EXPOSURE OF NG108-15 CELLS TO ILOPROST ALTERS RESPONSIVENESS TO STIMULATORS AND INHIBITORS OF ADENYLATE CYCLASE

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Prolonged stimulation of prostacyclin (PGI_2) receptors in platelets reduces subsequent PGI_2 and adenosine A_2 mediated activation of adenylate cyclase (Edwards et al., 1987). The NG108-15 neuroblastoma hybrid cell line also expresses receptors for both PGI_2 and adenosine which mediate adenylate cyclase activation (Kenimer & Nirenberg, 1981), as well as opiate receptors which inhibit adenylate cyclase (Klee & Nirenberg, 1976). In the present study, we investigated whether prolonged activation of PGI_2 receptors in NG108-15 cells with the stable PGI_2 analogue iloprost, would alter the subsequent responsiveness to agonists that activate or inhibit adenylate cyclase in these cells.

Cells of the NG108-15 and NCB-20 cell lines (passage 16-25) were grown in 80cm^2 flasks to confluency. Following incubation with drugs, the cells were washed before freezing at -80°C. Adenylate cyclase activity was measured in the cell homogenates (unwashed membranes) as described previously (Edwards et al., 1987).

In NG108-15 cell homogenates, iloprost activated adenylate cyclase with an EC_{50} of about 15nM; 1μM iloprost produces a 7 to 10-fold increase over basal activity. Likewise, the adenosine A_2 selective agonist 5'-(N-ethyl)-carboxamidoadenosine (NECA) activated adenylate cyclase with an EC_{50} of about 450nM. There was a maximal 3 to 4-fold increase above basal enzyme activity with 10μM NECA. Preincubation of cells with iloprost (10μM; 17h) reduced the subsequent responsiveness to both iloprost and NECA. Adenylate cyclase activity in the presence of 1μM iloprost was reduced by 72±2% (n=6) and that in the presence of 10μM NECA by 29±4% (n=5). Responses to both NaF and forskolin were also reduced by 20-40% in iloprost pretreated NG108-15 cells. In contrast, iloprost pretreatment of NCB-20 cells did not reduce adenylate cyclase activation by NECA, NaF or forskolin. There was however homologous loss of sensitivity to iloprost.

In NG108-15 cells, morphine inhibited iloprost (1μM) stimulated adenylate cyclase activity with an IC_{50} of around 1μM. Maximal inhibition of iloprost stimulation by morphine was 36±1% (n=3). Following iloprost desensitization, the IC_{50} for morphine inhibition of subsequent iloprost (1μM) stimulated adenylate cyclase was unchanged. However, iloprost desensitization enhanced the maximum morphine inhibition from 36±1% to 48±1% (n=3). In control cells, the % inhibition of adenylate cyclase by morphine is independent of the initial level of enzyme activity. It is unlikely therefore that the enhanced sensitivity to morphine following iloprost pretreatment relates to the lower levels of adenylate cyclase activity in these cells.

In conclusion, there are clear differences between the effects of iloprost pretreatment on NG108-15 and NCB-20 cells. The heterologous desensitization observed in NG108-15 cells is likely to be due to modulation of receptor(s) or G-proteins, and we propose to exploit these cell lines to identify the biochemical mechanisms involved.

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LACK OF SYNERGISM OF ILOPROST AND SODIUM NITROPRUSSIDE ON RABBIT VASCULAR SMOOTH MUSCLE

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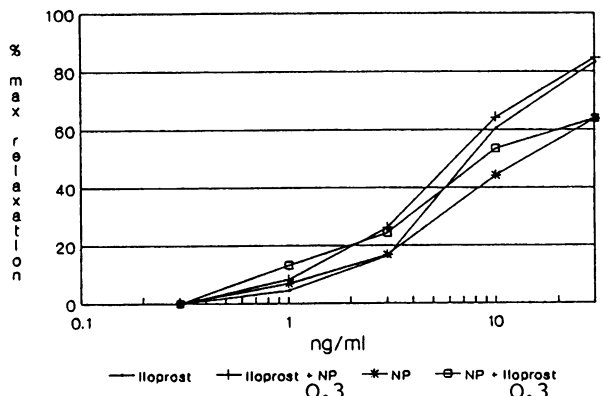
Endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) from porcine cultured endothelial cells (EC) is nitric oxide (NO; Palmer et al., 1987). Radomski et al. (1987) have shown that prostacyclin (PGI_2) synergizes with NO to inhibit platelet aggregation. Levin et al. (1982) have also shown that PGI_2 and sodium nitroprusside (NP) synergize as inhibitors of platelet aggregation. Here we report the lack of synergism between iloprost and NP on rabbit vascular smooth muscle.

New Zealand white rabbits (2.5kg) were anaesthetized and exsanguinated. The coeliac (RbCA) and mesenteric (RbMesA) arteries were freed in situ of any adipose tissue and strips were spirally cut from the vessels (Bunting et al., 1976). The strips were then suspended and superfused in cascade (Vane, 1964) with warmed (37°C) and gassed (95%O_2-5%CO_2) Krebs' solution containing indomethacin (5.6 μM) at a flow rate of 5ml/min. The strips were contracted with U46619 (30nM). Iloprost, PGI_2 and NP were infused (0.05 ml/min) for 5 min in order to achieve a steady-state. Dose-response curves were constructed with each agonist and then a sub-threshold dose of either iloprost or NP was used to repeat the dose-response curve. Results are expressed as % of maximum relaxation.

Figure 1



There was no synergism between iloprost and NP observed using the RbCA (Figure 1) or RbMesA. Using single bolus injection of nitric oxide, no synergism was observed between nitric oxide and subthreshold infusions of PGI_2.

Prostacyclin and its analogues act via stimulation of adenyl cyclase (Gorman et al., 1977) whereas nitric oxide and the nitrovasodilators act via stimulation of guanyl cyclase. Whether this lack of synergism on vascular smooth muscle is consistent across species remains to be established.

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INHIBITION OF FMLP-INDUCED AGGREGATION OF RABBIT NEUTROPHILS BY NITRIC OXIDE

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The vascular endothelium releases on unstable factor, endothelium-derived relaxing factor (EDRF), now characterised as nitric oxide (NO) (Palmer et al, 1987). The antiaggretatory activity of EDRF or NO on platelets is potentiated by the selective inhibitor of c-GMP phosphodiesterase, M&B 22948 and by superoxide dismutase (Radomski et al, 1987). Indirect studies have suggested that EDRF can inhibit A23187-induced leucocyte aggregation in vitro (Bhardwaj et al, 1988). In the present study, we have investigated the effects of NO on aggregation of rabbit polymorphonuclear leucocytes in vitro induced by formyl-methionyl-leucyl-phenylalanine (FMLP), and have studied the actions of superoxide dismutase (SOD) and M&B 22948.

Neutrophils were prepared from freshly collected citrated rabbit whole blood by red cell sedimentation with 2.5% methylcellulose followed by centrifugation (230g for 30 minutes) on Ficoll-Hypaque (Pharmacia). After hypotonic lysis of any remaining red cells, the neutrophils (>95% pure) were resuspended in Tyrode's solution and adjusted to a final concentration of 5×10^6 cells ml^{-1}. Leucocyte aggregation in 0.4 ml aliquots (37°C) of the stirred suspension, induced by FMLP (10^{-6}M) was measured in a Payton dual channel aggregometer.

Incubation with NO (1.32 - 132µM) induced a concentration-related inhibition of neutrophil aggregation. However, the small inhibition, observed after 30 sec incubation with NO (13µM) of 8 ± 3% (n=5), probably reflects the rapid breakdown of NO, particularly in the presence of neutrophils which can release oxygen radicals. Pre-incubation of the leucocytes with SOD (15 units ml^{-1}) alone for 30 sec or 60 sec inhibited leucocyte aggregation by 3.3 ± 1.7% and 12 ± 2% respectively (n=3). However, pre-incubation of the leucocytes with SOD (15 units ml^{-1}) substantially potentiated the inhibitory action of NO (13 µM), reaching 40 ± 5% inhibition (n=5, $P<0.001$) after 30 sec, which was maintained at 60 sec. Likewise, SOD (15 units ml^{-1}) significantly ($P<0.001$) augmented the effects of a 30 sec incubation with lower concentrations of NO (1.3 µM; from 2.3 ± 1.8% to 36 ± 4% inhibition, n=6), as well as those of threshold concentrations of NO (0.13µM; from 0.5 ± 0.5% to 28 ± 4% inhibition). Pre-incubation with M&B 22948 (3µM), which itself did not inhibit leucocyte aggregation, potentiated ($P<0.001$) the effects of NO (132µM), from 15 ± 2% to 36 ± 7% inhibition, which was further augmented to 67 ± 7% (n=4) inhibition in the presence of SOD (15 units ml^{-1}).

These findings indicate that NO can inhibit rabbit neutrophil aggregation in vitro, an effect which can be potentiated by SOD. The finding that this inhibitory effect of NO, like that exerted on platelets, can be significantly augmented by the selective c-GMP phosphodiesterase inhibitor M&B 22948, suggests that this action in leucocytes is mediated via activation of guanylate-cyclase.

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SELECTIVE ANTI-PLATELET SYNERGISM BETWEEN NITRODILATORS AND THE PROSTACYCLIN MIMETIC RS93427

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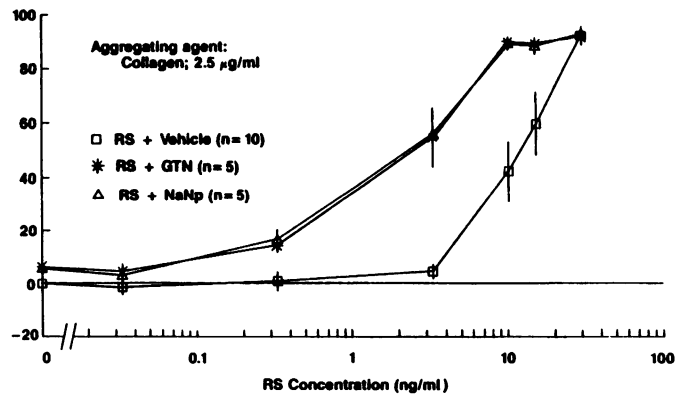
Vascular endothelial cells liberate nitric oxide (NO), whose actions, via increased intracellular cyclic GMP, are mimicked by "nitrodilator" drugs (Palmer et al; Fridovitch, 1987). PGI_2, acting via increased intracellular cyclic AMP produces similar responses, including vasodilatation and inhibition of platelet aggregation, but differs by its lack of potency for inhibiting the adhesion of platelets to surfaces such as collagen microfibrils. In addition, NO is synergistic with PGI_2 in inhibiting collagen-induced platelet aggregation, but is not synergistic with PGI_2 for inhibiting platelet adhesion (Radomski, 1987a,b).

Citrated human platelet-rich plasma (PRP) was induced to aggregate by addition of collagen suspension (2.5 $\mu g\ ml^{-1}$; Chronolog) or ADP (1.6 $\mu g\ ml^{-1}$, Sigma). The platelet inhibitory effects of RS93427-017 (RS) were examined in the presence of glyceryl trinitrate (GTN), sodium nitroprusside (NaNp), or vehicle. The minimal inhibitory concentrations of GTN (6-25 $\mu g\ ml^{-1}$) and NaNp (0.3-1.3 $\mu g\ ml^{-1}$), which gave <14% inhibition when added alone, were determined for PRP of each donor prior to their use in conjuction with RS. For collagen-induced aggregation (Fig.1), dose-response inhibition by RS in the presence of either nitrodilator demonstrated a 9-fold reduction in ED_{25} and 5-fold reduction in ED_{50} concentrations of RS ($p<0.01$ for each comparison, 2-tailed paired Student's t-test). By contrast, inhibition by RS of ADP-induced aggregation was not potentiated by GTN or NaNp. In anesthetized rats, intravenous administration of RS induced a dose-dependent depressor response, with a threshold of 0.3 $\mu g\ kg^{-1}$ and maximal response of 37+ 6mm Hg at 30 $\mu g\ kg^{-1}$ (8 rats per group). Intravenous infusion of GTN at a minimal effective dose of 1 $\mu g\ kg^{-1}\ min^{-1}$ did not alter the hypotensive dose-response curve for RS.

Thus, coadministration of a "nitrodilator" with a PGI_2 mimetic, as exemplified by RS93427-017, may render the latter compound relatively more selective for inhibiting thrombus formation than for producing dilatation.

Figure 1



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EFFECTS OF ACETYLCHOLINE AND NITRIC OXIDE ON COUPLING MECHANISMS UNDERLYING CONTRACTION AND RELAXATION IN RAT ISOLATED AORTIC RINGS

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Endothelium-dependent vascular smooth muscle relaxation induced by acetylcholine (ACh) is mediated via stimulation of soluble guanylate cyclase and increased levels of cyclic guanosine monophosphate (cGMP) (Rapoport & Murad, 1983). However, ACh-induced vascular relaxation is also dependent on the nature of the contractile agonist used to raise tone and this is reflected in an altered ability to increase levels of cGMP (Morrison & Pollock, 1988). The mechanism underlying these effects remains unclear. This study, therefore, re-examined the phenomenon by comparing responses of rat isolated aortic rings to ACh and nitric oxide (NO), which has been identified as the endothelium-derived relaxing factor (EDRF) (Palmer et al, 1987). Aortic rings were contracted with noradrenaline (NA) or phorbol-12-myristate-13-acetate (PMA). NA-induced contraction is associated with increased rate of phosphatidylinositol (PI) turnover, whereas PMA-induced contraction is mediated via direct stimulation of protein kinase C. The effects of ACh and NO on tone, cGMP levels and PI turnover were examined.

Aortic rings (2-3 mm in length) prepared from the descending thoracic aorta of male Wistar rats (250-300 g) were suspended under an initial resting tension of 2 g, between wire hooks in 25 ml organ baths containing Krebs' bicarbonate buffer, gassed with a mixture of 95% O_2/5% CO_2. Isometric tension was recorded with Statham force displacement transducers and displayed on a Grass polygraph. Tone was raised with either NA (EC_{70}: 2×10^{-7} M) or PMA (EC_{70}: 5×10^{-7} M) and inhibitory responses obtained to ACh and NO. At intervals following the addition of these vasodilator agents, tissues were frozen in liquid nitrogen then homogenised and extracted in trichloracetic acid (5% v/v). The cGMP content of an aliquot of each extract was determined by radioimmune assay. In separate experiments, agonist-induced hydrolysis of PI was monitored by measuring the formation of phosphatidic acid (Ptd OH) in aortic rings prelabelled with [^{32}P]-orthophosphate. Lipids were extracted according to Lloyd et al (1972).

ACh and NO both produced powerful dose-dependent relaxations of aortic rings precontracted with NA (2×10^{-7} M) but were less able to relax aortic rings precontracted with PMA (5×10^{-7} M). ACh (10^{-5} M) and NO (3×10^{-6} M) increased cGMP levels associated with inhibition of NA-induced tone (control: 30.8 ± 6.2 pmol.g^{-1}; ACh: 282.0 ± 20.8 pmol.g^{-1}; NO: 3102.0 ± 475.4 pmol.g^{-1}; mean ± S.E. mean, n = 4, $P < 0.001$). The increases in cGMP levels induced by ACh (10^{-5} M) and NO (3×10^{-6} M) in aortic rings precontracted with PMA (5×10^{-7} M) were much smaller. ACh (10^{-5} M) and NO (3×10^{-6} M) both inhibited PI hydrolysis stimulated by NA (2×10^{-7} M) (NA: 1188.4 ± 188.7 cpm.mg^{-1}; ACh: 485.0 ± 82.0 cpm.mg^{-1}; n = 4, $P < 0.01$; NA: 501.0 ± 72.5 cpm.mg^{-1}; NO: 296.2 ± 19.1 cpm.mg^{-1}; n = 4, $P < 0.05$). This study suggests that PMA inhibits both endothelium-dependent and endothelium-independent relaxation via stimulation of protein kinase C in vascular smooth muscle cells.

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CYCLOSPORIN MEDIATED CHANGES IN ARACHIDONIC ACID RELEASE FROM MEMBRANE PHOSPHOLIPIDS IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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The mechanism of acute cyclosporin (CS) nephrotoxicity is unclear, although it appears to be haemodynamically mediated and associated with renal vasospasm (Murray et al 1985). Prostacyclin (PGI_2), a potent vasodilator, plays a central role in the regulation of renal blood flow and hence inhibition of PGI_2 by CS may account for some aspects of CS nephrotoxicity and the associated vascular injury. We have previously reported that CS inhibits PGI_2 formation by cultured human umbilical vein endothelial cells (HUVEC) (Brown et al 1987). The aim of this study was to investigate the effects of CS on liberation of arachidonic acid (AA) from membrane phospholipids. Such effects could contribute to the observed decrease in PGI_2 formation.

HUVEC were isolated and cultured using standard techniques (Jaffe et al 1973). Cells $2x10^5$/ml were plated into 6-well tissue culture plates. Confluent cells were incubated for 24h at 37°C in the presence of 1 ml medium M199+20%FCS containing 0.5uCi of [^{3}H]-AA. After three washes with medium, monolayers were cultured for 24h with either 1ml of CS (5ug/ml), vehicle control (absolute alcohol) or media alone (M199+20%FCS). Lipids were then extracted, separated and quantified using a method described by Emilsson and Sundler (1985).

Cyclosporin caused a significant elevation in AA-associated radioactivity when compared with vehicle control (table). The rise in AA was accompanied with an increase in the phospholipid phosphatidylethanolamine (PE) and a decrease in phosphatidylcholine (PC). No significant changes were observed in the other two lipid species examined, namely phosphatidylinositol and phosphatidylserine.

	AA	PC	PE	PI	PS
Vehicle control (n)	79 ± 12* (5)	115 ± 1* (3)	100 ± 13* (3)	99 ± 5 (4)	83 ± 5 (3)
CS 5ug/ml (n)	124 ± 25* (5)	84 ± 6* (5)	143 ± 28* (4)	107 ± 14 (4)	96 ± 19 (5)

Results are expressed as percentage of appropriate control (i.e. vehicle compared with untreated cells, CS compared with vehicle control. *denotes ($p < 0.05$), (n) denotes number of experiments.

Our previous findings that CS inhibited PGI_2 following AA (20uM) stimulation at 24h suggested that CS might have an inhibitory effect on cyclo-oxygenase synthesis or activity, if an effect on liberation of AA from membrane phospholipids could be excluded (Brown et al 1987). Our present study provides further evidence that CS inhibits PGI_2 production in HUVEC by partially affecting cyclo-oxygenase activity rather than by inhibiting liberation of AA from membrane phospholipids.

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THE EFFECTS OF PAF-ANTAGONIST WEB 2086 ON PAF-INDUCED RESPONSES IN GUINEA-PIG ISOLATED HEARTS AND IN CARDIAC ANAPHYLAXIS

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The putative role of platelet activating factor (Paf) in the regulation of coronary blood flow, myocardial contractility and in the development of rhythm disturbances under some pathological conditions such as angina pectoris and myocardial infarction is a subject of much current interest. Recently, Paf has also been implicated as one of the important mediators in cardiac anaphylaxis (CA) (Levi et al. 1984, Koltai et al. 1986). In the present study, we describe the action of a thienotriazolodiazepine, WEB 2086 (Casals-Stenzel, 1987), on Paf-induced responses in isolated perfused heart. WEB 2086 was also used to investigate the possible role of Paf in cardiac anaphylaxis.

Male guinea-pigs (350-400g) were killed by cervical dislocation, the hearts removed and perfused with oxygenated Tyrode's solution under constant flow of 8 ml/min at 37°C and containing different concentrations of WEB 2086. In other experiments male guinea-pigs (200-250g) were sensitized with ovalbumin (100mg i.p and 100mg sc). After 3-4 weeks, the animals were killed and hearts prepared as described above. CA was induced by a bolus injection of ovalbumin (100μg) into the fluid perfusing the sensitized hearts. The effects of increasing concentration of WEB 2086 on cardiac anaphylaxis was studied. In each of these experiments the heart effluent was collected at 1 min intervals on ice, leukotriene C_4 (LTC_4) and thromboxane B_2 (TxB_2) were quantitated by radioimmunoassay. The release of LTC_4 was also assayed by bioassay on superfused guinea-pig ileum smooth muscle.

Bolus injection of Paf (50pmol) elicited responses similar to CA which were characterized by a sustained increase in coronary perfusion pressure (Cpp) and a decrease in cardiac developed tension (Cdt). Paf-induced increases in Cpp and decreases in Cdt were antagonized by WEB 2086 (0.03-1.0μM) in a selective, reversible and dose dependent manner. At this dose range WEB 2086 also antagonized the antigen-induced increases in Cpp and decreases in Cdt. Both Paf and antigen triggered the release of LTC_4 (1.38 ± 0.19 and 6.02 ± 0.89 pmol/min, respectively) and ir-TxB_2 (1.13 ± 0.22 and 4.90 ± 0.47 pmol/min, respectively) WEB 2086 markedly reduced the antigen- and Paf-induced release of LTC_4-like material (60.02 ± 0.38°/o and 35.62 ± 2.02°/o, respectively) and WEB 2086 totally inhibited Paf-induced TxB_2 release (100°/o) but did not significantly inhibit antigen-induced TxB_2 release. In addition, the increase in Cpp and decrease in Cdt elicited by LTC_4 (30pmol) and TxA_2-mimetic agent, U44069 (100 pmol) were not affected by WEB 2086, reflecting its selectivity on Paf receptors.

These results show that WEB 2086 is a potent and selective Paf-receptor antagonist in guinea-pig heart, and suggests that the cardiac actions of Paf may be partly mediated through the release of vasoactive arachidonic acid metabolites. Paf, mimics many of the features of CA, and the observations that the attenuation of CA by WEB 2086 provide further evidence that Paf plays a role in cardiac anaphylaxis.

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BW A868C: A NOVEL, HIGHLY POTENT AND SELECTIVE DP-RECEPTOR ANTAGONIST

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Prostaglandin D_2 (PGD_2) and BW245C are reported to cause inhibition of human platelet aggregation through the DP-receptor (for review see Giles & Leff 1988). A definitive study of the receptor type(s) involved in other PGD_2-mediated events has been hampered due to the lack of a selective DP-receptor antagonist. We now report the effects of BW A868C (3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexyl-2-hydroxyethylamino)-hydantoin) on inhibition of platelet aggregation and relaxation of vascular smooth muscle by BW245C and PGD_2, and on contraction of guinea-pig trachea by PGD_2.

Human platelets were washed according to the method of Vargas et al., (1982) and resuspended in modified Tyrode's buffer containing indomethacin (5μg ml^{-1}) and fibrinogen (400μg ml^{-1}). Inhibition of ADP (50μM)-induced aggregation by BW245C and PGD_2, in the presence and absence of BW A868C (3nM-1μM), was measured as change in light transmission in Payton aggregometers. Rings of rabbit jugular vein (R.J.V.) were suspended in modified Krebs buffer containing BM13.177 (30μM) to abolish effects of PGD_2 at the TP-receptor, and indomethacin (1μg ml^{-1}). Tissues were contracted with histamine (1μM) and cumulative concentration-effect curves to BW245C and PGD_2 were constructed, and measured as changes in isometric force. Following washout, a second curve was constructed in the presence of BW A868C (10nM-10μM).

BW A868C behaved as a simple competitive antagonist in the platelet assay. Anti-aggregatory concentration-effect curve to BW245C were displaced in a parallel manner and the shifts accorded with a Schild plot slope of unity and a pK_B of 9.26 ± 0.1 (n=4). Inhibition of platelet aggregation by PGD_2 was antagonised with a similar potency, as were the relaxation effects of BW245C and PGD_2 in R.J.V.. BW A868C could, therefore, be classified as a DP-receptor antagonist. Furthermore, action of BW A868C at other prostaglandin receptors (IP, EP_1, EP_2, TP and FP) were excluded at concentrations up to 1,000x higher than the DP-receptor affinity in appropriate assays.

Analyses of BW245C- and PGD_2-mediated effects were complicated by additional agonist-receptor interactions which were revealed by BW A868C. For example, in R.J.V. a resistant phase of agonism became detectable, indicating that both agonists exerted effects through another receptor (possibly EP_2). Also, PGD_2, in addition to its anti-aggregatory effect on platelets, demonstrated a pro-aggregatory action in the presence of BW A868C. The contractile effects of PGD_2 in guinea-pig tracheal strips were studied; their resistance to 10μM BW A868C indicated that they were not mediated through DP-receptors. To our knowledge this is the first report of a well-classified and selective competitive antagonist at the DP-receptor. Its potency and selectivity make it an important new tool in prostanoid receptor classification and identification.

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PROSTAGLANDIN STIMULATION OF NORADRENALINE RELEASE FROM BOVINE ADRENAL CHROMAFFIN CELLS

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In a number of cell types prostaglandin (PGE_1) is known to stimulate the synthesis and accumulation of cyclic AMP. We have previously shown that PGE_1 can influence stimulus secretion coupling in cultured bovine adrenal chromaffin cells by reducing the release of catecholamines in response to nicotine (Marriott et al. 1988). Here we show that prostaglandins acting alone can stimulate release of catecholamines from these cells.

Chromaffin cells were prepared from bovine adrenal medulla by collagenase digestion as described previously (Marriott et al. 1988) and were purified by differential plating (Waymire et al. 1983) prior to culture. Release was measured over a 5 min. period; noradrenaline in supernatant and cell extracts was measured by high pressure liquid chromatography with electrochemical detection. Cyclic AMP was measured by a protein binding assay and inositol phosphate accumulation was in lithium treated cells prelabelled with [^{3}H]myo-inositol.

Release was increased in the presence of PGE_1 in a dose-related manner (0.1-30 μM). A similar response was seen to PGE_2 and $PGF_{2\alpha}$ (Table 1).

Table 1 Release of noradrenaline and cell content of cyclic AMP

	Control	PGE_1 (10 μM)	PGE_2 (10 μM)	$PGF_{2\alpha}$ (10 μM)	Iloprost (1 μM)	Forskolin (5 μM)
% Release of noradrenaline	1.71 ± 0.18	5.80 ± 0.30	5.56 ± 0.44	6.19 ± 0.37	2.59 ± 0.23	2.95 ± 0.29
Cyclic AMP (pmol/well)	0.36 ± 0.07	2.44 ± 0.19	2.40 ± 0.11	1.35 ± 0.08	1.06 ± 0.11	3.89 ± 0.45

Mean ± S.E.M. (n = 4).

Neither iloprost nor forskolin produced substantial or reproducible stimulation of release, despite an increase in cyclic AMP. PGE_1 enhanced the formation of inositol phosphate about 1½ fold, compared to about 10 fold by 100 μM histamine, which produced a similar stimulation of release. PGE_1-stimulated release was dependent on external Ca^{++}, and was reduced by prior incubation of cells for 1-2 h in calcium-free medium in the presence of PGE_1.

These results suggest that PGE_1 is stimulating release by acting on a cell-surface receptor, permitting entry of calcium into the cell by a mechanism not mediated by cyclic AMP accumulation. Furthermore, the comparison with histamine may indicate that the stimulation of phosphoinositide breakdown cannot fully account for the release in response to prostaglandin.

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BRADYKININ STIMULATION OF CATECHOL RELEASE FROM CHROMAFFIN CELLS IS SENSITIVE TO DIHYDROPYRIDINE AGONISTS BUT NOT ANTAGONISTS

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Adrenal chromaffin cell cultures provide a means of studying neuronal function and secretion mechanisms (Livett et al. 1983). Bradykinin has been shown to cause catecholamine release from the adrenal gland in situ (Feldberg & Lewis, 1964) and give increases in inositol phosphate levels in cultured chromaffin cells (Plevin & Boarder, 1988). Here we show that bradykinin causes catecholamine release from these cells and investigate the characteristics and dihydropyridine sensitivity of this release.

Bovine adrenal chromaffin cells were prepared by collagenase digestion (Marriott et al. 1988) and purified by centrifugation and differential plating (Waymire et al. 1983) before being cultured as monolayers onto 'Primaria' multiwell plates. Release experiments were carried out 3-7 days later, using a 3 min stimulation period, followed by analysis of supernatants and cell contents, by high pressure liquid chromatography and electrochemical detection, for noradrenaline. Where appropriate calcium channel drugs were included in the 3 min stimulation and were also present for a 12 min preincubation period.

Initial results showed that bradykinin caused release of catecholamines in a dose-dependent manner (EC_{50} 1-3 nM), with an initial burst of release (up to 3 min) followed by a slow steady rise. Experiments suggested that the release was mediated via a B_2 type receptor and was largely dependent on external calcium. As shown in Table 1, addition of the calcium channel agonist Sandoz 202-791 (+) caused potentiation of bradykinin-stimulated release but the antagonist Sandoz 202-791 (-) had no effect. However, release caused by depolarisation was sensitive to both. Similar results were also obtained with the agonist BAY-K-8644 and the antagonist nitrendipine. The dihydropyridine agonists alone produce a small increase in release in some experiments but not in others. However, this increase, if observed, was not sufficient to account for the increase seen when combined with bradykinin. The antagonists alone produced no effects.

Table 1 Noradrenaline release from chromaffin cells

	Bradykinin (10 nM)	Potassium (45 mM)
No channel drug	10.0 ± 0.3	17.6 ± 0.9
202-791 (+) (1 μM)	15.1 ± 0.1	25.8 ± 0.3
202-791 (-) (1 μM)	9.9 ± 0.5	7.1 ± 0.4

Results are % of cell content released ± S.E.M. (points in quadruplicate). Basal release was 2.7 ± 0.3. Experiment was repeated 4 times with similar results.

These results suggest that bradykinin causes catecholamine release by a mechanism, which is dependent on external calcium, but is independent of the L type channel. However, they also suggest that bradykinin may sensitise this channel to the action of agonists.

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EFFECT OF PHORBOL ESTER ON HUMAN NEUTROPHIL β-ADRENOCEPTOR RESPONSIVENESS AND CELL SURFACE RECEPTOR DENSITY

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Tumour promoting phorbol esters elevate adenylate cyclase(AC) activity (Yoshimasa et al.,1987) and internalise the beta-adrenoceptor(BAR) (Nambi et al.,1985) in certain cell types. Here we have examined the effect of phorbol 12-myristate 13-acetate(PMA) on $beta_2$-adrenoceptor responsiveness and cell surface receptor density in neutrophils from drug free healthy males.

Neutrophils were isolated by dextran sedimentation and purification with percoll. AC responses were measured in intact cells in the presence of medium 199, ascorbic acid(1mM), RO 201724(0.1mM) and DMSO(0.005%) from adenosine 3',5'-cyclic monophosphate(cyclic AMP) accumulation over 5 minutes at 37°C. BAR internalisation was measured following incubation for 15 minutes at 37°C in the presence or absence of ISO(1μM). Cell surface and total receptor density were measured by displacement of ^{125}I-iodopindolol binding with hydrophylic (CGP 12177,10nM) and lipophylic (ICI 118551,1μM) BAR antagonists in lysates(Hertel et al.,1986). Preincubation with PMA or protein kinase C inhibitor 1-(5-isoquinolinylsulfonyl)-2-methyl piperazine (H-7) was for 10 and 15 minutes respectively.

PMA enhanced basal, isoprenaline(ISO) and forskolin(FO) stimulation of AC (table). H-7 did not affect AC activity significantly, but abolished the effects of PMA (table). Results are presented as percent of the control state in the absence of ISO and FO.

Table		Basal	ISO(1μM)	FO(10μM)
cyclic AMP (% control+ SEM)	control	100	156+15*	267+89*
	PMA(0.1μM)	281+56~	552+157~	419+101~
	H-7(50μM)	156+31	237+72*	167+35
	PMA + H-7	170+16*^	275+55*^	179+43*^
	n	6	5	5

*, P<0.05 cf control in the absence of ISO and FO; ~, P<0.05 cf control; ^, P<0.05 cf PMA alone.

ISO significantly decreased cell surface BAR density(84.7+3.1 to 60.2+8.6 % total BAR density + SEM, P<0.01, n=5). PMA did not significantly reduce cell surface BAR density(73.0+8.3% +SEM) and did not affect isoprenaline induced internalisation(PMA + ISO, 61.2+7.3% + SEM).

These data demonstrate elevation of neutrophil BAR responsiveness by PMA without a significant effect on BAR distribution. Marked attenuation of PMA action by H-7 suggests involvement of protein kinase C in neutrophil adenylate cyclase sensitisation by PMA in man.

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EFFECT OF ACTIVATED HUMAN NEUTROPHILS ON HISTAMINE RESPONSE OF RABBIT SECONDARY BRONCHI SMOOTH MUSCLE IN VITRO

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Asthma is associated with an inflammatory infiltrate in the bronchial wall and with airway hyperresponsiveness to histamine. The products of activated neutrophils have been implicated as a cause of that hyperresponsiveness. We have studied the effect of activated human neutrophils on the histamine responsiveness of rabbit bronchial smooth muscle in vitro.

Male New Zealand White rabbits (2.0-2.5 kg) were killed by cervical dislocation and exsanguination. The lungs were removed en bloc and secondary bronchi dissected free from extraneous tissue. Isometric tension was recorded from rings (2-3 mm diameter) suspended under 1 g initial tension in a 5 ml organ bath containing Krebs-Henseleit solution aerated with 5% CO_2 in O_2 at 37°C. The rings were washed every 15 min over a period of 60-90 min. A submaximal concentration of histamine (30 μM) was added to the organ bath to test the responsiveness of the smooth muscle rings to histamine. After washing and recovery of resting tension, a first cumulative concentration response curve (CCRC) to histamine (0.03-1000 μM) was performed on all preparations after which untreated preparations were used as time controls and others exposed to the supernatant of activated human neutrophils. Human neutrophils were prepared from heparinised whole blood by dextran sedimentation of red cells followed by separation of neutrophils from mononuclear cells over a density gradient (1.077 g/ml). Neutrophils were washed, suspended in Krebs-Henseleit solution at $2x10^7$/ ml and activated by incubation for 5 min at 37°C with opsonised zymosan. The mixture was centrifuged and the supernatant used to replace the Krebs-Henseleit solution in the organ baths of test preparations. A second histamine CCRC was performed after 5 min exposure of the tissue to this supernatant without further washing.

Time controls showed a shift to the right of the CCRC to histamine: pD2 mean (SEM) - 1st CCRC = 5.57 (0.12), 2nd CCRC = 5.42 (0.12); $p<0.05$. The addition of the supernatant from activated neutrophils did not alter the resting tension. The histamine CCRC (pD2 1st CCRC = 5.48 (0.10), 2nd CCRC = 5.46 (0.10)) was not altered by the supernatant from activated neutrophils when corrected for the effect of time.

The above data indicate that the products from zymosan-activated human neutrophils do not influence the in vitro histamine responsiveness of rabbit secondary bronchi smooth muscle.

CONTROL OF HUMAN ALVEOLAR MACROPHAGE: ROLE OF ADENYLATE CYCLASE

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Alveolar macrophages make up the majority of luminal cells in normal and asthmatic people. There is increasing evidence for a role of these cells in allergic and inflammatory diseases (Fuller et al., 1986). β_2-adrenoceptor stimulants are potent inhibitors of mast cell activation, however, their role as anti-inflammatory agents in asthma is controversial. We have examined the effect of the β_2 stimulant isoprenaline on adenylate cyclase activity and phagocytic activation of human macrophages, and found no evidence of any inhibitory action of this drug (Fuller et al., 1987). Further investigation has been carried out to quantify the level of adenylate cyclase activity in these cells, using forskolin, a diterpene which directly activates the enzyme.

Alveolar macrophages isolated by culture from patients undergoing diagnostic bronchoscopy were studied. Cells from 11 patients were incubated with Ro20-1724 (2.5×10^{-4}M in 0.25%, EtOH) in the presence or absence of zymosan (opsonized with pooled human serum (1mg/ml)), and forskolin (10^{-7} to 10^{-4}M in 0.1% EtOH). Cells from 5 patients were incubated at 37°C and 5% CO_2 for 1 hour; the medium was then harvested for measurement of thromboxane B_2 (TXB_2) by radioimmunoassay and N-acetyl-glucosaminidase (NAG) by specific enzymic assay. Cells from a further 6 patients were assessed for their ability to release superoxide anion (SO), determined spectrophotometrically by reduction of ferricytochrome C.

In all experiments there was activation of the cells by zymosan. Incubation with forskolin significantly ($p<0.05$) inhibited zymosan-induced TXB_2 release in a dose dependent manner to a maximum of 14 ± 9% of the control value. However in the same patients no change was observed in the NAG release. Zymosan-stimulated SO release was also significantly ($p<0.05$) inhibited by forskolin (10^{-5} M) to 73 ± 15% of the control value.

	CONTROL	ZYMOSAN	FORSKOLIN (M)		
			10^{-6}	10^{-5}	10^{-4}
TxB_2	3.4 ± 0.8	15.4 ± 1.2	13.9 ± 1.5	7.6 ± 0.5	5.3 ± 0.8
NAG	42.0 ± 25.7	42.8 ± 25.7	37.4 ± 16.1	36.6 ± 19.1	39.2 ± 22.4
SO	0.8 ± 0.6	13.8 ± 2.7	-	10.7 ± 2.3	-

Results are expressed as mean ± s.e.mean; TXB_2 (ng/mg); NAG (μmol/min/mg); SO (nmol cyt C reduced/20min/ 10^6 cells)

Human alveolar macrophages possess adenylate cyclase. Stimulation of adenylate cyclase by forskolin results in an increase in cAMP which inhibits the membrane associated events TXB_2 and SO production, but does not affect NAG release. Therefore, it seems likely that agents which stimulate adenylate cyclase may modify activation of the human alveolar macrophage.

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INDUCTION OF PGE_2 FORMATION IN HUMAN SYNOVIAL CELLS BY TNFα

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Tumour necrosis factor alpha (TNFα) is a protein released by activated macrophages and has a wide range of pro-inflammatory effects. These effects include induction of prostaglandin E_2 (PGE_2) release by synovial cells, an activity which is shared by the protein interleukin-1 (IL-1; Nathan, 1987). We have previously characterised the ability of IL-1 to induce PGE_2 release by synovial cells (O'Neill et al, 1987) and have now gone on to investigate the effect of TNFα in this respect.

Cells were grown in macrowell plates ($1.2x10^5$ cells/well) and PGE_2 estimated as described previously (O'Neill et al,1987). TNFα (100ng/ml) caused a time-dependent increase in PGE_2 output, from control levels of<0.3ng/ml to 2±0.5ng/ml (n=3) at 8h and 12±1.5 ng/ml (n=3) after 24h. If exogenous arachidonic acid (AA; 10uM) was added to the cells, no increase in PGE_2 release occurred, but when AA was added to TNFα-stimulated cells (100ng/ml), 8±1ng/ml (n=3) PGE_2 was released at 8h and 20.5± 1.5ng/ml (n=3) at 24h, indicating that TNFα could increase the metabolism of exogenous AA to PGE_2 from 8h. The response to TNFα could be blocked with the protein synthesis inhibitors, cycloheximide (10ug/ml) and actinomycin D (10ug/ml) which reduced PGE_2 levels to 2.4±0.5ng/ml (n=3) and 1.5±0.6ng/ml (n=3) respectively. This implied a protein synthesis-dependency. Like IL-1 (O'Neill and Lewis, 1988), TNFα (100ng/ml) pretreatment of cells for 24h could potentiate the ability of bradykinin (1-1000nM) and IL-1β (0.25-1ng/ml) to stimulate PGE_2 release, from media-pretreated controls of<0.6ng/ml (n=6) to 10-15ng/ml for bradykinin and 8-11ng/ml for IL-1β (n=3).

Apart from the longer lag phase, these results were similar to, although quantitatively less than, those obtained with IL-1 (O'Neill et al, 1987; O'Neill and Lewis, 1988). The possibility therefore arose that TNFα was inducing IL-1 which was then mediating the effect (as has been shown in other systems [Le et al, 1987]). Investigations with specific antibodies to TNFα revealed that anti-TNFα antiserum inhibited PGE_2 release induced by TNFα but not that by IL-1 . On the other hand, a 50:50 mixture of anti-IL-1α/anti-IL-1β antisera prevented both IL-1 and IL-1β-induced PGE_2 release but did not affect TNFα-induced PGE_2. Thus it appeared that IL-1 and TNFα induced PGE_2 release independently.

These results therefore implied that TNFα, like IL-1, caused PGE_2 release by inducing cyclo-oxygenase (O'Neill et al, 1987). Antibody studies suggested that TNFα was having a direct effect, which did not involve induction of IL-1. A similar result was obtained with chondrocytes (Merluzzi et al, 1987) and implied that in some instances the two mediators may act independently but via a similar mechanism.

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INHIBITION BY STAUROSPORINE OF MITOGEN- AND ANTIBODY-INDUCED CALCIUM MOBILISATION IN HUMAN T LYMPHOBLASTS

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Stimulation of T cells via the antigen/CD3 receptor complex using mitogens or monoclonal antibodies specific to CD3 such as UCHT1 (O'Flynn et al, 1984) results in phosphatidylinositol metabolism and elevation of cytosolic free calcium concentration ($[Ca^{++}]_i$, Imboden et al, 1985; Isakov et al, 1986). Elevated $[Ca^{++}]_i$ in association with protein kinase C activation may contribute to IL-2 receptor expression, IL-2 production and ultimately, T cell proliferation. The aim of this study was to determine the role of protein kinase C (PKC) in the modulation of T cell $[Ca^{++}]_i$ following stimulation of the antigen/CD3 T cell receptor.

Human T lymphoblasts were produced essentially according to the method of Smith and Cantrell (1985). Loading of the T-lymphoblasts with Fura 2, the fluorescent indicator of $[Ca^{++}]_i$, (Grynkiewicz et al, 1985) and calibration of the signal was essentially as described previously (Ward & Westwick, 1988). Addition of UCHT1, 2.4-24 µg/ml, Concanavalin A (Con A) 3-300 µg/ml and phytohaemagglutinin (PHA) 0.6-60 µg/ml, to Fura-2-loaded human T lymphoblasts suspended in HEPES buffered Tyrode solution containing 1 mM extracellular calcium concentration, produced an elevation of $[Ca^{++}]_i$ above basal levels of 166±17 nM (n=30) to approximately 200-2000 nM depending on dose, which was preceded by a delay of approximately 30 sec. Sub-optimal doses of UCHT1 (8 µg/ml), Con A (30 µg/ml) and PHA (20 µg/ml) were selected which elevated $[Ca^{++}]_i$ to around 600 nM. One minute pretreatment with staurosporine (9-900 nM), an agent which inhibits PKC (Tamaoki et al, 1986), resulted in a dose-dependent inhibition of sub-optimal UCHT1-, Con A- and PHA-induced elevation of $[Ca^{++}]_i$. The IC_{50} values for inhibition of UCHT1, Con A and PHA were 66±18 nM, 91±7 nM and 82±10 nM respectively.

Pretreatment with the PKC activator, tetradecanoylphorbol acetate (TPA, 48 nM) for 10 min, enhanced and accelerated the peak elevation of $[Ca^{++}]_i$ in response to all 3 agonists. Basal $[Ca^{++}]_i$ was unaffected by staurosporine (9-900 nM) or TPA (48 nM).Furthermore, one-minute pretreatment with TPA (48 nM) partially reduced the inhibitory effect of staurosporine (90 nM) on PHA-induced elevation of $[Ca^{++}]_i$. Pretreatment with sodium nitroprusside (150 µM), 8-bromo-cAMP (50 µM) or PGE_2 (1 µM) for periods of up to 10 minutes, had no effect on the calcium mobilisation induced by UCHT1, Con A or PHA

In T lymphoblasts, it would appear that protein kinase C activation results in a unique positive feedback regulation of receptor-operated calcium mobilisation as demonstrated by staurosporine inhibition and TPA-induced potentiation of mitogen-induced elevations of $[Ca^{++}]_i$. This is in contrast to PKC-mediated negative feedback on calcium mobilisation in other cell types such as platelets (Poll & Westwick, 1986). Furthermore, mitogen-induced elevations of $[Ca^{++}]_i$ were insensitive to agents known to modulate levels of cAMP and cGMP.

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ACTION OF ICI 198615, A PEPTIDE LEUKOTRIENE ANTAGONIST, ON RECEPTOR-OPERATED CALCIUM MOBILISATION IN U937 CELLS

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We have previously demonstrated that the human pre-monocytic cell line, U937, can be used for characterising putative platelet-activating factor (Paf) receptor antagonists, as these cells possess Paf and leukotriene (LT) B_4 receptors which are coupled to calcium mobilisation (Ward & Westwick,1988; Ward et al,1987). We now report that cells express peptide LT receptors which are coupled to calcium mobilisation and can be used to characterise peptide LT receptor antagonists. Culture of the U937 cells, loading of U937 cells with Fura 2 the fluorescent indicator of cytosolic calcium ($[Ca^{++}]_i$) and calibration of the signal were performed as described previously (Ward & Westwick, 1988).

Addition of LTD_4 (30pM-30nM, n=3), LTC_4 (3-300nM, n=3), LTB_4 (3-900nM, n=3) produced a very rapid ($\leq$1sec), dose-related elevation of $[Ca^{++}]_i$ of 50-700nM above basal values. In contrast, LTE_4 at 1uM or above produced a very small increase ($\leq$50nM) in $[Ca^{++}]_i$. The concentrations of LTD_4, LTB_4, LTC_4 to produce an elevation of 100nM $[Ca^{++}]_i$ above basal were 486$\pm$96pm, 5$\pm$2nM and 14$\pm$6nM respectively. LTB_4, LTC_4, and LTD_4 induced a very short lived elevation in $[Ca^{++}]_i$ ($\leq$20s) compared to that induced by sub-maximal doses of Paf or ATP ($\geq$240secs). A repeat application of sub-maximal doses of the above agonists demonstrated homologous desensitisation, e.g. LTD_4 and LTD_4, LTB_4 and LTB_4, or Paf and Paf, or ATP and ATP; but heterologous desensitisation, e.g. LTD_4 and LTB_4, or LTB_4 and LTD_4, or Paf and LTB_4, or Paf and LTD_4, was absent with respect to elevation of $[Ca^{++}]_i$.

The structural specificity of the U937-LTD_4 receptors was compared with that of the receptors present on other human cells by using ICI 198,615 which is a very potent and selective antagonist of LTC_4/LTD_4-induced contraction of human bronchi and pulmonary veins (Snyder et al, 1987). Pre-incubation of U937 cells for 1 min with ICI 198,615 produced IC_{50}s of 18$\pm$8nM, 330$\pm$60uM, $\gg$100uM and $\gg\gg$100 uM, respectively, against submaximal LTD_4 (9nM), LTB_4 (300nM), Paf (10nM) and ATP (5uM)-induced elevation of $[Ca^{++}]_i$. Therefore ICI 198,615 is a very potent antagonist at the LTD_4 receptor on U937 cells, and is very selective when examined against non-LTD_4-induced responses in the same cells.

In conclusion, the U937 cells possess receptors coupled to calcium mobilisation for three inflammatory mediators: PAF, LTD_4 and LTB_4 and thus offer a convenient and appropriate system by which to examine the mechanisms of receptor-operated calcium mobilisation, and the role of these mediators in human monocyte maturation and differentiation.

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EFFECT OF ANTI-C5A ANTIBODIES ON OEDEMA FORMATION AND PMN LEUOCYTE ACCUMULATION IN ALLERGIC INFLAMMATION IN THE RABBIT

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The mediators responsible for inducing oedema formation and polymorphonuclear leukocyte (PMN) accumulation in a passive cutaneous anaphylactic (PCA) in rabbit skin have previously been investigated but are still unknown (Hellewell et al, 1987). In contrast, it is well established that in a reversed passive Arthus reaction (RPA), oedema formation is PMN dependent and is suppressed in complement depleted animals (Humphrey, 1955). Furthermore, high levels of C5a have been detected in a RPA reaction in the peritoneal cavity of the rabbit (Jose et al, 1983). Taken together, the evidence suggests that endogenous C5a may be an important mediator in the RPA. In this study we have used anti-C5a antibodies (Jose & Williams, 1987) to assess the contribution of endogenous C5a to oedema formation and PMN accumulation in both the PCA and RPA reactions in rabbit skin. The effects of the antibodies on inflammatory responses induced by zymosan activated plasma (ZAP, as a source of C5a des Arg), FMLP and PAF were also assessed.

Rabbit PMN were isolated from whole blood and labelled with ^{111}In. PMN accumulation and oedema formation in skin were simultaneously measured as the local accumulation of intravenously (i.v.) injected ^{111}In-PMN and ^{125}I-rabbit serum albumin. Mediators were injected intradermally (i.d.) in 0.1ml volumes into the shaved dorsal skin. PCA reactions were induced by i.d. injection of IgE antibodies 2-3 days prior to antigen challenge (bovine gamma globulin, 1μg/site). RPA reactions were induced by i.d. injection of purified IgG antibodies immediately followed by i.v. injection of antigen (5mg/kg). PCA and RPA reactions were measured over 30 min and 4 hours respectively. Goat anti-rabbit anti-C5a IgG antibodies were purified and injected i.d. at a dose of approximately 300μg total IgG/site (Jose & Williams, 1987). IgG purified from preimmune goat serum was used as control.

Intradermal injection of anti-C5a, but not preimmune IgG, effectively inhibited both oedema formation (92±5% inhibition, mean±SEM, n=4) and ^{111}In-PMN accumulation (92±4%) induced by ZAP at 4 hours. Responses to FMLP (5×10^{-11}moles) and PAF (10^{-9}moles) were unaffected. In the same group of animals, oedema formation in the RPA was inhibited by 82±5% in the presence of anti-C5a. However, ^{111}In-PMN accumulation was only partially suppressed (57±10%); an additional dose of anti-C5a into the RPA at t=2 hours did not produce futher inhibition. In the PCA reaction measured over 30 min, oedema formation and ^{111}In-PMN accumulation were unaffected by anti-C5a; responses in the presence of preimmune IgG and anti-C5a were 64±9 μl vs. 65±6 μl and 565±88 vs. 597±78 ^{111}In-PMN respectively (means±SEM, n=6 sites). In the same animal, oedema and ^{111}In-PMN accumulation induced by ZAP+PGE_2 (3×10^{-10}moles/site) were inhibited by 100% and 99% respectively in the presence of anti-C5a and responses to FMLP and PAF were unaffected.

Our results demonstrate that anti-C5a antibodies can selectively inhibit C5a-induced inflammation in rabbit skin. As predicted, the results indicate that in the RPA, oedema formation is induced by C5a/C5a des Arg, however this mediator only partly contributes to PMN accumulation. This latter observation is intruiging and suggests the existence of an another endogenous mediator in the RPA which induces PMN accumulation but not oedema formation. In the PCA reaction there was no evidence to suggest the involvement of C5a in either oedema formation or PMN accumulation and the mechanisms underlying this reaction remain to be elucidated.

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SUPEROXIDE ANIONS POTENTIATE THROMBIN-INDUCED PLATELET ADHESION TO INERT SURFACES

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The aggregation of blood platelets and their surface-adhesive properties are important in haemostasis and diseases such as atherosclerosis. Activated platelets release several mediators such as platelet activating factor, platelet derived growth factor, and thromboxane A_2. In common with most aerobic cells they also have the capacity to generate superoxide anions (Marcus 1979). As platelets are present with leukocytes in certain inflammatory conditions where oxygen derived free radicals are generated, we have investigated the effects of free radical scavengers on platelet adhesion to gelatin-coated plastic.

Plastic 24 well tissue culture plates coated with 0.1% gelatin were washed twice with Krebs-Ringer-Bicarbonate (KRB) containing 1mM Ca^{2+}. The free radical scavengers, superoxide dismutase (SOD), catalase or mannitol were added to the appropriate wells, followed by 450μl of indomethacin (10uM) treated [^{3}H]-adenine labelled washed human platelets. The platelet suspension was stimulated with thrombin (0.02-0.32 U/ml) in a final volume of 530μl and incubated at 37^{o}C for 20 min. The platelet suspension was removed, the wells washed with 3 x 1 ml KRB and the residual radioactivity was released with 500μl water and counted for [^{3}H].

Neither catalase (500U/ml) nor mannitol (1mM) modified thrombin-stimulated platelet adhesion, suggesting that if H_2O_2 or OH^- were present, they did not influence platelet adhesion.

The influence of SOD on thrombin-stimulated platelet adhesion is shown in Table 1. Adhesion is expressed as the ratio of platelet adhesion induced by thrombin divided by the control adhesion in the absence of thrombin. Results are expressed as mean ± S.E.M. for (n) experiments

Table 1

Thrombin (U/ml)	Without SOD (n=9)	SOD (60U/ml) (n=6)
0.00	1	0.89
0.02	17 ± 6	6 ± 3
0.04	49 ± 13	12 ± 3
0.08	84 ± 15	31 ± 7
0.16	85 ± 18	29 ± 7
0.32	75 ± 16	24 ± 6

Thus, SOD (60U/ml) significantly reduced adhesion of thrombin-stimulated platelets at all concentrations of thrombin used, suggesting that superoxide anions were present and that they increased platelet adhesion. Stimulation of the platelet suspension with f-met-leu-phe (10^{-8}-10^{-4}M) did not increase adhesion, indicating that the suspension was not contaminated by leukocytes which would liberate superoxide anions.

We conclude that platelet adhesion is increased by superoxide anions and suggest that at sites of inflammation where superoxide anions are generated, they will modulate the response of blood platelets to activating stimuli.

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LOCAL INTRAVASCULAR ADMINISTRATION OF OXYGEN METABOLITES INDUCED RAT GASTRIC LESIONS

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Local arterial administration of the superoxide-generating system, hypoxanthine-xanthine oxidase, induces vascular permeability and erythrocyte extravasation in the rat stomach and cat intestine, and such oxygen metabolites have been implicated in the pathogenesis of ischaemia-induced mucosal damage (Parks et al, 1984; Ito & Guth, 1985, Wadhwa & Perry, 1987). In the present study, we have investigated the macroscopically apparent haemorrhagic damage to the rat gastric mucosa following local intra-arterial infusion of either hydrogen peroxide (H_2O_2) or the enzyme-substrate mixture, hypoxanthine-xanthine oxidase. In addition, the effects of catalase, which reduces H_2O_2 and of superoxide dismutase (SOD) on such damage have been evaluated.

Male rats (230-250g) were anaesthetised with pentobarbitone, the stomach exposed and the left gastric artery cannulated with a 23g teflon cannula (Esplugues & Whittle, 1988). After ligating the oesophagus and the pyloric sphincter, 3ml of 0.1M HCl in saline were instilled into the gastric lumen via a 25g needle inserted through the forestomach wall.

Close-arterial infusion of H_2O_2 (1.3 μmole kg^{-1} min^{-1}; 10 μl min^{-1}) for 10 min, with the stomach being removed 20 min later, induced haemorrhagic damage affecting 39 ± 13% (n=5) of the total mucosal area, as determined macroscopically by computerised planimetry. Pretreatment with catalase (50,000 I.U. kg^{-1} i.v. bolus, followed by 2,000 I.U. kg^{-1} min^{-1} i.v.) reduced the extent of H_2O_2-induced gastric damage to 12 ± 5% of the total area (n=5, $P<0.05$). Oxygen metabolites were also generated by separately infusing (20μl min^{-1}) xanthine oxidase (0.1 IU ml^{-1}) and hypoxanthine (1.4 mg ml^{-1}) into a mixing micro-chamber immediately prior to the intra-arterial teflon cannula. Local infusion of this superoxide generating system for 30 min, with the stomachs being removed 20 min later, induced haemorrhagic damage affecting 59 ± 16% (n=6) of the mucosa, significantly ($P<0.05$) greater than that following the vehicle alone (5 ± 2%, n=6). Introduction of a 10 min delay between the mixing of the enzyme-substrate system and its reaching the arterial cannula reduced the extent of damage to 18 ± 15% (n=4). Furthermore, concurrent local infusion of SOD (96 IU kg^{-1} min^{-1}) reduced the damage induced by the superoxide generating system to 11 ± 9% (n = 4; $P<0.05$).

Previous in vivo studies have suggested that both the superoxide anion and the hydroxyl radical can induce gastric mucosal damage, although subsequent in vitro studies on cultured mucosal cells have suggested that H_2O_2 is more toxic (Hiraishi et al, 1987). The present studies in the rat confirm that both intravascular H_2O_2 and local superoxide generation can induce acute gastric mucosal lesions.

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INVOLVEMENT OF GLUTATHIONE IN THE STIMULATION OF MITOGEN-INDUCED INTERLEUKIN-2 PRODUCTION BY ANTI-RHEUMATIC DRUGS

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Studies reporting inhibitory actions of auranofin (AF) and D-Penicillamine (D-Pen) on mononuclear cell functions have used concentrations unlikely to be free *in vivo* (Griswold *et al*, 1985). We report a stimulatory effect of AF and D-Pen on mitogen-induced interleukin-2 (IL-2) release. The glutathione (GSH) synthesis inhibitor buthionine sulfoximine (BSO) reversed the stimulatory effect of AF and D-Pen on IL-2 release.

Splenocytes (SCS) from BKW mice (Fidelus & Tsan, 1986) were suspended in RPMI 1640 medium with 5% foetal calf serum, and incubated with AF or D-Pen, for 12 hours at 37°C in a humidified 5% CO_2 atmosphere; followed by 1μg/ml Concanavalin-A (Con-A). 48 hours later the supernatants were assayed for IL-2 on an IL-2 dependent murine cytotoxic T-cell line (CTLL), Tada *et al*, 1986. BSO 0.2mM was pre-incubated with SCS for 10 hours prior to the 12 hours incubation with AF or D-Pen.

Treatment	CTLL response (Absorbance units)	
Medium only	1	
Con-A 1μg/ml	40±2	
BSO 0.2mM+Con-A	44±2	
Con-A + D-pen (μM)	no BSO	with BSO 0.2mM
10	48±3*	30±3**
100	58±2*	28±1**
1000	77±1*	52±1**
Con-A + AF (nM)		
3	51±5*	47±1
10	57±2*	32±2**

Results are absorbance units of the dye 3-(4,5-dimethylthiazol-2-yl)-2, 5-dipenyltetrazolium bromide (MTT) used to measure growth of CTLL's in response to IL-2. Each value is the mean ± S.E.M: n=3, in one experiment, at a supernatant dilution of 1/16. * significant increase above Con-A alone response $P<0.05$; ** significant decrease of Con-A + D-Pen/AF response $P<0.05$. In 5 experiments enhancement of IL-2 production by AF 10nM and D-Pen 10μM was 33±5% and 37±6% respectively. BSO 0.2mM reversed the effect of AF and D-Pen in 3 separate experiments. Above 100nM AF significantly inhibited IL-2 release. In the absence of Con-A, AF and D-Pen at these concentration did not induce IL-2 release. At the concentrations used, AF, D-Pen and BSO did not affect the basal or submaximal recombinant IL-2- induced CTLL proliferation.

The concentrations of AF and D-Pen shown here to be stimulatory are those likely to be free *in vivo*. GSH levels in rheumatoid patients are depressed and agents affecting GSH levels modulate lymphocyte proliferative responses (Fidelus & Tsan, 1986). Some anti-rheumatic drugs elevate GSH levels in rheumatoid patients prior to a therapeutic effect (Munthie & Jellum, 1980). Stimulation of IL-2 production, possibly through increases in intracellular GSH levels, could be involved in the action of AF and D-Pen.

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THE EFFECTS OF DEXAMETHASONE ON INFLAMMATORY RESPONSE IN RABBIT SKIN

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Phospholipase A_2(PLA_2) is an enzyme which hydrolyses membrane phospholipids leading to the production of mediators of inflammation e.g. prostaglandins (PG),leukotrienes (LT) and platelet activating factor (PAF) (Chang et al 1987). Glucocorticosteroids have been suggested to exert at least part of their anti-inflammatory effects by inducing the production of a PLA_2 inhibitory protein, lipocortin (Di Rosa et al 1984). We have therefore injected PLA_2 into rabbit skin to produce an acute inflammatory response and studied the effect of dexamethasone on mediator production and the inflammatory response.

PLA_2 (acidic isoenzyme from Naja mocambique [Sigma]) or other inflammatory agents were injected intradermally into rabbit skin (New Zealand White) and plasma exudation was measured over 2 hours by accumulation of intravenously administered [^{125}I]-human serum albumin. In some experiments the injection of [^{125}I]-HSA was omitted and animals were either killed after 10 minutes for measurement of LTB_4 and PGE_2 by RIA or after 2 hours for histological assessment of PMN number (Aked and Foster 1987).

Injection of PLA_2 (200U) or arachidonic acid (100µg) produced increases in [^{125}I] HSA extravasation and eicosanoid production.

	µl plasma	LTB_4(ng)	PGE_2(ng)
saline	7±2	0.5±0.2	17±7
PLA_2 (200U)	92±28	3.0±1.2	50±28
AA (100µg)	54±23	2.8±1.0	83±37

mean ± sd, n = 6-9

Co-injection of the cyclooxygenase/lipoxygenase inhibitor BW 755c (10µg) or the PAF antagonist WEB 2086 (10µg) produced 56% and 28% inhibition respectively of PLA_2-induced [^{125}I] HSA extravasation. Dexamethasone (2mg/kg i.v. 4 hours prior to injection of inflammatory agents) inhibited [^{125}I]-HSA extravasation caused by PLA_2, AA, LTB_4 + PGE_2 and PAF + PGE_2 and PMN influx in response to PLA_2 and AA (Table 2). However, dexamethasone had only a minor influence on LTB_4 and PGE_2 production.

	Mean % Inhibition (n=4)			
	[^{125}] HSA	PMN	LTB_4	PGE_2
LTB_4 + PGE_2 (50ng)	57*	-	-	-
PAF + PGE_2 (50ng)	69*	-	-	-
PLA_2 (200U)	52*	62*	15	33
AA (100ug	67*	72*	0	24

* P <0.05 Student's t-test

These results suggest that PLA_2 induced inflammation in rabbit skin is mediated by LTB_4 + PGE_2 and possibly PAF. The anti-inflammatory effects of dexamethasone in this model appear to be due to inhibition of mediator induced increases in vascular permeability and/or inhibition of PMN influx.

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FPL 62064: A NOVEL DUAL INHIBITOR OF ARACHIDONIC ACID METABOLISM IN SKIN

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FPL 62064, N-(4 methoxyphenyl)-1-phenyl-1H pyrazol-3-amine is one of a series of compounds reported to be dual inhibitors of 5-lipoxygenase (5-LO) and cyclo-oxygenase (CO) enzymes (Appleton et al., 1987: IC_{50} vs RBL-1 cytosol 5-LO = 4μM; IC_{50} vs bovine seminal vesicle microsomal prostaglandin synthetase = 3μM). FPL 62064 was rapidly and extensively metabolised following intravenous administration to rats and was inactive by the oral route at doses up to 100 mg kg^{-1} against carrageenin foot oedema in rats (Fisons internal report). The compound had local anti-inflammatory activity against immune complex induced peritoneal inflammation in the mouse (Blackham et al., 1985) when given intraperitoneally as a fine suspension in saline 15 min before challenge. The mean contents of leukotriene C_4 (LTC_4) and prostaglandin E_2 (PGE_2) were measured in 2ml of saline washings from the peritoneal cavities of each of 6 drug or vehicle treated mice using radioimmunoassays (RIA, New England Nuclear). The amount of extravasated dye in each washing (each mouse received 0.25 ml 0.5% pontamine sky blue in saline intravenously just prior to immune complex challenge) was measured spectrophotometrically [A^{630}]. ID_{50}'s (95% confidence limits) vs LTC_4, PGE_2 and dye extravasation 7.5 min post challenge were 11.4 (7.8 - 19.7), 6.1 (1.5 - 9.2) and 18.1 (12.8 - 43.7) mg kg^{-1}, respectively.

Doses of FPL 62064 in acetone (20 μl) applied to the right (R) ear of a mouse dose dependently inhibited ear inflammation induced by prior application of 2 mg arachidonic acid (AA) in acetone (20 μl) to the same ear. Application of the same dose of FPL 62064 in acetone to the left (L) ear following topical application of 20 μl of acetone (AA vehicle) had no irritant effect. ID_{50}'s (95% confidence limits) vs AA induced increases in LTC_4 and PGE_2 (RIA of methanolic extracts of homogenised ears) and ear thickness (oedema) at 30 min were 28.3 (22.1 - 36.6), 3.7 (1.7 - 5.9) and 40.8 (32.1 - 55.0) μg/ear respectively, (42 FPL 62064-dosed groups, n = 6). Topical application of FPL 62064 in acetone (20 μl) also inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA 2 μg) induced ear inflammation in mice, see Table below.

	MEAN % INHIBITION + (n=6 - 12)			
Dose of FPL 62064 μg/ear (R+L ears)	Increase in Ear thickness (oedema) (3h)	Increase in Neutrophils (MPO activity) (6h)	Increase in Epidermal area (48h)	Increase in Epidermal cell number (48h)
250	52***		8	38*
500	76***		29	44*
1000	95***	49***	53*	63**

+ Compared with vehicle treated mice (20μl acetone to both ears).
All mice received 2μg TPA in 20μl acetone to R ear and 20μl acetone to L ear.
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ Student's unpaired t test.

Single applications of FPL 62064 (-0.5h) inhibited ear oedema and myeloperoxidase (MPO, neutrophil marker enzyme) activity and three applications of drug (-0.5, 6 and 24h) inhibited epidermal thickness and epidermal cell proliferation (histological sections of ears stained with eosin and haematoxylin were assessed blind; mean of 10 low power fields per section). The profile of FPL 62064, i.e. topical and local anti-inflammatory activities combined with rapid systemic clearance, may be suited to the treatment of inflammatory dermatoses in man.

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THE ISOLATION OF GRANULES FROM DOG MASTOCYTOMA CELLS

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We have established permanent lines of dog mastocytoma cells that are morphologically and functionally stable and resemble normal dog skin and human mast cells in their functional and chemical characteristics (Lazarus et al 1986). Furthermore the mastocytoma cells release histamine and proteases concomittantly in response to immunologic and non-immunologic stimulation (Caughey et al 1988). In order to study the packaging, storage and mechanisms of release of granule-associated mediators we have developed a preparative method to isolate pure granules from dog mastocytoma cells.

Cells were enzymatically disagregated as previously described (Lazarus et al 1986), washed in Ca^{++} Mg^{++}-free Tyrode's Solution and resuspended in sucrose (0.34 M), containing EDTA (1 mM), DNase (10 ug/ml) and HEPES (10 mM). The cells ($7.8x10^8$ in 78 ml) were disrupted by N_2 cavitation at 400 psi for 15 min to cause >90% cell disruption. The disrupted cells were then centrifuged at 300 x g for 10 min at 4°C, to yield a pellet and supernatant. The supernatant was layered onto continuous sucrose gradients (20-50%) containing 4% Ficoll and then centrifuged for 12 h at 82,500 x g. Fractions were removed from the gradient to measure histamine, tryptase and chymase activities (granules), succinic dehydrogenase (mitochondria), lactate dehydrogenase (cytosol), bicinchoninic acid (protein) and DNA (nucleus).

Observation by light microscopy showed that the 300 x g pellet consisted of whole mast cells and nuclei. Three major bands were obtained on the sucrose gradient corresponding to densities of 1.09 g/cm^3 (band 1), 1.15 g/cm^3 (band 2) and 1.23-1.25 g/cm^3 (band 3) respectively. The major peak of histamine was located in band 3 and accounted for 63% of the total histamine. In addition 50% of the total tryptase activity and 60% of the total chymase activity recovered on the gradient were associated with this band. These markers suggest the presence of intact granules in band 3. The second band contained mitochondria as demonstrated by the presence of succinic dehydrogenase activity, (60% of the total recovered on the gradient). A small amount of tryptase (14%) and chymase (2.4%) activity was also found in band 1 but no histamine, suggesting that these enzymes were associated with granules without intact membranes.

The distribution of enzyme markers and histamine in band 3 suggests the presence of granules with intact membranes. These granules can be isolated in preparative quantities making it feasible to use biochemical techniques to investigate the interactions between histamine, proteases and proteoglycans. This preparation will be useful for studying the regulation of mast cell granules in a non-rodent species.

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ESTIMATION OF THE ATP UTILIZATION OF RAT MAST CELLS DURING AND AFTER ANAPHYLACTIC HISTAMINE SECRETION

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Anaphylactic histamine secretion is an energy-dependent mechanism (Chakravarty, 1977; Johansen, 1987), and it seems necessary that energy is produced as the secretion takes place (Johansen, 1981). During secretion the cellular content of adenosine triphosphate (ATP) decreased (Johansen & Chakravarty, 1975; Johansen, 1979) and aerobic glucose metabolism was stimulated (Svendstrup & Chakravarty, 1977). This may indicate an increased utilization of ATP during the time period of secretion. After completion of the secretory process there was a further decrease of the cellular content of ATP for 2½-5 min, and no recovery was observed after prolonged incubation for up to 2 hours (Johansen & Chakravarty, 1975; Johansen, 1979).

The aim of this study was to estimate the amount of ATP used for anaphylactic histamine secretion and to examine if the prolonged decrease of the cellular ATP content after the secretion was associated with an increased cellular ATP utilization.

Wistar rats were sensitized to egg albumin and their mast cells were isolated as described earlier (Johansen, 1979). The ATP content of the cells was determined by the bioluminescence technique using luciferin-luciferase from firefly tails (Johansen & Chakravarty, 1975).

Depletion of the mast cell ATP content by incubation of the cells with both glycolytic and respiratory inhibitors apparently followed a first order reaction. This was used to estimate the cellular utilization of ATP in relation to histamine secretion. Taken together with the observed decrease in the mast cell ATP content during and immediately after the secretion, it was calculated that the consumption of energy of the mast cells was increased during the time period of histamine secretion by 0.51 pmol $(10^3 \text{ cells})^{-1}$ $(40 \text{ s})^{-1}$. The increased energy consumption gradually levelled off. After 2 hours incubation with antigen the persistent decrease of the cellular ATP content may be explained by a decreased rate of oxidative ATP synthesis.

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THE UPTAKE PROCESS FOR CATECHOLAMINES IN ENDOTHELIAL CELLS IN RAT PERFUSED LUNGS IS THE SAME AS UPTAKE$_1$ IN NORADRENERGIC NEURONES

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The aim of the present study was to investigate the claim (Gillis & Pitt, 1982) that uptake of catecholamines (CAs) in the lungs occurs by a process with some properties of both neuronal uptake (Uptake$_1$) and extraneuronal uptake (Uptake$_2$).

Rats were pretreated with pargyline (75 mg/kg, i.p., 18 h and 2 h prior to the experiment) to inhibit monoamine oxidase. The lungs were isolated and perfused at 10 ml/min with Krebs solution containing 5% bovine serum albumin at 37°C for 20 min, and then with 2 nM [^{3}H]-CA ((-)-noradrenaline, NA, (-)-adrenaline, ADR or (±)-isoprenaline, ISO) for 2 min. U-0521 (10 µM) was included in the perfusion solution throughout the experiments to inhibit catechol-O-methyltransferase. Inhibitors were added to, or changes made to the ionic composition of, the perfusion solution throughout the experiments, where necessary.

The effects of inhibitors of Uptake$_1$ and Uptake$_2$ were examined on the rate of uptake of NA in the lungs (5.63 ± 0.39 pmol g^{-1} min^{-1}, n=5, controls with no inhibitor). The rate of uptake was significantly ($P<0.001$, n=4 or 5) reduced by the Uptake$_1$ inhibitors, 10 µM cocaine (88% inhibition) and 10 µM imipramine (88% inhibition), but was unaffected by the Uptake$_2$ inhibitors, 10 µM corticosterone and 10 µM 3-O-methylisoprenaline. Normetanephrine (10 µM) reduced the rate of uptake of NA to a small extent (24% inhibition, $0.05>P>0.01$, n=5), compatible with its low affinity for Uptake$_1$, and not with its much higher affinity for Uptake$_2$. Phenylephrine (30 µM) and the adrenergic neurone blocking drug, debrisoquine (30 µM), also reduced the rate of uptake of NA (72% and 79% inhibition, respectively, $P<0.001$, n=3). These results are as predicted from their IC$_{50}$ values for Uptake$_1$ in rat vas deferens (Langeloh *et al.*, 1988).

Uptake of NA in the lungs was dependent on Na^+ ions, in that replacement of 118 mM Na^+ by 118 mM Li^+ caused 77% inhibition of uptake. Uptake of NA was not affected by increasing the K^+ ion concentration from the normal level of 5.9 mM to 10.9 mM or 20.9 mM. These ionic requirements are the same as those for Uptake$_1$, and not Uptake$_2$.

NA and ADR are substrates for cocaine-sensitive uptake in the lungs, with rates of uptake of 5.63 ± 0.39 pmol g^{-1} min^{-1} and 1.45 ± 0.11 pmol g^{-1} min^{-1}, respectively, in the absence of cocaine, and 0.68 ± 0.10 pmol g^{-1} min^{-1} and 0.62 ± 0.05 pmol g^{-1} min^{-1}, respectively, in the presence of cocaine (n=4 or 5). There was no cocaine-sensitive uptake of ISO in the lungs. This substrate profile is the same as that of Uptake$_1$. It has previously been shown that the K_m values of NA and ADR for uptake in the lungs are also very similar to those for Uptake$_1$ (Bryan & O'Donnell, 1987).

These studies present conclusive evidence that the process for the uptake of CAs in the lungs of the rat has properties indistinguishable from Uptake$_1$. Its properties do not parallel those of Uptake$_2$. Hence, the uptake of CAs in the pulmonary endothelial cells appears to occur by the same process as Uptake$_1$ into noradrenergic neurones.

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THE INTERACTION BETWEEN SALMETEROL AND β-ADRENOCEPTOR BLOCKING DRUGS ON GUINEA-PIG ISOLATED TRACHEA

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Salmeterol is a β_2-adrenoceptor agonist with a long duration of action both in vitro and in vivo (Ball et al., 1987a and b). On preparations of isolated, superfused, electrically-stimulated, guinea-pig trachea (ESGPT), responses to salmeterol are well-maintained for at least seven hours after removal of the compound, despite continuous superfusion of the tissues (Ball et al., 1987). We have suggested that the mechanism of this sustained action involves salmeterol binding to exo-sites within access of the β-adrenoceptors, rather than it being irreversibly bound to the β-adrenoceptors themselves (Bradshaw et al., 1987).

We have now further investigated this proposal. Thus salmeterol was infused into the fluid superfusing preparations of ESGPT until equilibrium responses were obtained, and then the infusion stopped. We then studied the effects of β-adrenoceptor blocking drugs (β-antagonists) on the responses of ESGPT to salmeterol that persist after removal of the agonist from the superfusion fluid. For comparison, in parallel preparations, similar experiments were carried out against inhibitory responses to isoprenaline present in the superfusion fluid throughout the experiment. The concentrations of salmeterol and isoprenaline used (10-100nM) were those sufficient to cause 75-90% maximal inhibition of electrically-induced responses. The concentrations of the β-antagonists (Table 1) correspond to approximately a 100-times their pA_2 values (10-times for atenolol) at β_2-adrenoceptors.

Table 1.

	conc.	Salmeterol			Isoprenaline		
β-Antagonist	(μM)	% reversal	RT_{50}(min)	n	% reversal	RT_{50}(min)	n
atenolol	10	47±8	4.7±0.9	3	81±9	11±6	3
sotalol	10	96±3	22±3	3	83±4	19±4	3
timolol	0.1	92±13	164±22	4	100	166±82	3
propranolol	0.1	82±5	259±49	3	97±2	178±23	4

RT_{50} - time to 50% reassertion of agonist response.
Results are mean ± SEM of n observations.

All of the β-antagonists reversed the inhibitory effects of salmeterol and isoprenaline. The time of onset of this reversal was rapid, with 50% maximal effect being achieved in less than 15 min. In each case, after removal of the antagonists from the superfusion fluid, responses to salmeterol were eventually reasserted. While the rates of reassertion differed between antagonists (most rapid after atenolol and slowest after propranolol), they were similar to the corresponding rates for the control isoprenaline responses (Table 1).

The rapid reversal by β-antagonists of responses to salmeterol is consistent with competitive binding of salmeterol to β-adrenoceptors, but the ability of salmeterol to reassert its inhibitory effect after removal of β-adrenoceptor blockade supports the concept of local exo-site binding.

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BLOCKADE OF PUTATIVE ADRENOCEPTOR IN GUINEA-PIG ILEUM BY ALPRENOLOL

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We have described a putative adrenoceptor in guinea-pig ileum which functions to inhibit longitudinal muscle tension development and which is pharmacologically distinct from currently defined alpha- and beta-adrenoceptors (Bond & Clarke, 1987; Bond et al., 1988). The putative adrenoceptor responds to BRL 37344 (Arch et al., 1984) > isoprenaline > noradrenaline > adrenaline > fenoterol, despite total alpha- and beta-adrenoceptor blockade (eg: with phentolamine, 3μM, and propranolol, 5μM). Thus, characterization of the putative site with agonists suggests a 'beta-like' rather than an 'alpha-like' adrenoceptor but further definition has been hampered by the lack of a potent antagonist. Currently, nadolol, at 1000 times its equilibrium dissociation constant for beta-adrenoceptors, is the only drug known to block the putative site ($pA_2 = 4.5$). We now report on studies with atenolol (a preferential $beta_1$-adrenoceptor antagonist) and alprenolol (a non-selective beta-adrenoceptor antagonist).

The preparation used was the transmurally stimulated guinea-pig ileum (0.1 Hz; 1.5 ms pulse duration; supra- maximal voltage) from which the cholinergically mediated 'twitch' response was recorded. Inhibitory responses to isoprenaline were measured. Ileal segments from reserpine treated pigs (5 mg/kg, i.p. for 18h) were set-up in Krebs solution bubbled with 5% CO_2 and 95% O_2 (pH 7.4). The Krebs solution was of the following composition (mM): NaCl 118, $CaCl_2$ 2.6, KCl 4.9, $NaHCO_3$ 25, NaH_2PO_4 1, $MgSO_4$ 1.2, glucose 11.7, choline 0.2 and also contained ascorbic acid (110 μM), cocaine (30 μM), corticosterone (30 μM), and phentolamine (3 μM) to inhibit the auto-oxidation of amines, neuronal uptake, extraneuronal uptake, and alpha-adrenoceptors respectively.

Atenolol (0.3 μM and 3 μM) interacted competitively with isoprenaline at beta-adrenoceptors (apparent $pA_2 = 6.6$). However, higher concentrations of atenolol (10 μM to 100 μM) did not shift the concentration effect curve to isoprenaline further indicating a lack of affinity of atenolol for the putative adrenoceptor. Alprenolol (0.03 μM-10 μM) gave a Schild regression which was the resultant of antagonism at beta-adrenoceptors and the putative adrenoceptor (x intercept = 8.4; slope = 0.78, 95% CL:0.69-0.86). However, in the presence of propranolol (5 μM), to saturate beta-adrenoceptors, alprenolol (1 μM-10 μM) gave a Schild regression with a slope of 1 (95% CL:0.8-1.2) and a pA_2 value of 6.5. Thus, alprenolol is the most potent antagonist so far discovered for the putative site and, in presence of beta-adrenoceptor blockade, may be a useful probe for the identification of the putative adrenoceptor in tissues.

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EFFECTS OF CATECHOLAMINES ON HUMAN COLONIC CIRCULAR SMOOTH MUSCLE

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The adrenergic pharmacology of the smooth muscle of the human colon has only partially been elucidated. It is known that there exist inhibitory α- and β-adrenoceptors on the longitudinal smooth muscle, the taenia coli (Bucknell and Whitney, 1964). It has also been suggested (Gagnon *et al*, 1972) that excitatory α-adrenoceptors exist on both the circular and longitudinal smooth muscle of the human sigmoid colon. The present experiments were carried out to characterise (by means of agonist and antagonist studies) the adrenoceptors present in the circular smooth muscle layers of the human colon.

Circular strips of macroscopically normal segments of human colon (obtained fresh from operation and stored overnight at 4°C) were suspended in Krebs' PSS containing cocaine (3μM), EDTA (30μM) and ascorbic acid (30μM). After an initial equilibration period of approximately 30 minutes, these strips exhibited spontaneous phasic activity, typically of around 2g amplitude. Neither the frequency nor the amplitude of this activity were affected by tetrodotoxin (1μM) or atropine (3μM), suggesting that this activity is myogenic in nature. Therefore, any responses observed to adrenoceptor agonists were likely to be due to actions on post-junctional adrenoceptors.

Isoprenaline, noradrenaline and adrenaline inhibited the activity of these strips (pIC_{50}'s: 7.6 ± 0.4, n=8; 7.1 ± 0.24, n=12; 6.9 ± 0.2, n=11; respectively). Phentolamine (0.7μM) significantly shifted the noradrenaline concentration / response curve to the right (pIC_{50} NA, 5.74 ± 0.27, n=13; $p < 0.01$) giving an apparent pA_2 value of 7.5. Phentolamine did not significantly shift the isoprenaline or adrenaline concentration / response curves (pIC_{50}: ISO, 7.6 ± 0.2, n=7; ADR, 6.4 ± 0.4, n=7; $p > 0.05$). Propranolol (1μM) significantly shifted the concentration / response curve to isoprenaline to the right (pIC_{50}: 5.9 ± 0.26, n=6; $p < 0.05$) with an apparent pA_2 value of 7.7, but did not affect the responses to noradrenaline or adrenaline (pIC_{50}: NA 7.0 ± 0.1, n=9; ADR, 6.8 ± 0.17, n=8; $p > 0.05$).

Further experiments with noradrenaline and a combination of blockers gave a similar shift with phentolamine (1μM) after propranolol (1μM) as previously with phentolamine alone giving a mean pA_2 value for phentolamine of 7.45 ± 0.25 (n=6). In the presence of phentolamine (1μM), propranolol (1μM) produced a significant shift in the noradrenaline concentration / response curve giving a mean pA_2 value for propranolol of 6.17 ± 0.16 (n=6).

In conclusion, both α- and β- inhibitory adrenoceptors are present post-junctionally in human colonic circular smooth muscle. No evidence for excitatory α-adrenoceptors was found. Noradrenaline appears to act mainly on the α-adrenoceptors whereas isoprenaline acts mainly on the β-adrenoceptors. Adrenaline (as suggested by the failure of either antagonist alone to shift the concentration / response curve) appears to be acting on both the α- and β-adrenoceptors. The low pA_2 value for propranolol against noradrenaline (6.2) may be evidence for an atypical β-adrenoceptor (Coleman *et al*, 1987; Bond *et al*, 1988), but as propranolol produced a much better antagonism of isoprenaline (pA_2 7.7), the low pA_2 value could be explained by noradrenaline overcoming phentolamine block.

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EFFECTS OF CLONIDINE AND NORADRENALINE ON THE NERVE TERMINAL IMPULSE IN POSTGANGLIONIC SYMPATHETIC NERVES

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The concept of local regulation of transmitter release at the sympathetic neuroeffector junction developed from studies of the effects of α-adrenoceptor agonists and antagonists on electrically evoked transmitter release (Starke, 1977). However, little is known about the precise site of action of these agents. Recently, we reported a method which allows simultaneous measurement of both the nerve impulse and transmitter release from postganglionic sympathetic nerve terminals (Brock & Cunnane, 1987). The effects of clonidine and noradrenaline (NA) have now been investigated using this technique.

The methods used were essentially the same as those previously described (Brock & Cunnane, 1987) and involved the placing of a small suction electrode on the surface of the guinea-pig vas deferens to record simultaneously the nerve terminal impulse and the excitatory junction current (e.j.c.), which was taken as a measure of evoked transmitter release.

Clonidine (0.1 μM) and NA (0.1 - 1 μM) applied by internal perfusion of the suction electrode inhibited transmitter release without altering the nerve terminal impulse. Locally applied yohimbine (0.1 - 1 μM) antagonized this inhibitory action of both clonidine and NA. We presume therefore that this effect is mediated through α_2-adrenoceptors. However, when NA was applied to the bath (1 - 10 μM) a marked decrease in size of the nerve terminal impulse was also observed (n = 6 experiments). This effect was not antagonized by yohimbine (1 μM) (Fig. 1), was only slowly reversible by wash, and was not normally observed with concentrations of clonidine up to 1 μM.

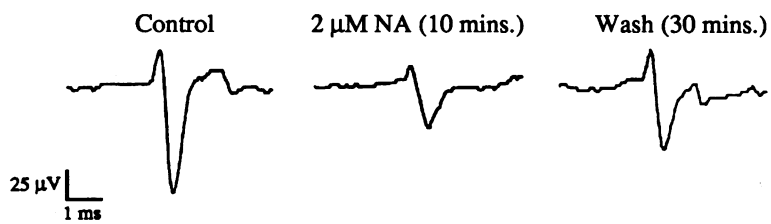

Fig. 1. Inhibitory effects of NA on the nerve terminal impulse. Averages of 25 responses at 1 Hz before, in the presence of NA and after 30 minutes wash are shown.

In conclusion, NA has at least two actions on sympathetic nerve terminals, one on the configuration of the nerve terminal impulse and the other on depolarization-secretion coupling.

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EFFECTS OF MORPHINE ON ELECTRIC ACTIVITY IN SYMPATHETIC NERVE TERMINALS OF THE MOUSE VAS DEFERENS

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It has long been known that morphine inhibits transmitter release from varicose nerve terminals (Hughes *et al*, 1975) although the precise mode of action is unclear. In the present study a suction electrode was used to record extracellularly the nerve terminal impulse and transmitter release in the mouse vas deferens to determine whether morphine interferes with impulse conduction in sympathetic nerve terminals.

The methods used were largely the same as those previously reported for the guinea-pig vas deferens (Brock & Cunnane, 1987). Briefly, vasa were removed from CB1 mice and mounted in an organ bath which was placed on the stage of a Zeiss ACM microscope. A bevelled glass electrode (tip diameter < 50 μm) was applied to the muscle surface to record electrical activity extracellularly. The organ bath was perfused continuously at 1-3 ml/min with Krebs solution at 36-37 °C. The vas deferens was electrically stimulated indirectly by field stimulation of the prostatic end (pulse width 0.01 - 0.1 ms, 7-30 V). The excitatory junction current (e.j.c.) was taken as a measure of evoked transmitter release.

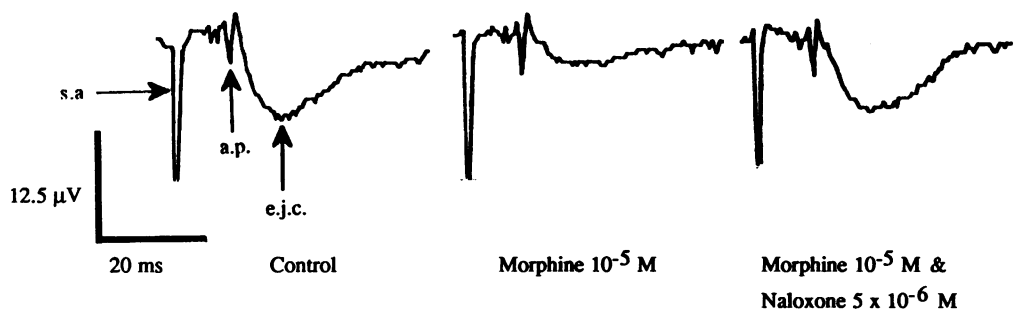

Fig. 1. Inhibitory effects of morphine on e.j.c.s and reversal by naloxone. The averages of 40 evoked responses at 1 Hz are shown; stimulus artifact (s.a.) action potential (a.p.).

Morphine (10^{-7} - 10^{-5} M) inhibited e.j.c.s without any detectable effects on the nerve terminal impulse or on spontaneous e.j.c.s. Inhibition of transmitter release by morphine was reversed by the addition of naloxone (5 x 10^{-6} M) to the organ bath (Fig 1).

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ACTION OF BRADYKININ IS INHIBITED BY VASOACTIVE INTESTINAL PEPTIDE IN GUINEA-PIG LUNG *IN VITRO*

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We have previously shown that vasoactive intestinal peptide (VIP) inhibits the generation of cyclo-oxygenase products induced by leukotriene D_4, both in guinea-pig lung parenchymal strip (GPP) and in isolated perfused lung *in vitro* (Samhoun et al, 1987, Conroy et al, 1988). In this study, we have used both preparations to study the effect of VIP on bradykinin (Bk), another bronchoconstrictor agent known to induce formation of cyclo-oxygenase products (see Piper & Samhoun, 1982).

Lungs from male guinea pigs (Dunkin-Hartley, 300-600g) were removed and perfused via pulmonary artery with oxygenated Tyrode's solution (5ml/min) at 37°C. Spirally-cut strips of rabbit aorta (RbA) were superfused with lung effluent. Bk (3-30 nmol) was administered either by i.a. bolus injection or directly over RbA. VIP (10^{-8}M; Peninsula) was given as a continuous infusion over RbA during i.a. administration of Bk and then into lungs, while doses of Bk were repeated. Effluent (30 ml) was also collected, partially purified, using C18 Sep-Paks (Waters) and levels of thromboxane (Tx)B_2, 6-keto-prostaglandin (PG)$F_{1\alpha}$ and PGE_2 measured by radioimmunoassay. Strips of GPP were prepared as described (Piper & Samhoun, 1982). Actions of Bk (0.1-3nmol) were studied in the absence and presence of VIP (10^{-8}M).

Bk caused dose-related contractions of GPP, which were reduced in the presence of VIP. Contractions due to 1 and 3 nmol of Bk were inhibited by 57±3% and 28±4%, respectively (n=4). Following i.a. administration of Bk, RbA contracted in a dose-related manner, suggesting the release of mainly TxA_2 from the perfused lung. VIP infused into lungs reduced the dose-related contractions of RbA caused by Bk. Bk had little or no direct effect on RbA. Further, as shown in Table 1, Bk caused release of 6-keto-$PGF_{1\alpha}$ and TxB_2 and a smaller release of PGE_2.

Table 1. Generation of cyclo-oxygenase products induced by i.a. administration of Bk in the perfused lung (control) and its inhibition by VIP (10^{-8}M; treated)

	6-keto-$PGF_{1\alpha}$		TxB_2		PGE_2	
BK	Control	Treated	Control	Treated	Control	Treated
3nmol	143± 30	51±17	92± 21	28± 7	10±2	5±1
10nmol	319± 50	150±32	251± 49	71±17	23±1	10±1
30nmol	637±238	178±51	785±231	203±41	20±1	10±2

Results expressed as mean ng/30ml effluent ± s.e.m.; n = 4-7

In the presence of VIP, levels of all cyclo-oxygenase products measured were reduced, e.g. release of 6-keto-$PGF_{1\alpha}$, TxB_2 and PGE_2 induced by 10nmol Bk were inhibited by 48±12%, 65±11% and 55±6% respectively. Preliminary results indicate that VIP has no effect on contractions of RbA obtained after i.a. administration of exogenous arachidonic acid (AA). This suggests that VIP does not inhibit the generation of TxA_2 caused by exogenous AA and that VIP may be inhibiting Bk-induced release of cyclo-oxygenase products by affecting a phospholipase.

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CHARACTERIZATION OF KININ RECEPTORS ON BOVINE AORTIC ENDOTHELIAL CELLS

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Bradykinin (BK) among other vasoactive agents is a potent stimulant of the release of endothelium-derived relaxing factor (EDRF) from bovine (Cocks et al., 1985; de Nucci et al., 1988) and porcine (Gryglewski et al., 1986) aortic endothelial cells. Previous studies have shown that BK and homologues induce endothelium-dependent relaxation of porcine and canine arterial rings via B_2 receptors (Regoli et al., 1985) and B_1 receptors in rabbit mesenteric artery strips (Deblois and Marceau, 1987). Here we investigated the type of kinin receptors involved in the release of EDRF and PGI_2 from BAEC.

Bovine aortic endothelial cells (BAEC) were cultured on microcarriers. The cell column and the bioassay cascade were assembled as described by Gryglewski et al. (1986). Briefly, a column containing 10-20 x 10^6 BAEC on microcarrier beads was perfused (5ml min^{-1}) with Krebs' buffer gassed with 95%O_2/5%CO_2 at 37°C, containing indomethacin (5.6 μM) and superoxide dismutase (10U/ml). The column effluent superfused a cascade (Vane, 1964) of four de-endothelialized rabbit aortas (RbA). The delay between the BAEC in the column and the consecutive RbAs was 1, 4, 7 and 10 s, respectively. The bioassay tissues were contracted by either U46619 (30-60 nM) or noradrenaline (100-500 nM). PGI_2 was monitored by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$.

Bolus injections of BK (2.5-25 pmoles) induced dose-dependent release of EDRF and PGI_2 from BAEC. The infusion of a B_2 antagonist,[D-Arg^0,Hyp^3,$Thi^{5,8}$,D-Phe7]-BK over the tissues (O.T.), did not alter the response of the RbAs to the EDRF released by BK, AA or ADP, neither did the antagonist have a releasing effect by itself when it was infused through the column (T.C.) (n=5). Two concentrations of the B_2 antagonist infused T.C. (7 and 20x 10^{-7}M) totally abolished the release of EDRF and PGI_2 from BAEC induced by injections of lower doses of BK injected T.C., and significantly reduced the responses to the higher concentrations of BK (30-100 pmoles). The B_2 antagonist at these concentrations did not affect the release of EDRF and PGI_2 induced by AA or ADP. The inhibition observed with the BK induced release was reversible with the removal of the B_2 antagonist infusion. The release of EDRF and PGI_2 induced by BK was not affected by an infusion of a B_1 antagonist, [Leu^8]-des-Arg^9-BK (n=6). $Desarg^9$-BK, A B_1 agonist, induces the release of PGI_2 and EDRF via a different receptor population namely B_{1_0} receptors. The B_2 antagonist (10^{-6}M) did not affect the response to $desArg^9$-BK whereas the B_1 antagonist (10^{-5}M) abolished the response to $desArg^9$-BK.

These results show that the release of EDRF and PGI_2 from BAEC induced by kinins is due to activation of both B_1 and B_2 receptors and the release of EDRF and PGI_2 is coupled at the level of the receptors.

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RELEASE OF EDRF AND PGI_2 FROM BAEC INDUCED BY INFUSIONS OF ADP, BRADYKININ, ARACHIDONIC ACID, L-ARGININE AND ALKALINE BUFFERS

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Prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF) are both released from endothelial cells by a variety of vasoactive substances including acetylcholine, adenine nucleotides, bradykinin (BK) and vasopressin. We have recently shown that the receptor-mediated release of EDRF and prostacyclin from bovine aortic endothelial cells (BAEC) is coupled and the initial step of this coupled release is activation of phospholipase C (De Nucci et al., 1988). Here we report the different patterns of release of EDRF and PGI_2 from BAEC induced by infusions of BK, ADP, arachidonic acid (AA), L-arginine, D-arginine and alkaline buffers.

BAEC were cultured on microcarriers. The cell column and the bioassay cascade were assembled as described by Gryglewski et al. (1986). Briefly, a column containing 10-20 x 10^6 BAEC on microcarrier beads was perfused (5ml/min) with Krebs' buffer gassed with 95%O_2/5%CO_2 at 37°C containing superoxide dismutase (10U/ml). The column effluent superfused a cascade (Vane, 1964) of four de-endothelialized rabbit aortas (RbA). The delay between the BAEC in the column and the consecutive RbAs was 1, 4, 7 and 10 s, respectively. The bioassay tissues were contracted by either U46619 (30-60 nM) or noradrenaline (100-500 nM). Indomethacin (5.6 μM) was infused over the tissues (O.T.). Agonists (BK, ADP, AA, L-arginine, Na_2CO_3 buffer, Trizma buffer) were infused O.T. for 10 min (0.1 ml/min) and then through the column of endothelial cells (T.C.). PGI_2 production was monitored by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$.

BK (10 and 30nM) infused T.C. provoked releases of EDRF and PGI_2 neither of which were maintained during the infusion. ADP (1.6 and 4 μM) infused T.C. provoked a sustained release of EDRF. The release of PGI_2 was not maintained throughout the infusion. AA (30 and 90 μM) infused T.C. provoked a sustained and prolonged release of both EDRF and PGI_2. L-arginine or D-arginine (0.5-2 mM) infused T.C. provoked a sustained and prolonged release of EDRF, but there was no consistent release of PGI_2 (release in 2 out of 4 experiments). Alkaline buffer infusions which gave the same increase in pH as the arginine infusions released EDRF but also did not consistently release PGI_2 (release in 3 out of 4 experiments).

Our findings that the release of EDRF caused by D- or L-arginine or alkaline buffer infusions is not necessarily coupled to the release of PGI_2 suggest that these substances act by a different mechanism for release of EDRF from BAEC.

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SPECIFIC RECEPTOR SITES FOR THE SYMPATHETIC CARDIAC CHEMOREFLEX INDUCED BY BRADYKININ IN THE DOG

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Bradykinin (BK) and related kinins can exert a variety of biological effects through their interactions with two different receptor site, designated B_1 and B_2 (Regoli & Barabé, 1980). The present study was designed to determine whether activation of specific B_1 and/or B_2 receptor sites is involved in the initiation of reflex increases in arterial blood pressure and heart rate known to result from epicardial application of bradykinin in dogs (Staszewska-Barczak et al., 1976). Experiments were performed on open-chest dogs, anaesthetized with intravenous (IV) thiopentone sodium (20 mg/kg) and then α-chloralose (80 mg/kg initially and 10 mg/kg/h), treated with pancuronium bromide (0.01 mg/kg IV) and mechanically ventilated. Application of bradykinin (0.03 - 1 μg) to the exposed epicardium of the left ventricle in 10 dogs, consistently induced dose-related reflex pressor effects and tachycardia. Similar effects on arterial pressure and heart rate were induced by epicardial application of either KCl (13 μmol) or capsaicin (1 μg). In contrast, epicardial application of des-Arg^9-BK(1 - 100 μg), a selective B_1-receptor agonist, failed to cause any cardiovascular response.

Treatment of the ventricular epicardium with a selective B_1-receptor antagonist, (Leu^8)-des-Arg^9-BK(50 - 100 μg/min) had no effect on the magnitude of reflex increases in the arterial pressure or heart rate evoked by epicardial application of bradykinin (0.03-0.1 μg). However, when the epicardium was treated with a selective B_2-receptor antagonist, D-Arg^0-(Hyp^3-Thi^{5,8},D-Phe^7)-BK (10-25 μg/min), the reflex tachycardia and pressor effects occurring with epicardial application of 0.03 μg or 0.1 μg of bradykinin were suppressed and the responses to 1 μg of BK were reduced by 50 - 70% ($P < 0.02$). The inhibitory effect observed with this B_2-receptor antagonist was reversible and specific, since reflex pressor and heart rate responses evoked by epicardial application of either capsaicin (1 μg) or KCl (13 mmol) were not affected. These findings indicate that the initiation of the excitatory cardiac chemoreflex by bradykinin involves its interaction with B_2 receptor sites that are probably located on terminals and/or axons of sympathetic afferent neurons supplying the dog's heart.

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CORONARY EFFECTS OF BRADYKININ BEFORE AND AFTER PHOSPHOLIPASE C TREATMENT IN THE ISOLATED RAT HEART

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Bradykinin (BK) produces coronary dilation in the rat heart, an action which may be mediated via prostacyclin (PGI_2) or endothelial derived relaxant factor (EDRF) (Vane et al 1987). In this communication we show that BK induced coronary dilation is biphasic and is not due to PGI_2 release. In contrast, following phospholipase C (PLC, C. Welchii) treatment BK produces coronary constriction which is associated with PGI_2 release.

Hearts from male Wistar rats were perfused by the Langendorff technique at 37° and 10ml min^{-1} using Krebs Henseleit solution containing 3.2 mM K^+. Perfusion pressure (PP) changes were used to monitor coronary tone while apex displacement was used to measure developed tension (DT). Bolus injections of BK (0.01-10 nmoles) produced a dose dependent biphasic coronary dilation which was not affected by flurbiprofen (2μM), superoxide dismutase (20 $u.ml^{-1}$), catalase (300 $u.ml^{-1}$) or methylene blue (1μM). Release of PGI_2 as measured by radioimmune assay of 6-Keto $PGF_{1\alpha}$ was not enhanced by BK (n = 3).

Using a recirculating perfusion system (25 mls) addition of PLC (0.03 $u.ml^{-1}$) caused coronary constriction and a fall in DT. Routinely when DT was reduced by 50% the PLC was washed out using an open circuit perfusion. Following the wash out of PLC, PP returned to control values while DT remained depressed. At this time BK injections produced coronary constriction with no vasodilator component, paradoxically this constriction was associated with 6-Keto $PGF_{1\alpha}$ release. Flubiprofen (1μM) prevented this BK induced 6-Keto $PGF_{1\alpha}$ release while having no effect on the vasoconstrictor response.

These results show that PLC can reverse the effects of BK on the coronary ciruclation of the rat possibly as a consequence of endothelial cell damage. The lack of effect of drugs expected to modify EDRF release suggests that EDRF is not an important mediator of BK induced coronary dilation in the rat, alternatively these drugs may not reach the sites of formation and action of EDRF in this model.

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DIFFERENT EFFECTS OF CHRONIC LITHIUM TREATMENT ON MUSCARINIC RECEPTOR RESPONSES IN RAT BRAIN

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Muscarinic receptor stimulation provokes a number of biochemical responses in rat brain; e.g. inositol phospholipid hydrolysis is enhanced in most brain areas and in the striatum dopamine D_1 receptor mediated cyclic AMP (cAMP) production is inhibited (Kelly & Nahorski, 1986). Since we have shown previously that chronic lithium treatment reduces muscarinic stimulated inositol phospholipid hydrolysis (Kendall & Nahorski, 1987) we were interested to see whether another functional response was similarly affected.

Male Sprague-Dawley rats (200-250 g) were fed a diet containing 0.4% ($^{w}/w$) LiCl and were killed 13 days after the start of feeding. Lithium levels measured by flame photometry were 1.32±0.11 (cortex) and 1.63±0.28 (striatum) m.Equiv/Kg wet wt. ^{3}H-inositol phosphate (^{3}H-IP) accumulation and ^{3}H-cAMP formation in brain slices were measured as described by Brown et al (1984) and Shimizu et al (1969) respectively. In some experiments LiCl (1 mM) was included in the incubation medium throughout the procedure, and in others it was added 5 min prior to agonists.

^{3}H-IP accumulation in the cortex in the presence of the muscarinic agonist carbachol (1 mM) was lower (1845±99 dpm/50 µl slices) when lithium was present throughout the procedure compared with its addition 5 min before the agonist (2919±246 dpm). Chronic LiCl feeding reduced the maximum effect of carbachol in the cortex by 30±2% only after preincubation of slices (30 min) with unlabelled myo-inositol (2.5 mM) followed by a 30 min washout before ^{3}H-inositol labelling. LiCl feeding did not significantly affect carbachol stimulated ^{3}H-IP accumulation in striatal slices.

Dopamine D_1 receptor-mediated ^{3}H-cAMP formation in striatal slices stimulated by dopamine in the presence of the D_2 antagonist sulpiride (50 µM) was not significantly different in the presence of LiCl (1 mM). Carbachol reduced D_1 stimulated ^{3}H-cAMP formation in a concentration dependent manner (60±5% reduction in the presence of 10^{-5}M dopamine) and this was significantly potentiated in the presence of LiCl (80±4% reduction with 10^{-5}M carbachol). Chronic feeding with LiCl did not result in any significant ex vivo changes in the muscarinic inhibition of D_1-stimulated ^{3}H-cAMP accumulation in striatal slices.

Thus, in vitro, LiCl reduced muscarinic stimulated ^{3}H-IP accumulation in cerebral cortex slices and there was a further reduction ex vivo following chronic LiCl feeding in cortical but not striatal slices. In contrast LiCl potentiated the inhibition of D_1-mediated cAMP formation due to carbachol in vitro, but there was no effect of chronic LiCl treatment on this response.

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PURINE RELEASE FROM THE HYPOXIC RAT CEREBRAL CORTEX: EFFECTS OF THE XANTHINE OXIDASE INHIBITORS ALLOPURINOL AND OXYPURINOL

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Periods of hypoxia or ischemia result in an enhanced catabolism of ATP and the efflux of adenosine and its metabolites, inosine, hypoxanthine, xanthine, and uric acid into the interstitial spaces of the brain (Phillis et al., 1987). Under ischemic conditions, the degradation of hypoxanthine and xanthine, catalyzed by xanthine oxidase, results in the formation of superoxide radicals which have been implicated in ischemia-reperfusion tissue damage. Inhibitors of xanthine oxidase, such as allopurinol and its active metabolite oxypurinol, have been shown to protect against reperfusion injury in rat brain (Itoh et al., 1986).

Release of purines from the normoxic and hypoxic rat cerebral cortex was studied with the cortical cup technique. Male rats, maintained under methoxyflurane anesthesia, were subjected to a series of three, 10 min periods of 5% oxygen in nitrogen inhalation, with drug administration between the first and second hypoxias. Normoxic collections of cup perfusate preceded and followed each hypoxic challenge. Basal, normoxic, predrug perfusate concentrations of purines as determined by HPLC were (mean ± s.e. mean): adenosine-28.1 ± 3.8 nM, inosine-76.4 ± 10.4 nM, hypoxanthine-233.3 ± 52.3 nM, xanthine-536.9 ± 122.7 nM, and uric acid-1678.8 ± 559.2 nM. Comparable levels of these purines are present in rat cerebrospinal fluid withdrawn from the cisterna magna (Walter et al., 1988). Administration of allopurinol (100 mg/kg i.v.) resulted in a peak perfusate allopurinol concentration of 0.02 mM, that declined during the course of the experiment as the levels of its metabolite, oxypurinol, gradually increased to 3553 ± 566 nM, a value comparable to the levels reached (4386 ± 556 nM) following the administration of oxypurinol (20 mg/kg i.v.). Both allopurinol and oxypurinol reduced uric acid to 10% of predrug levels, indicating an effective block of xanthine oxidase activity. Perfusate concentrations of xanthine and hypoxanthine, substrates for xanthine oxidase, were increased. Hypoxia-evoked adenosine and inosine release from the cortex was significantly elevated by treatment with oxypurinol. Allopurinol initially depressed hypoxia-evoked adenosine and inosine release, but during the second post-drug challenge purine release was enhanced.

In addition to a protective action of reduced superoxide formation due to the block of xanthine oxidase, the effects of allopurinol or oxypurinol administration on adenosine release from the hypoxic rat cerebral cortex suggest other possible benefits. Adenosine depresses the release of the excitotoxic amino acids glutamate and aspartate, which are partially responsible for ischemic damage. It is a potent cerebrovascular dilator and an increase in the release of adenosine into the interstitial fluid would serve to increase local blood flow and partially counteract the effects of the ischemia/hypoxia, especially in the ischemic penumbra. By blocking the metabolism of hypoxanthine and xanthine, allopurinol and oxypurinol should exert a purine sparing effect, enhancing the recovery of ATP levels that have been depleted by the ischemic event.

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ARACHIDONIC ACID METABOLITES ARE NOT INVOLVED IN α-ADRENOCEPTOR POTENTIATED cAMP FORMATION IN RAT BRAIN

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Despite having no direct effect alone, α-adrenoceptor stimulation potentiates the formation of cyclic AMP (cAMP) due to β-adrenoceptor activation in brain slices. It has been suggested that this potentiation is mediated by prostaglandin formation (Partington et al, 1980) and more recently that a similar mechanism exists for α-adrenoceptor potentiation of VIP in mouse brain (Schaad et al, 1987). We have therefore examined the effects of a number of agents which modify the formation and metabolism of arachidonic acid to re-evaluate this hypothesis.

cAMP formation in cerebral cortex slices from male Sprague/Dawley rats in response to noradrenaline (NA) was measured by the ^{3}H-adenine prelabelling method of Shimizu et al (1969). The α-adrenoceptor potentiation was calculated as the difference between the maximum stimulation due to the β-adrenoceptor-agonist isoprenaline (ISO) 10^{-5}M and that due to ISO + NA (10^{-4}M) (α + β stimulation). Isoprenaline typically produced a 12-fold increase in ^{3}H-cAMP accumulation which was increased a further 3-fold by NA.

The following oxygenase inhibitors (with enzyme substrates shown in parenthesis) at maximum concentrations of 3 x 10^{-5}M were employed; indomethacin (inhibits cyclo-oxygenase), nordihydroguaiaretic acid, NGA (inhibits 5-, 12- and 15-lipoxygenase), BW755C, 3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline (inhibits cyclo-oxygenase, 5- and 12-lipoxygenase), 5,8,11,14 eicosatetraynoic acid, ETYA (inhibits cyclo-oxygenase & 5-lipoxygenase). None of these agents significantly reduced the α-adrenoceptor potentiation. (ANOVA $P>0.05$, n=3 or more.)

Using a different approach, attempts were made to mimic the α-adrenoceptor mediated potentiation. Arachidonic acid (10^{-4}M) alone or in the presence of BW755C, (3 x 10^{-5}M) to prevent arachidonate metabolism, failed to alter the response to isoprenaline.

The bee venom peptide mellitin stimulates phospholipase A_2 (PLA_2) and thereby releases arachidonic acid in a number of cell types. At a concentration of 10 μg/ml, mellitin stimulated ^{3}H cAMP formation (270±47% control) and in combination with isoprenaline caused a greater than additive stimulation (128±15% of isoprenaline plus mellitin individually). However this synergistic response was not affected by the dual oxygenase inhibitor BW755C.

The PLA_2 inhibitor quinacrine has been shown to inhibit α-adrenoceptor potentiation; however, in our experiments this inhibition was surmountable by increasing the NA concentration in the incubation medium suggesting that it was due to α-adrenoceptor blockade.

In conclusion, we have produced no evidence that the α-adrenoceptor potentiation of cAMP production following β-adrenoceptor stimulation is mediated by metabolism of arachidonic acid.

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EFFECTS OF SELECTIVE PHOSPHODIESTERASE INHIBITORS ON CYCLIC AMP HYDROLYSIS IN RAT CEREBRAL CORTICAL SLICES

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A major determinant of cellular cyclic AMP concentration is its rate of hydrolysis. Several isoenzymic forms of cyclic nucleotide phosphodiesterase (PDE) have been characterised (Strada et al. 1984), however, few studies have investigated the regulation of cyclic nucleotide hydrolysis in situ. The present work was undertaken to investigate the effects of various selective PDE inhibitors on the rates of cyclic AMP hydrolysis in rat cortical brain slices.

Slices were washed in 20 ml Krebs-Ringer bicarbonate (KRB) medium gassed with O_2:CO_2 (95:5) for 3 x 20 min. at 37°C. The medium was then replaced by 20 ml KRB containing various concentrations of PDE inhibitors and incubated for 30 min. Slices were allowed to sediment and aliquots of packed slices were added to identical medium containing 10 μM isoprenaline, gassed and incubated at 37°C. Preliminary work showed that elevation of tissue cyclic AMP levels was maximal after 10 and 20 min. in the absence and presence of PDE inhibitors, respectively. At these points a β-adrenoceptor antagonist (20 μM timolol) was added. Cyclic AMP metabolism was stopped by addition of HCl (to a final concentration of 1 M) at various times after timolol addition and cyclic AMP extracted at 4°C for 30 min. Samples were neutralised and cyclic AMP determined using a competitive protein-binding assay.

In the absence of PDE inhibitors, isoprenaline caused a two-fold increase in tissue cyclic AMP concentration (basal: 7.1 ± 0.7; isoprenaline: 14.3 ± 1.4 pmol/mg prot). Addition of timolol caused a rapid decrease in cyclic AMP to basal levels ($t_{\frac{1}{2}}$ < 1 min). Denbufylline, a selective inhibitor of a Ca^{++} independent, low K_m cyclic AMP PDE found in cerebrum (Nicholson and Wilke, 1987), increased both basal and isoprenaline-stimulated cyclic AMP concentrations (at 10^{-5} M: basal: 25.7 ± 1.2; isoprenaline: 81.8 ± 4.1 pmol/mg prot). Addition of timolol to isoprenaline-stimulated slices in the presence of 10 μM denbufylline caused a less rapid decrease to basal levels ($t_{\frac{1}{2}}$ > 6 min). IBMX, a non-selective PDE inhibitor, also elevated basal and isoprenaline-stimulated cyclic AMP levels (at 10^{-3} M: basal: 18.2 ± 1.0; isoprenaline: 35.3 ± 2.1 pmol/mg prot), but was less effective in decreasing the rate of decrease in tissue cyclic AMP concentration upon termination of the β-adrenoceptor agonist stimulation ($t_{\frac{1}{2}}$ = 5 min). SKF 94120, a selective inhibitor of one of two low K_m cyclic AMP PDE isoforms present in ventricular tissue (Reeves et al. 1987), had only a small effect on tissue cyclic AMP concentration (at 10^{-4} M: basal: 10.4 ± 1.5; isoprenaline: 18.2 ± 1.5 pmol/mg prot) and had no effect on the rate of decrease of cyclic AMP in isoprenaline-stimulated slices upon addition of β-adrenoceptor antagonist ($t_{\frac{1}{2}}$ = 1 min).

The results indicate that, although the major cyclic AMP PDE activity present in brain is a Ca^{++}-dependent, high K_m isoform (Strada et al. 1984), selective inhibition of the low K_m cyclic AMP PDE isoform present in cortical slices is sufficient to elevate the cyclic AMP concentration to higher steady-state levels. Furthermore, selective inhibition of this PDE isoform has a profound effect on the rate of cyclic AMP hydrolysis upon termination of adenylate cyclase activation by stimulatory agonists.

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TEMPORAL CHANGES IN THE CALCIUM-DEPENDENCE OF THE POTENTIATION OF CYCLIC AMP ACCUMULATION PRODUCED BY HISTAMINE IN GUINEA-PIG BRAIN

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Histamine can elevate cyclic-AMP (cAMP) levels in brain slices by two mechanisms. It can stimulate adenylate cyclase directly via H_2-receptors or it can act through H_1-receptors to augment the cAMP responses to H_2- or adenosine A_2-receptor stimulation (Al-Gadi & Hill, 1987). The augmentation of the adenosine response is rapid in onset and requires a maintained stimulation of H_1-receptors; addition of the H_1-antagonist mepyramine causes a rapid fall in cAMP levels to that obtained with adenosine alone (Donaldson et al., 1987). Since calcium ions have been implicated in this H_1-receptor action (Schwabe et al., 1978 Al-Gadi & Hill, 1987), we have investigated the effect of a sudden reduction in external Ca concentration on H_1-receptor-mediated cAMP responses.

cAMP accumulation was measured in slices of guinea-pig cerebral cortex labelled with ^{3}H-adenine and incubated at 37°C with magnetic stirring in Krebs-Henseleit medium containing 2.5 mM $CaCl_2$ and gassed with O_2/CO_2 (95:5), as described previously (Donaldson et al., 1987). In all experiments, the H_2-receptor antagonist tiotidine (30 µM) was included in the incubation medium to eliminate the H_2-receptor-mediated cAMP response to histamine. The stable adenosine analogue 2-chloroadenosine (2CA) was used instead of adenosine as the direct cAMP stimulus, so that effects of endogenous adenosine could be eliminated with adenosine deaminase (1.2 U/ml). At various times before and after stimulation of H_1-receptors with histamine at 2CA steady state, extracellular Ca was rapidly reduced to below 0.1 µM by addition of alkaline EGTA to a final concentration of 5 mM (final pH=7.5). Tissue Ca content was measured by atomic absorption spectrophotometry (in the presence of 1% $LaCl_3$) in trichloroacetic acid extracts of slices washed in ice-cold Ca-free Krebs.

Addition of EGTA after cAMP had reached a steady state in the presence of 2CA (30 uM) produced a small increase in cAMP level (1.4 $\pm$ 0.1 fold, n=5) but greatly attenuated the increase in cAMP produced by subsequent addition of histamine (0.1 mM). When EGTA was added 2 min before histamine, the peak of the histamine-induced increase in cAMP accumulation was only 25 $\pm$ 11% (n=4) of that measured in the absence of EGTA. When EGTA was added 20 min before histamine, the cAMP response to histamine was abolished (n=3). If, however, EGTA was added during the cAMP steady state in the presence of 2CA (30µM) + histamine (0.1mM), cAMP levels fell by only 3.3 $\pm$ 2.3% over 10 min (n=5) even though a significant fall ($p < 0.05$: 44.1 $\pm$ 4.5% over 10 min, n=3) was obtained following addition of mepyramine (1µM). Addition of EGTA either just before histamine or in the cAMP steady state with 2CA + histamine, caused a fall in tissue Ca content of 50-70 % (n=3 in each case) within 2 min.

These studies show that the histamine-induced augmentation of cAMP accumulation becomes less sensitive to a reduction in extracellular free Ca as the response progresses, while the response of tissue Ca content apparently remains the same. This suggests that the roles of intracellular and extracellular Ca in the histamine H_1-receptor-mediated cAMP response in slices of guinea-pig cerebral cortex change with time.

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