Table 1). The combined treatment also inhibited Rx-induced PPE in the small intestine. These results indicate that the inhibition of cytotoxic-induced inflammation by an NK_1 antagonist can be improved by dexamethasone in the ferret..

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Table 1. Major effects of GR205171 and dexamethasone on cytotoxic-induced PPE in ferrets (Values are means and s.e. mean, n=6-7 per group).

	Time†	Tissue	Saline	GR205171	Dexamethasone	GR205171 + dex.
CYP (125mg kg^{-1})	1h	Bladder	141±14.8	80±13.0**	84±18.1*	46±11.7**
Rx (2Gy)	2h	Duodenum	119±18.6	112±11.6	88±5.2	60±9.7**
Rx (2Gy)	2h	Jejunum	81±10.1	71±14.5	71±6.0	58±10.8

†Time between administration of cytotoxic agent and exsanguination. * $p<0.05$, ** $p<0.01$ compared with control, unpaired Student's t test.

152P ADENOSINE A_3 RECEPTORS MEDIATE A MAST CELL-DEPENDENT PLASMA PROTEIN EXTRAVASATION IN RAT SKIN

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The present study has investigated the effects of intradermal (id) administration of adenosine receptor agonists on plasma protein extravasation (PPE) in the skin of conscious rats.

Wistar rats of either sex (c. 300g) were anaesthetised (NO_2/isoflurane) and injected intravenously with 5μCi [^{125}I] human serum albumin (in 0.3mls). Following shaving, the skin on the back of the rat was injected (id) at nine sites with 50μl of different doses of adenosine receptor agonists and the animals allowed to recover from anaesthesia. Thirty minutes later, the rats were given an intraperitoneal overdose of sodium pentobarbitone, a blood sample taken by cardiac puncture, the skin removed and the injection sites cut out. Blood plasma and skin injection site radioactivity was determined and PPE in these sites calculated (as μl of plasma).

N^6-(3-iodobenzyl)-5-(N-methylcarboxamido-adenosine) (IB-MECA, 0.01-10μg, n=8), N^6-2-(4-aminophenyl) ethyl adenosine (APNEA, 0.1-10μg, n=6) and N-[(2-methylphenyl) methyl] adenosine (metrifudil, 0.03-10μg, n=7) each produced dose-dependent increases in PPE. In contrast, 5'-N-ethyl carboxamidoadenosine (NECA, 0.1-10μg, n=6), N^6-cyclopentyl adenosine (CPA, 0.1-100μg, n=6) and 2-[-p-(-carboxyethylphenylethylamine]-5-N-ethylcarboxamidoadenosine (CGS21680, 0.1-100μg, n=6) were weak or inactive. The adenosine A_3 receptor agonist, IB-MECA (Gallo-Rodriguez *et al.*, 1994), was the most potent compound in rat skin with an EC_{100} [dose producing a PPE of 100μl (± 95% confidence limits)] value of 36 (8-87) ng and a maximum PPE of 247 ±48μl at 3μg id. The rank order of agonist potency (equipotent dose ratio at EC_{100} level and percentage maximum response where IB-MECA = 1 and 100% respectively) was IB-MECA (1, 100%) > APNEA (14, 76 ±10%) = metrifudil (28, 40 ± 28%) > NECA (>2500, 34 ±8%) > CPA (>2500, 25 ±9%) > CGS21680 (>2500, 10 ± 1%).

The PPE produced by IB-MECA was not affected by the non-selective A_1/A_2 receptor antagonists, 8-phenyltheophylline (3mgkg^{-1} iv, n=4) or 9-fluro-2-(2-furyl)-5,6-dihydro-[1,2,4]-triazolo-[1,5-c]-quinazin-5-imine (CGS15943, 1mgkg^{-1} iv, n=5) (Collis *et al.*, 1985, Rollins, *et al.*, 1994), but was abolished by cyproheptadine (0.1mgkg^{-1} iv, n=4) and in rats pretreated for 4 days with compound 48/80 (1.2mgkg^{-1} day^{-1} ip, n=4).

These results show that certain adenosine receptor agonists produce a mast cell dependent PPE in rat skin which apparently is mediated via stimulation of adenosine A_3 receptors.

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153P SILDENAFIL, A POTENT SELECTIVE INHIBITOR OF TYPE 5 PHOSPHODIESTERASE, ENHANCES NITRIC OXIDE-DEPENDENT RELAXATION OF RABBIT CORPUS CAVERNOSUM

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The neuronal nitric oxide (NO)/ cyclic GMP (cGMP) system is believed to be the main pathway mediating penile erection (Burnett, 1995). Agents that inhibit cGMP hydrolysis may therefore enhance the erectile process. Sildenafil (Viagra™, 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d] pyrimidin-5-yl) phenylsulphonyl]-4-methylpiperazine) is a novel and selective inhibitor of the cGMP specific phosphodiesterase type 5 enzyme (PDE5) found in human corpus cavernosum; IC_{50} 4nM (Ballard *et al*, 1996). Sildenafil is currently undergoing clinical evaluation as an oral treatment for male erectile dysfunction. Here, we report the effects of sildenafil on relaxation of pre-contracted rabbit corpus cavernosal tissue induced by electrical field stimulation (EFS) and by the NO-donor, sodium nitroprusside (SNP).

Corpus cavernosal tissue strips from male New Zealand White rabbits (2-3kg) were mounted under a resting tension of 2g, in organ baths containing Krebs buffer at 37°C, plus 1μM atropine and 5μM guanethidine, gassed with 95% O_2 / 5%CO_2. Following equilibration, these tissues were contracted with phenylephrine (10μM). The relaxation responses of tissues to EFS were determined at sequential frequencies of 2, 4, 8 and 16Hz (10volts, 0.2msec pulse width delivered as 10sec trains). Relaxation to EFS was then determined after treatment of tissues with sildenafil (1nM-1μM). Similar cavernosal tissue preparations were used to determine the effects of sildenafil on relaxation responses to SNP (0.01-100μM).

EFS-induced relaxation of rabbit corpus cavernosal tissue strips was completely blocked by tetrodotoxin (1μM) and inhibited by at least 87 % by the NO-synthase inhibitor, Nω-nitro-L-arginine (100μM). The guanylate cyclase inhibitor, methylene blue (30μM), inhibited relaxation by 29-46%. These data confirmed the role of the neuronal NO/cGMP pathway in corpus cavernosal relaxation. Sildenafil (1-100nM) enhanced the relaxation of rabbit corpus cavernosal tissue elicited by EFS in a concentration-dependent manner. At 2Hz, the mean relaxation response in the presence of 100nM sildenafil was increased to 2.8-fold the pre-treatment response. The maximum effect concentration for sildenafil appears close to 100nM as there was only a slight increase of effect at 1μM. Sildenafil (1μM) also significantly reduced the geometric mean EC_{50} for sodium nitroprusside-induced relaxation of cavernosal tissue from 6.15μM to 0.77μM, representing an 8-fold increase in sensitivity (Table 1).

Table 1. Effect of sildenafil on geometric mean EC_{50} values for SNP-induced relaxation of rabbit corpus cavernosal tissue.

	Sildenafil concentration			
	Pre-treatment	10nM	100nM	1μM
EC_{50} (μM)	6.15	4.94	1.07***	0.77***
95% C.I.	(3.04, 12.46)	(3.24, 7.53)	(0.73, 1.56)	(0.44, 1.36)

*Results are geometric mean EC_{50} and 95% confidence interval (C.I.) (n=7-10 tissues). Significant differences from pre-treatment, by Students t-test, are shown as:- *** $P < 0.001$*

This study shows that the selective PDE5 inhibitor, sildenafil, enhances relaxation of corpus cavernosal smooth muscle induced by neuronal NO and an NO-donor. Overall sensitivity to NO may be increased up to 8-fold. Sildenafil, therefore, enhances the endogenous pathway leading to corpus cavernosal relaxation and penile erection.

Ballard, S. A. *et al* (1996), J. Urol. (in press).
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154P EFFECTS OF THE NOVEL PHOSPHODIESTERASE TYPE 5 INHIBITOR, SILDENAFIL, ON METHACHOLINE-INDUCED RELAXATION OF RABBIT ISOLATED CORPUS CAVERNOSUM

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Sildenafil (VIAGRA™, 1-[4-Ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)phenylsulphonyl]-4-methyl piperazine), is a novel and selective inhibitor of the human cyclic GMP (cGMP) specific phosphodiesterase type 5 enzyme (PDE5) found in human corpus cavernosum; IC_{50} 4nM (Ballard *et al*, 1996). Sildenafil is undergoing clinical evaluation as an oral treatment for male erectile dysfunction. Its mechanism of action is believed to be via enhancement of the nitric oxide (NO)/cGMP pathway, mediating penile erection. Potential sources of this NO drive include cavernosal endothelial cells and non-cholinergic/non-adrenergic neurons (Burnett, 1995). In this study, we examined the effects of sildenafil on endothelium-dependent relaxation of rabbit corpus cavernosum elicited by the selective muscarinic receptor agonist, methacholine.

Corpus cavernosal strips, from male New Zealand White rabbits (2.5 - 3 kg) were mounted in organ bath chambers under a resting tension of 2g, and perfused with Krebs buffer at 37°C gassed with 95% O_2 / 5% CO_2. Following equilibration, these tissues were pre-contracted with phenylephrine (10μM). The subsequent response was allowed to stabilise over 15 min prior to constructing serial concentration response curves to methacholine. Tissues were then washed and methacholine concentration responses curves repeated after addition of sildenafil (1nM - 1μM).

Methacholine elicited concentration-dependent relaxation responses in corpus cavernosal tissues, which were inhibited by atropine. Endothelial cell removal and treatment with the NO-synthase inhibitor, Nω-nitro-L-arginine, also inhibited relaxation. Thus, methacholine-induced responses are mediated through muscarinic receptors, leading to the release of NO from endothelial cells, which causes relaxation of the cavernosal smooth muscle.

Sildenafil potentiated relaxation induced by methacholine in a concentration-dependent manner. At 1μM, sildenafil reduced the methacholine EC_{50} to 25% of the control value (Table 1). Sildenafil (1μM) also enhanced the maximum response to methacholine by 74 ± 14% (mean ± SEM, n=7) above pre-treatment.

Table 1. Effects of sildenafil on methacholine-induced relaxation of phenylephrine contracted rabbit corpus cavernosum.

	Concentration of Sildenafil				
	Control	1nM	10nM	100nM	1μM
EC_{50} (nM)	43.7	23.4	20.0*	11.7**	9.8**
(95% C.I.)	(36-54)	(11-54)	(9-42)	(6-22)	(6-16)

*EC_{50} = concentration of methacholine producing relaxation equivalent to 50% of the pre-treatment maximum relaxation response. Values are geometric means ($n \geq 6$) with 95% confidence intervals (95% C.I.). Significant differences from pre-treatment by Students t-test are:- * = $P < 0.05$, ** = $P < 0.001$.*

This study shows that sildenafil enhances the relaxation of corpus cavernosal tissue induced by a muscarinic receptor agonist, via a mechanism dependent on release of NO from cavernosal endothelium. Thus, the efficacy of sildenafil in men with erectile dysfunction, may in part be mediated by potentiation of the relaxant effects of NO released from cavernosal endothelium under acetylcholine stimulation.

Ballard, S. A. *et al* (1996), J. Urol. (in press).
Burnett, A.L. (1995), Biol. Repro. 52, 485 - 489.