INTERLEUKIN-1 PRETREATMENT OF SYNOVIAL CELLS POTENTIATES TUMOUR NECROSIS FACTOR AND BRADYKININ-INDUCED PGE_2 RELEASE

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Part of the pro-inflammatory effect of interleukin-1 (IL-1) is its ability to cause prostaglandin (PG) production by cells such as rheumatoid adherent synovial cells (RASC). We have shown that central to this response is cyclo-oxygenase induction by IL-1 (O'Neill et al, 1987) although there is also an activation of phospholipase A_2. We now go on to describe how tumour necrosis factor (TNF) and bradykinin (BK) interact with IL-1 in causing PGE_2 release by RASC.

RASC were cultured as previously described (Gordon & Lewis, 1984). Cells (grown to confluence in 24-well macrowell plates; $6x10^4$ cells/well) were first incubated with media (Dulbecco's modified Eagles medium + 10% heat-inactivated fetal calf serum), human recombinant (hr) IL-1α or hr IL-1ß for 24 h, washed 3 times, then incubated for a further 1 h with media +/- serum, hrIL-1α, hrIL-1ß, hrTNFα, BK or arachidonic acid (AA). PGE_2 levels were determined in the supernatant by radio-immunoassay. A range of concentrations of each mediator was used, the response to one concentration being shown in Table 1.

Table 1

24h pretreatment	1 h subsequent incubation ([PGE_2], ng/ml ± SEM, n = 3)						
	Media	Media+ serum	hrIL-1α 0.5ng/ml	hrIL-1ß	hrTNFα 25ng/ml	BK 1uM	AA 10uM
Media + serum	<0.3	<0.3	<0.3	1±0.4	<0.3	1.7±0.2	<0.3
hrIL-1α (0.5ng/ml)	4.2±0.2	6.7±0.3	20.0±2.0	22.3±3	25.0±2.0	23±3	15±2.4
hrIL-1ß (0.5ng/ml)	4.0±0.2	10.0±2.0	18.2±2.0	17.6±2	Not done	25.5+2.0	Not done

The results show that at 1 h, hrIL-1α ,hrIL-1ß, hrTNFα, BK and AA did not cause PGE_2 release in media pretreated cells. However, if cells were pretreated with hrIL-1α or hrIL-1ß and then treated for 1 h with hrIL-1α, hrIL-1ß, hrTNFα, BK or AA, there was a marked increase in PGE_2 synthesis although very little PGE_2 formation occurred when the IL-1-pretreated cells were incubated in media alone. As IL-1 and TNF will cause AA release under these conditions (Godfrey et al, 1987) and as exogenous AA causes a similar effect, this response could be due to these mediators activating phospholipase A_2. The resulting increased free AA is consequently metabolised by IL-1-induced cyclo-oxygenase to PGE_2. Thus any mediators which will increase free AA (such as those described here) will be potentiated in their ability to cause PG release, in cells exposed to IL-1. Such mediator interaction may have important consequences for PG production in the rheumatoid joint, where IL-1, TNF and BK are all detectable.

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A NEUTROPHIL CHEMOKINETIC FACTOR PRODUCED BY INTERLEUKIN-1-STIMULATED HUMAN SYNOVIAL FIBROBLASTS

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Neutrophil accumulation is an early event in the inflammatory response in vivo. We have previously reported (Watson et al,1987) that in contrast to its chemoattractant properties in vivo (Pettipher et al, 1986), the putative inflammatory mediator interleukin-1 (IL-1) does not influence neutrophil motility in an in vitro chemokinesis assay. We have now examined the possibility that human recombinant (hr) IL-1α stimulates the production of a neutrophil chemokinetic factor from cultured synovial fibroblasts.

Synovial cells were obtained from tissue removed from patients undergoing corrective surgery and cultured as described by Dayer et al (1979). Confluent subcultures (2nd - 5th passage) were incubated in 24-well macrowell plates ($6x10^4$ cells/well) together with hrIL-1α or its vehicle, bovine serum albumin (BSA, 0.025%), for 24h unless otherwise indicated. Supernatants were then collected and tested for chemokinetic activity on human neutrophils in the agarose microdrop assay (Smith & Walker, 1980). Supernatants from synovial cells that had been incubated with 2.8-28pM hrIL-1α (equivalent to 1-10 LAF units/ml) for 24h stimulated a neutrophil chemokinesis that was related to both the dose of IL-1 and dilution of supernatant used. For example, 1:30 diluted supernatant from synovial cultures incubated with 28pM IL-1 stimulated a migration of $1025\pm93\mu m$ (mean$\pm$SEM, n=8 neutrophil donors) which was significantly higher ($p<0.005$) than in the presence of 1:30 diluted supernatants from BSA treated synovial cells ($339\pm49\mu m$, n=7). This dose of IL-1 (28pM) and supernatant dilution (1:30) was used in subsequent experiments. The production of chemokinetic factor was found to be time-dependent, with no significant activity above the basal level of $432\pm66\mu m$ being detected in the supernatant 10, 30 or 120 min after IL-1 addition but stimulating 499 ± 14, 1004 ± 91 and $1324\pm92\mu m$ migrations after 4, 8 and 24 h incubation respectively; results are from triplicate determinations. Supernatant from synovial cells incubated for 24h with heat-treated (80°C, 15 min) hrIL-1α caused a migration of only $613\pm50\mu m$ in the same experiment. Indomethacin (5 μM) or BW755C (50 μM) did not reduce hrIL-1α-stimulated chemokinetic factor production,indicating that eicosanoids were not involved in its production, whereas actinomycin D (0.8 μM) significantly ($p<0.01$) reduced supernatant activity to $246^{+}53\mu m$ (n=3) compared with activity from cultures stimulated with IL-1 alone ($1025^{+}93\mu m$). Dexamethasone (added 2 h before IL-1) virtually abolished factor production, reducing migration to 430 ± 32 (n=3), 265 ± 33 (n=3) and 261 ± 48 (n=4) μm for 0.01, 0.1 and 1 μM respectively. None of these drugs modified neutrophil chemokinesis when added directly to the chemokinesis assay with the factor. Ultrafiltration of the active supernatant indicated that the factor had a molecular weight above 10,000 daltons.

We therefore conclude that hrIL-1α, whilst lacking direct activity on neutrophil chemokinesis, is capable of inducing the production of a chemokinetic factor from synovial fibroblasts. The production of this factor is dependent on protein synthesis and can be inhibited by dexamethasone. This factor may play an important role in the inflammatory actions of IL-1 in vivo.

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CHANGES IN SENSITIVITY TO ARACHIDONIC ACID OF PERFUSED HINDQUARTERS OF RATS WITH ALLOXAN-INDUCED DIABETES

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Changes in eicosanoid metabolism and vascular sensitivity may play a role in the cardiovascular complications of diabetes (Rosen & Hohl, 1984). In the present study, vascular responses to the eicosanoid precursor arachidonic acid were examined using both autoperfused and Krebs perfused hindquarters of diabetic rats.

Male Wistar rats (300-375g) were made diabetic by subcutaneous injection of alloxan monohydrate (175 mg kg^{-1}), controls being injected with saline. Only rats with urinary and blood glucose levels of > 60 and > 13.9 mmol l^{-1} (respectively) on days 2, 7 and 14 after injection were considered to be diabetic. Fourteen days after injection control and diabetic rats were anaesthetised with pentobarbitone sodium (60 mg kg^{-1}, i.p.), artificially ventilated, injected with heparinised saline (500u kg^{-1}, i.v.), and then the hindquarters were perfused with blood from the aorta at a constant flow (1.5-1.8 ml min^{-1}) using an autoperfusion technique (Brody et al, 1963; Boura et al, 1986). Perfusion pressure was recorded using a pressure transducer. The technique for Krebs perfusion was similar, except that animals were not ventilated, and Krebs solution (mM: NaCl, 118.4; KCl 4.7; $MgSO_4.7H_2O$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2.2H_2O$, 2.5; glucose 11.1) saturated with 95% O_2 and 5% CO_2 at 37°C was pumped into the caudal aorta (1.9 ml min^{-1}) whilst recording perfusion pressure. Statistical analysis was performed using unpaired Student's t tests.

Arachidonic acid (AA, 0.125-1.0 mg kg^{-1}) injected via the aorta into blood-perfused hindquarters caused dose-dependent increases in perfusion pressure. These responses were significantly greater in diabetic rats than in controls ($p < 0.01$, $n \geq 5$ per group), were inhibited by the thromboxane A_2 receptor antagonist AH23848 ([1α(Z), 2β, 5α]-(±)-7-[5[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid, 5 mg kg^{-1}, i.v., $P < 0.01$) or indomethacin (5 mg kg^{-1}, i.v., $P < 0.05$), and were markedly greater than responses obtained in Krebs perfused hindquarters ($P < 0.001$). In contrast, increases in perfusion pressure of blood-perfused hindquarters to the thromboxane-mimetic U46619 (0.5-8 μg kg^{-1}) were less in diabetics than in controls ($P < 0.01$, $n \geq 5$ per group). Doses of guanethidine(1 mg kg^{-1} i.v.) and pentacynium (1 mg kg^{-1} i.v.) which inhibited pressor responses to intravenous administration of the ganglion stimulants McNeil-A-343 (4-(m-chlorophenylcarbamoyloxy)-2-butynyl-trimethyl-ammonium chloride, 300 μg kg^{-1}) and dimethylphenylpiperazinium (300 μg kg^{-1}) respectively, did not affect responses to AA. In diabetic rats, responses of blood perfused hindquarters to 5-hydroxytryptamine (0.25-4 μg kg^{-1}) were reduced compared to controls ($P < 0.01$, $n \geq 8$ per group).

It is concluded that AA produces vasoconstriction in the blood-perfused hindquarters by conversion to an eicosanoid which acts on thromboxane A_2-like receptors. This constrictor effect is dependent upon a constituent of blood, and is augmented during alloxan-induced diabetes.

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RECEPTOR-OPERATED CALCIUM MOBILISATION IN A HUMAN PRE-MONOCYTIC CELL LINE, U937

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U937 cells are a continuous line of human cells of committed monocytic origin (for review, see Harris & Ralph, 1985). As part of our programme to characterise receptor-operated calcium mobilisation in immunocompetent cells, we have examined Fura-2-loaded U937 cells as a model of calcium mobilisation in monocytes.

Undifferentiated U937 cells were suspended at 10^7 cells/ml in RPMI-1640 medium supplemented with 10% HIFCS and incubated for 30 min at 37°C with 2.5μM Fura-2am. The cells were then pelleted, washed 3 times in calcium-free HEPES buffered Tyrode and finally suspended at $2x10^6$ cells/ml. Monitoring of the intracellular free calcium ion concentration ($[Ca^{++}]_i$) in the presence of 1 mM extracellular calcium was essentially as described previously (Poll & Westwick, 1986) except that the calibration of the calcium Fura-2 fluorescence signal was as described by Pollock et al (1986). The naturally occurring stereoisomer (R)-Paf (1-O-octadecyl-2-O-acetyl-sn-glycero-3-phosphoryl-choline, 0.01-300 nM) and the less hydrolysable racemic analogue PR1501 (10 nM-3μM) produced a dose related and rapid elevation in the $[Ca^{++}]_i$ of 100-1200nM above a basal value of 135±9nM (n=22), while the unnatural stereoisomer (S)-Paf and the natural stereoisomer lyso-(R)-Paf up to a concentration of 10 μM (n=6) did not modify basal $[Ca^{++}]_i$. Leukotriene B_4 (LTB_4, 28.5nM-2.85μM) also induced increases in $[Ca^{++}]_i$, but the responses were smaller and of shorter duration, compared to those induced by Paf. Agonist-specific desensitisation was demonstrated with a repeat addition of either Paf or LTB_4.

Pre-incubation of the U937 cells for one minute with the platelet Paf receptor antagonists, WEB 2086 (Casals-Stenzel et al, 1986), Ro19-3704 (Burri et al, 1985), L652,731 (Hwang et al, 1985), BN52021 (Braquet et al, 1985), or CV3998 (Terashita et al, 1983) inhibited sub-optimal Paf (10nM)-induced increase in $[Ca^{++}]_i$ with IC_{50}s of 48±2, 99±31, 317±100, 340±120, 2300±180 nM respectively (n=5-6 experiments). Pre-incubation of either of the above compounds at 10μM (n=6) did not affect either LTB_4 or ionomycin-induced elevation of $[Ca^{++}]_i$, indicating that the compounds do not modify the LTB_4 receptor or transmembrane coupling mechanisms involved in calcium mobilisation. These results confirm and extend the recent observations of Maudsley and Morris (1987) that Paf induces calcium mobilisation in U937 cells. Thus U937 cells provide a model system for characterising both the Paf receptor and calcium mobilisation of human monocytes.

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MODULATION OF RECEPTOR-OPERATED CALCIUM MOBILISATION BY PROTEIN KINASE C, BUT NOT CAMP OR CGMP-DEPENDENT SYSTEMS IN U937 CELLS

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We have demonstrated that Fura-2-loaded U937 cells provide a model system for characterising the PAF receptor that is closely coupled to calcium mobilisation in these cells (Ward & Westwick, this meeting). In this presentation, we have evaluated the potential role of cAMP, cGMP or protein kinase C-dependent systems in modulating both PAF-induced increase in cytosolic free calcium concentration ($[Ca^{++}]_i$) and the re-equilibration of the elevated $[Ca^{++}]_i$ in U937 cells. Loading the U937 cells with Fura-2, the fluorescent indicator of $[Ca^{++}]_i$, and monitoring of the signal is as described previously (Ward & Westwick, 1987).

Pre-incubation of Fura-2-loaded cells for 1 to 10 min with either PGE_1 (0.1-1μM, n=3), PGE_2 (0.1-1μM, n=3), 6-oxo-PGE_1 (0.1-1μM), Forskolin (10μM, n=3; 30μm, n=6; 100μM, n=3) or 8-bromo-cAMP (50μM, n=6) had no significant effect upon submaximal PAF (10nM)-induced calcium mobilisation. Similarly, pre-incubation with sodium nitroprusside (50μM, n=3, 150μM, n=6) or 8-bromo-cGMP (50μM, n=6) for 10 min had no significant effect ($p > 0.05$) on submaximal PAF-induced calcium mobilisation. The above results suggest that cAMP and cGMP-dependent systems do not modulate receptor-operated calcium mobilisation in these cells. However, 1 min pre-incubation of U937 cells with protein kinase C activators tetradecanoyl phorbol acetate (TPA), 1-oleoyl-2-acetyl-sn-glycerol (OAG; Castagna et al [1982]) or 1,2-dihexanoyl-sn-glycerol (DHG, Dawson et al., 1987) produced a dose-related inhibition of the suboptimal PAF-induced elevation of $[Ca^{++}]_i$ (Table 1). When protein kinase C activators were added at the peak of the elevated $[Ca^{++}]_i$, they all caused a dose-related acceleration of the decline in Ca^{++} signal down to pre-PAF levels (Table 1)

Agent		TPA nM				DHG μM			OAG μM			Vehicle
Concentration		1.6	4.8	16	48	6.8	21	34	25	75	250	
% Inhibition	mean	26	45	84	97	47	73	83	21	37	98	
	± s.e.	5	4	3	2	4	1	9	6	3	1	
Recovery	mean	144	109	88	n.d.	135	90	41	147	133	78	154
time (secs)	± s.e.	14	13	11		6	2	2	12	14	1	2

Pre-incubation of U937 cells for 1 min with the protein kinase C inhibitors H-7 (30-100μM; Hidaka et al, 1984) or staurosporine (30-300nM; Tamaoki et al, 1986) for 1 min prior to the addition of DHG (21μM, n=5) prevented DHG-induced inhibition of PAF-induced calcium mobilisation. Therefore these results suggest activation of protein kinase C by exogenous ligands can modulate both PAF-induced increase of $[Ca^{++}]_i$ and the mechanisms responsible for the return of elevated $[Ca^{++}]_i$ to basal levels in U937 cells.

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THE CALCIUM- AND IGE-DEPENDENT RELEASE OF EICOSANOIDS FROM HUMAN CUTANEOUS MAST CELLS

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This study examines the profile of eicosanoid release from human skin mast cells dispersed and purified as described by Benyon et al (1987). Cells were labelled by incubation for 2 hours at 37°C in modified Tyrodes buffer containing 250 μCi of [^{3}H] arachidonic acid. The cells were then washed and subjected to density gradient centrifugation (Benyon et al, 1987). Cells from each density interface were challenged with either A23187 (1 μM) or anti-IgE (25 μg ml^{-1}) in a final volume of 300 ul. The profile of radioactive eicosanoids formed was studied using C18 reversed phase high performance liquid chromatography and liquid scintillation counting. Method 1, for prostanoid analysis, used a mobile phase of acetonitrile:phosphoric acid (32.8:67.2 v:v), whereas method 2, for lipoxygenase product analysis, used a mobile phase of methanol:water:phosphoric acid (65:35:0.06). Histamine release was measured fluorimetrically. In further experiments the release of prostaglandin D_2 (PGD_2) from unlabelled cells was measured by radioimmunoassay.

Unfractionated cells contained 4-5% mast cells and gradient centrifugation yielded six fractions containing 0.1-80% mast cells. In the chromatograms of method 1 the net composition after A23187 activation of unfractionated cells was PGD_2 (2.2%), PGE_2 (1.2%), 9α,11β-PGF_2/TXB_2 (1.8%) and $PGF_{2\alpha}$ (0.4%). Net histamine release was 22.3%. The net composition according to method 2 was 8.1% leukotriene (LT) C_4 but with <1% each of other LTs. Only small amounts of PGD_2 and histamine were found in gradient fractions depleted of mast cells. In contrast, the composition of prostanoids from mast cells of 80% purity was as shown below:

Stimulus	Net Composition (%)				
	PGD_2	PGE_2	9α,11β-PGF_2/TXB_2	$PGF_{2\alpha}$	6ketoPGF$_{1\alpha}$
A23187	8.6	3.8	0.6	0.1	0.3
Anti-IgE	9.1	1.5	0.2	0.0	0.5

After stimulation with A23187 or anti-IgE, LTC_4 accounted for a net 13.2% and 14.3% respectively of the radioactivity in the chromatograms of method 2. No other lipoxygenase products were detected. In experiments with unlabelled cells in which all gradients fractions were analysed (mast cell purity 0.1-90%), it was found that there were significant correlations between net histamine and PGD_2 release with both ionophore ($r=0.75$, $n=33$, $p<0.001$) and immunological ($r=0.52$, $n=28$, $p<0.01$) activation. Furthermore, there were significant correlations between PGD_2 release and numbers of mast cells ($r=0.75$, $n=33$, $p<0.001$), but not nucleated cell numbers ($r=0.16$, $p>0.1$). The correlation between histamine and PGD_2 release with both secretagogues suggests that this prostanoid is derived from mast cells.

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THE EFFECT OF SKF 525A ON THE RELEASE OF NITRIC OXIDE FROM VASCULAR ENDOTHELIAL CELLS

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We have recently shown that the vascular relaxation induced by endothelium-derived relaxing factor (EDRF) is accounted for by the release of nitric oxide (NO) from endothelial cells (Palmer et al., 1987). The cytochrome P-450 inhibitor SKF 525A has been shown to attenuate endothelium-dependent relaxation of vascular tissues induced by acetylcholine, A23187 or arachidonic acid. As a result, it has been suggested that this compound is an inhibitor of EDRF (Singer et al., 1984). Inhibition by SKF 525A of endothelium-dependent relaxation induced by other agents has not been reported, and no confirmation of this suggestion has been obtained in experiments in which the release or the action of EDRF has been measured directly. Because of this we have now examined the effect of SKF 525A on the release of EDRF, NO and prostacyclin from porcine aortic endothelial cells in culture and on their actions on the bioassay tissues.

The culture of endothelial cells, preparation of the cell column and the cascade bioassay were carried out as described previously (Gryglewski et al., 1986). Prostacyclin was determined by specific radioimmunoassay of its stable breakdown product, 6-keto $PGF_{1\alpha}$ (Salmon, 1978). NO was prepared as solutions in He-deoxygenated water and measured by chemiluminescence as the product of its reaction with ozone as previously described (Palmer et al., 1987). Endothelium-dependent relaxation of precontracted rings of rabbit aorta was measured by the method of Furchgott and Zawadzki (1980) and lactate dehydrogenase (LDH) release by the method of Wroblewski and La Due (1955).

Bradykinin (20 nM, 1 min infusion) caused the release of EDRF, NO and prostacyclin when administered to the endothelial cells in the column (T.C.). The release of EDRF, NO and prostacyclin was not affected by SKF 525A (30 μM T.C., n=3). The action of EDRF and NO on the bioassay tissues was also unaffected by this concentration of SKF 525A. Prostacyclin has no effect on the rabbit aorta (Gryglewski, et al. 1986).

High concentrations of SKF 525A (30-200 μM, T.C., n=3) caused a concentration-dependent release of EDRF, NO and prostacyclin, but not of the cytoplasmic marker enzyme LDH, from the endothelial cells. The amounts of NO released by SKF 525A and bradykinin were sufficient to account for the relaxation of the bioassay tissues attributed to EDRF.

SKF 525A (30-200 μM, n=3) also caused concentration-dependent relaxations of rings of rabbit aorta, which were endothelium-dependent, and the release of prostacyclin into the organ bath.

Our results indicate that SKF 525A is not an inhibitor of the release or the actions of NO or prostacyclin from endothelial cells in culture. Furthermore, high concentrations of SKF 525A induce the release of NO, and prostacyclin by a mechanism not involving cell damage. These mechanisms require further investigation.

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THE FAILURE OF PLATELET ACTIVATING FACTOR (PAF ACETHER) TO PRODUCE DIAPEDESIS IN THE RAT DESPITE LEUCOCYTE STIMULATION

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Some of the changes produced in the blood and peritoneal cavities of rats by the intraperitoneal injection of PAF or antigen were compared. The rats had been given Sephadex particles intravenously to produce a blood eosinophilia and those given antigen were actively sensitised. (Spicer B.A. et al).

Materials injected in 1 ml.	Number of leucocytes millions/ml. (Means ± SEM) at various times after the injections.						
	Blood 4 hours			Peritoneal washings 5 min.	4hours		24hours
	Mono	Neutro	Eosino	Total	Neutro	Mono	Eosino
PAF							
Control	10.7±1.1	3.5±0.4 ***	0.4±0.04 ***	4.8±0.5 *	0.4±0.2	4.4±0.3	1.4±0.2
PAF 3.5×10^{-5}M	13.7±1.0	13.0±0.7	0.2±0.03	3.1±0.5	0.3±0.06	4.1±0.4	1.3±0.2
Antigen							
Control	10.6±1.4 ***	4.1±0.8 **	0.4±0.08 ***	5.5±0.2 **	0.2±0.2 ***	4.7±0.2 ***	1.7±0.1 ***
Sensitised	6.7±0.2	7.7±0.4	0.2±0.02	2.7±0.2	5.5±0.5	7.8±0.3	3.3±0.2

*p<0.05; **p<0.01; *** p<0.001.

The peritoneal cavities were washed with 5ml of saline and the mean volumes of the recovered washings were not significantly different from each other except for the antigen challenged group at 4 hours when it was 4.4±0.3ml* compared with controls 3.3±0.14ml.

There was an increase in the extravasation of plasma proteins into the peritoneal fluids collected 5 minutes after challenge, the optical density at 625 nM increased from 0.06±0.02 in the controls to 0.4±0.02*** after PAF and to 0.5±0.03*** after antigen. 5 minutes after antigen the concentration of histamine in the peritoneal fluid increased from 0.3±0.09 µg/ml in the control animals to 1.8±0.13 µg/ml** in the sensitised rats. PAF produced no increase in histamine concentration.

There was a blood neutrophilia and an eosinopenia 4h after either challenge with a decrease in numbers of blood mononuclear cells 4h after antigen. Antigen, but not PAF, produced a cellular infiltration into the peritoneal cavity with an increase in the peritoneal washings of numbers of neutrophils at 4h and of mononuclear cells and eosinophils at 24h. The failure of PAF to produce a cellular infiltration shows that its ability to produce extravasation and changes in numbers of leucocytes were insufficient to produce diapedesis. When PAF was injected intraperitoneally into actively sensitised rats together with antigen there was no effect on the numbers of leucocytes in the peritoneal washings at 4h and 24h as compared with rats given antigen alone. PAF does not therefore inhibit diapedesis in the rat.

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METHYLENE BLUE INHIBITS PROSTACYCLIN PRODUCTION BY PIG AORTIC ENDOTHELIAL CELLS

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Methylene blue (MB), an inhibitor of soluble guanylate cyclase, and haemoglobin (Hb) each block the actions of endothelium-derived relaxing factor (EDRF) (Martin et al, 1985). The possibility that MB or Hb might modulate the actions of the other major endothelium-derived vasodilator, prostacyclin (PGI_2), has not been tested. We therefore examined whether MB or Hb influenced basal as well as bradykinin (BK)-stimulated production of PGI_2 by primary cultures of pig aortic endothelial cells (PAEC).

Endothelial cells were isolated from pig aortae by collagenase treatment, seeded into 9.6 cm^2 multiwell plates and grown to confluence over the next 3-7 days. The tissue culture medium was removed and the cells incubated in Krebs solution at 37°C. After 60 min the Krebs was renewed and PGI_2 (measured by radioimmunoassay of 6keto $PGF_{1\alpha}$) produced in the following 30 min was determined.

Basal production of PGI_2 in the 30 min period was 439±99 $pg.\mu g^{-1}$ DNA (n=20). Bradykinin (1-100nM), added for the final 5 min of the 30 min period, increased PGI_2 production in a concentration-dependent manner: maximum production of 5.5 times the basal level was obtained at 100nM BK. MB (0.1-20μM) reduced both basal and BK (100nM)-stimulated PGI_2 production: IC_{50} concentration of MB was 0.5±0.1μM (n=6). Elevating PAEC cyclic GMP content by adding atriopeptin II (0.1μM) (Martin and White, 1987) or 8 bromo cyclic GMP did not reverse the MB (20μM)-induced inhibition of PGI_2 synthesis. Adding arachidonate (1μM) to PAEC for 5 min increased the production of PGI_2 to 4940±660 $pg.\mu g^{-1}$ DNA (n=12). Pretreating PAEC with MB (20μM) or the cyclooxygenase inhibitor, flurbiprofen (10μM), reduced the arachidonate (1μM)-induced production of PGI_2 to 118±32 pg. μg^{-1} DNA (n=6) and 12±3 $pg.\mu g^{-1}$ DNA (n=6), respectively.

MB appears to inhibit endothelial production of PGI_2 by a mechanism unrelated to inhibition of soluble guanylate cyclase. The ability to prevent endothelial synthesis of PGI_2 from arachidonate suggests that MB might inhibit cyclooxygenase. We would caution against the use of MB as an inhibitor of EDRF due to its additional ability to block PGI_2 production.

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DIFFERENTIAL PRODUCTION OF ENDOTHELIUM-DERIVED RELAXING FACTOR AND PROSTACYCLIN BY PIG AORTIC ENDOTHELIAL CELLS

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The production of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) by the vascular endothelium is believed to be Ca^{2+} dependent (Griffith et al, 1986; Brotherton et al, 1982). We have demonstrated previously that EDRF once released feeds back on the endothelium and elevates cyclic GMP levels by stimulating soluble guanylate cyclase (Martin and White, 1987). By measuring endothelial cyclic GMP content we have examined the time course and Ca^{2+} dependence of EDRF production and have compared this with the production of PGI_2 by pig aortic endothelial cells (PAEC).

Endothelial cells were isolated by collagenase treatment, seeded into 9.6 cm^2 multiwell plates and grown to confluence. The tissue culture medium was removed and the cells incubated in Krebs solution at 37°C. Cyclic GMP content and PGI_2 production (6keto $PGF_{1\alpha}$) were measured by radioimmunoassay.

In the presence of 2.5 mM extracellular Ca^{2+} the resting content of cyclic GMP in PAEC was 46±7 fmol.μg^{-1} DNA (n=6) and the resting production of PGI_2 measured over 40 min was 266±41 pg.μg^{-1} DNA (n=6). Bradykinin (BK) (0.1μM) induced a 3.2- fold (n=6) increase in cyclic GMP content that was maximal at 30 sec and began to decline after $\simeq$ 10 min. PGI_2 production, measured simultaneously, increased 5.2- fold (n=6) during the first 3 min but declined rapidly thereafter.

When extracellular Ca^{2+} was omitted (1mM EGTA added) the resting content of cyclic GMP fell significantly to 12±1 fmol.μg^{-1} DNA (n=6)($P<0.01$) and the resting production of PGI_2 measured over 40 min was 368±91 pg.μg^{-1} DNA (n=6). BK (0.1 μM) now induced a rise in cyclic GMP content that was transient: at 30 sec the rise was similar in magnitude to that seen in the presence of Ca^{2+}, but thereafter declined sharply and had returned to basal levels within 3 min. PGI_2 production, measured simultaneously, increased 4.9- fold (n=6) in the first 3 min but declined rapidly thereafter.

In conclusion, in the presence of Ca^{2+} BK-induced EDRF production is maintained for some time but production of PGI_2 is much more transient. Extracellular Ca^{2+} is required for the sustained production of EDRF but appears not to contribute to PGI_2 production. Release of an intracellular store of Ca^{2+} may control PGI_2 production and contribute transiently to EDRF production. The endothelium may therefore differentially control the production of EDRF and PGI_2 by utilising distinct Ca^{2+} pools.

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THE INHIBITORY EFFECT OF LIPOCORTIN ON EICOSANOID SYNTHESIS IS DEPENDENT ON Ca^{2+} IONS

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The lipocortins are glucocorticoid-induced anti-phospholipase proteins with inhibitory actions on eicosanoid production (cf Di Rosa et al 1984). Human recombinant lipocortin 1 is a protein with a mw of 37 kd, and several authors (cf Saris et al 1986) have commented upon the presence of a four-fold repeating subunit structure also found in other proteins known to bind Ca^{2+} ions and lipids. We now present evidence that lipocortin - or indeed steroids themselves - cannot inhibit eicosanoid formation in the absence of extracellular Ca^{2+}.

Three types of experiments were performed. In the first type, male Wistar rats (150-200 g) were injected s.c. with either saline or dexamethasone sodium phosphate (1mg/kg). After 3h, the animals were killed and the peritoneal leukocytes (~80% macrophages) were collected by lavage (using Krebs' solution + 5U/ml heparin). The two pools of cells (steroid and non-steroid treated) were pelleted and then washed (3 x 5 ml) in either saline + 1mMCa^{2+}, saline alone or saline + 1mM EDTA before being resuspended in Krebs' solution at a density of 10^7/ml. Aliquots of the cells were incubated with opsonised zymosan (100 µg/ml) for 5 min at 37°C and the amount of PGI_2 produced measured by platelet aggregometry: 3 separate experiments (2-5 determinations for each) were performed. When washed with saline + Ca^{2+} there was a reduction in PGI_2 production by cells taken from dexamethasone-treated rats (mean 63.6 vs 122.2 ng $PGI_2/10^6$ cells: 47.9% inhibition: $p<0.05$; paired t-test), no inhibition was observed when cells were first washed with saline alone or saline + 1mm EDTA (109.6 vs 95.4 ng $PGI_2/10^6$ cells and 105.4 vs 83.2 ng $PGI_2/10^6$ cells respectively).

In a second type of study, the saline "washings" from the above experiments were concentrated and added to untreated pools of cells in Krebs' solution and the PGI_2 production measured as before. Saline washings from cells obtained from dexamethasone-treated animals produced a dose-related inhibition of PGI_2 formation with 200 µl giving 53.8% inhibition (mean of duplicates from 2 experiments). An equivalent amount of saline washings from control cells gave only 5.4% inhibition.

In the final study, 10 µg (an approximate IC_{50}) authentic human recombinant lipocortin 1 (a gift from Biogen) was added to one pool of cells and vehicle to another. Both groups of cells were washed with 3x5 ml of either saline, or saline + 1mMCa^{2+}, resuspended in Krebs' solution and PGI_2 production measured as before. When cells were washed in saline + Ca^{2+}, the inhibitory action of lipocortin persisted (343.6±22.7 ng $PGI_2/10^6$ cells compared to the controls 507.3±29.5 ng $PGI/10^6$ cells (n=3-8): 32.3% inhibition: $p<0.05$), but did not when cells were washed in saline alone (550.3±12.9 ng $PGI_2/10^6$ cells vs 557.3±58.0 ng $PGI_2/10^6$ cells).

These experiments show that (a) the acute inhibitory action of steroids on PGI_2 formation ex vivo is prevented if cells are washed in a Ca^{2+}-free medium, and that (b) this is caused by displacement of the effector protein(s) from the cell surface. Also, that (c) authentic lipocortin itself will only 'attach' to cells and produce its inhibitory effect if there is Ca^{2+} present in the medium.

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ARE PAF AND LTB_4 MEDIATORS OF NEUTROPHIL ACTIVATION?

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Activation of neutrophils is induced by a variety of stimuli including the chemotactic tripeptide, formyl-methionyl-leucyl-phenyl alanine(FMLP); the calcium ionophore, ionomycin; the ether lipid, platelet activating factor (PAF) and the 5-lipoxygenase metabolite, leukotriene B_4 (LTB_4). All stimuli induce a similar series of cellular responses including aggregation, superoxide generation and degranulation. Also released, via activation of phospholipase A_2, are LTB_4 and PAF which thus have the potential to act as endogenous amplifiers of neutrophil reactivity. Indeed previous studies have implicated products of phospholipase A_2 as mediators of neutrophil responses induced by other agonists (Smith et al.,1986; Smolen & Weissmann,1980). In the present study we have employed a specific PAF receptor antagonist, kadsurenone, and a specific lipoxygenase inhibitor, REV5901A, to investigate the role of PAF and LTB_4 in neutrophil activation.

Neutrophils were isolated from the blood of healthy adult volunteers by gelatin sedimentation followed by hypotonic lysis of contaminating erythrocytes (Henson,1971). Aggregation was monitored photometrically and degranulation was detected by measuring the release of the lysosomal enzymes β-N-acetyl glucosaminidase (NAG) and lysosyme. NAG was monitored fluorimetrically and lysosyme was detected photometrically by the rate of lysis of the bacterium Micrococcus lysodeicticus. LTB_4 generation was determined by specific radio-immunoassay (Forder & Carey,1986).

FMLP, PAF and LTB_4 produced concentration-dependent neutrophil aggregation which was rapid and reversible. Each receptor-directed agonist also induced concentration-dependent release of NAG and lysosyme, which required pre-incubation with cytochalasin B. Ionomycin also induced cellular activation but aggregation was irreversible and cytochalasin B was not necessary for degranulation. The calcium ionophore induced LTB_4 generation (EC_{50}=20nM) whereas FMLP and PAF induced barely detectable levels of LTB4. REV5901A inhibited ionomycin-induced LTB_4 formation (IC_{50}=2μM) but at concentrations up to 100μM did not attenuate aggregation or degranulation elicited by ionomycin, FMLP, PAF or LTB_4. Kadsurenone inhibited PAF-induced aggregation (IC_{50}=800nM), NAG release (IC_{50}=40nM) and lysosyme release (IC50=10nM) but at concentrations up to 10μM failed to inhibit neutrophil responses induced by the other agonists.

These observations suggest that PAF and LTB_4 are potent independent activators of neutrophils. In contrast to previous studies which employed non-specific inhibitors of 5-lipoxygenase or phospholipase A_2 (Smith et al., 1986; Smolen & Weissmann, 1980), our data with kadsurenone and REV5901A indicate that release of PAF and LTB_4 is unlikely to contribute to activation induced by other agonists.

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HUMAN MONOCYTE PREPARATIONS PRODUCE A 10-30KD NEUTROPHIL CHEMOKINETIC COMPOUND WHICH IS DISTINCT FROM INTERLEUKIN 1

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Interleukin 1 (IL 1) preparations derived from monocyte and epidermal cell suspensions have been reported to possess neutrophil chemoattractant properties *in vitro* (Luger *et al.*, 1983) and *in vivo* (Granstein *et al.*, 1986). Recombinant IL 1 has however been reported to produce little activity in an agarose microdroplet neutrophil chemokinesis assay (Watson *et al.*, 1987). We have therefore re-evaluated the relationship between the IL 1 and neutrophil chemoattractant activity released by lipopolysaccharide (LPS) stimulated human monocytes.

Human monocytes were purified from National Blood Transfusion Service buffy coat preparations by Ficoll-Hypaque density gradient centrifugation and plastic adherence (1 h). Adherent cells (approximately 80% monocytes) were cultured for 48 h as monolayers in RPMI 1640 containing 5% heat inactivated foetal calf serum and E. coli LPS (serotype 055:B5, Sigma, 1 $\mu g ml^{-1}$). After 48 h medium was successively ultrafiltered through Amicon YM30 and YM10 membranes to obtain a 10-30 kD fraction. In preliminary experiments, samples derived from 4-6 ml monocyte supernatant were purified by reversed phase h.p.l.c. (Nucleosil 5 C18 column eluted with a linear 0.1% trifluoroacetic acid/acetonitrile gradient, 80:20 to 20:80 over 32 min, at 1 $ml min^{-1}$). Analysis of evaporated 1 min fractions by an agarose microdroplet neutrophil chemokinesis method (Smith and Walker, 1980) showed a discrete peak of activity eluting at 12-13 min (n=2), which could not be due to LPS since up to 10 $ug ml^{-1}$ produced only minimal activity in the chemokinesis assay. Subsequently a 10-30 kD fraction, prepared by successive ultrafiltration of bulked monocyte supernatant (450 ml), was purifed by the above reversed phase h.p.l.c. system and a portion (10 µl) of each 1 min fraction assayed for neutrophil chemokinetic activity and for IL 1 in a bioassay incorporating EL-4 NOB-1 and CTLL cell lines (Gearing *et al.*, 1987). A discrete peak of chemokinetic material eluting at 12-13 min, which was partly resolved from a broader peak of IL 1 activity (13-16 min), was again seen. Further purification of the chemokinetic material by anion exchange h.p.l.c. (TSK-DEAE-5-PW column, eluted with a 0.1 M Tris acetate gradient, pH 8 to pH 4 over 30 min, at 1 $ml min^{-1}$) and analysis of a portion (30 µl) of each 1 min fraction showed a single peak of chemokinetic activity eluting at 20 min (approximate pI of 5) containing a trace of IL 1 activity. Repurification of this material by use of the reversed phase h.p.l.c. system and analysis of 0.5 min fractions now showed a peak of activity (12 min) which was free of detectable IL 1 and which produced dilution related effects in the chemokinesis assay.

These results indicate that human monocyte preparations produce a neutrophil chemokinetic compound which is distinct from IL 1, but which may contaminate monocyte derived IL 1 which is not rigorously purified. The neutrophil chemoattractant properties ascribed to such IL 1 preparations may in part be due to this novel chemokinetic compound.

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EVIDENCE THAT 12(R)-HETE IS A MORE ACTIVE NEUTROPHIL CHEMOATTRACTANT THAN ITS 12(S) EPIMER IN HUMAN SKIN

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Racemic 12-hydroxy-5,8,10,14-eicosatetraenoic acid [12(R,S)-HETE] elicits dose related erythema, a mixed dermal leucocyte infiltrate and collections of intraepidermal neutrophils on application to human skin (Dowd et al., 1985). Since 12(R)-HETE is more potent than 12(S)-HETE as a chemoattractant for human neutrophils in vitro (Cunningham et al., 1986), and is the major stereoisomer of 12-HETE in lesional psoriatic scale (Woollard, 1986), the chemoattractant activities of 12(R)- and 12(S)-HETE have now been compared in human skin.

The 12-HETE enantiomers were resolved by chiral h.p.l.c. from 12(R,S)-HETE, prepared by photooxidation of arachidonic acid. 12(R)- and 12(S)-HETE (0.5 - 20 µg/site) in ethanol were applied topically in a randomised manner to the forearm skin of 5 healthy volunteers. 12(R)- and 12(S)-HETE (5 µg each) were also applied to the opposite forearm. The ethanol was evaporated and the sites occluded. After 6h two vertical diameters of any erythematous responses were measured and blood flow changes recorded using a laser Doppler flowmeter. Measurements were repeated at 24h when 3mm punch biopsies were also obtained from the sites of application of the 5 µg doses. Paraffin sections were stained with haematoxylin and eosin and leucocyte counts performed by use of light microscopy.

Both 12(R)- and 12(S)-HETE produced dose related erythematous responses which were similar in area and duration. Dose related blood flow changes of similar magnitude were also obtained with both enantiomers at 6h. At 24h responses to 5 and 20 µg 12(R)-HETE appeared greater, although the differences did not achieve statistical significance (107 ± 62 vs 215 ± 88 mV for 5 µg 12(S)- and 12(R)-HETE respectively $p=0.17$; 372 ± 180 vs 738 ± 225 mV for 20 µg 12(S)- and 12(R)-HETE respectively $p=0.053$; means $\pm$ s.e. means, n=5, paired t test). Biopsies revealed marked differences in neutrophil responses to the two enantiomers.

	n	Neutrophils/high power field epidermis	Neutrophils/high power field dermis	Mononuclears/high power field epidermis	Mononuclears/high power field dermis
12(R)-HETE	5	*25.2 ± 13.0	*13.2 ± 5.1	0.4 ± 0.2	20.5 ± 5.4
12(S)-HETE	5	0.02 ± 0.02	1.02 ± 0.7	0.3 ± 0.3	26.0 ± 9.9

Results are expressed as mean ± s.e. mean; *$p < 0.05$ when compared to responses to 12(S)-HETE; paired t test

These results suggest that 12(R)-HETE is a more active neutrophil chemoattractant than 12(S)-HETE in human skin, although mononuclear cell responses to the two enantiomers were similar, and that the neutrophil infiltrates seen 24h after application of 12(R,S)-HETE to human skin (Dowd et al., 1985) were largely due to the 12(R)- component of the racemic mixture. 12(R)-HETE may therefore be of potential importance as a mediator of inflammation in man.

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STUDIES ON RELATIONSHIP BETWEEN INTESTINAL LESIONS AND ALTERATIONS OF HEPATIC AND RENAL ENZYME ACTIVITY IN THE RAT

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Non steroidal anti-inflammatory agents, indomethacin in particular, have been recently reported to induce severe multisystem toxicity in the g.i. tract, liver and kidney in animals and humans (Patmas et al, 1984; Falzon et al, 1984).

The aim of this paper was to elucidate the relationship between intestinal lesions and hepatic and renal enzyme activity alterations. Fed female Sprague-Dawley rats (200-250 g) were divided in groups of 8, treated with an ulcerogenic oral dose of indomethacin (16 mg/kg) and killed by cervical dislocation 24 or 48 hours later. Organs were removed immediately: intestine was examined macroscopically for the presence of lesions and liver and kidney were prepared for microsome, where cytochrome P450 activity was assayed as described by Falzon et al, 1984. Indomethacin caused a marked reduction in cytochrome P450 microsomial activity (expressed as n moles/mg protein) in the kidney and the liver, smaller and shorter in the former than in the latter, as shown in the following table:

TREATMENT	TIME (hr) OF AUTOPSY	CYTOCHROME P450 ACTIVITY (MEAN ± SE) HEPATIC	RENAL
CONTROL	24	0.66 ± 0.03	0.12 ± 0.02
	48	0.70 ± 0.08	0.23 ± 0.09
INDOMETHACIN	24	0.10 ± 0.02*	0.05 ± 0.01*
	48	0.38 ± 0.07*	0.14 ± 0.02

* $P < 0.05$ as compared to controls

To determine whether the presence of intestinal lesions might be responsible of organ toxicity and whether this phenomenon might be extended to other enzyme activities besides the cytochrome P450, a further series of experiments was carried out. Indomethacin (10 mg/kg/die) was given orally for three days and animals were then killed by cervical dislocation 24 h after the last administration. Livers were then removed and pooled on the basis of presence or absence of intestinal lesions. Samples were handled as described above. Hepatic cytochrome activities in ulcerated animals were significantly ($P < 0.05$) lower (0.06 ± 0.01 for cytochrome b_5 and 0.33 ± 0.03 for cytochrome P450 expressed as n moles/mg protein) than in unulcerated rats (0.27 ± 0.04 and 0.41 ± 0.01). These findings indicated that indomethacin induces rat intestinal lesions which, in turn, can cause significant enzyme pattern alterations in other organs such as liver and kidney.

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EFFECT OF A23187 AND ISCHAEMIA-REPERFUSION ON LEUKOTRIENE RELEASE FROM RAT ISOLATED PERFUSED HEART

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Published evidence suggests that leukotrienes (LTs) may play a role in myocardial infarction (e.g. Mullane & Moncada, 1982). There is little information, however, concerning the type(s) of LT synthesised by the heart or the nature of stimuli inducing release. We have therefore assessed the ability of the calcium ionophore, A23187 and global ischaemia, followed by reperfusion, to release LTs from the rat isolated perfused heart.

The effect of the calcium ionophore, A23187 (10^{-7}-10^{-5}M) was studied in hearts from male AHA rats perfused by the Langendorff technique with Krebs containing glucose (11mM) at 7ml/min. Global ischaemia was induced by perfusing hearts with Krebs containing acetate (5mM) (Strong et al, 1979) at 0.7ml/min for either 1 or 4 hours, followed by reperfusion (15 min and 60 min respectively) at a mean aortic pressure of 50mmHg. Following reperfusion, hearts were perfused with 10^{-6}M A23187. Samples of effluent were collected throughout both protocols and assayed for the prostacyclin metabolite, 6-Keto-$PGF_{1\alpha}$ ($6KPGF_{1\alpha}$) and for LTC_4/D_4 and LTB_4 by radioimmunoassay.

At 10^{-7}M, A23187 induced little release of eicosanoids over basal: $6KPGF_{1\alpha}$ 0.14 ± 0.06 ng/min LTC_4/D_4 0.32 ± 0.16ng/min; LTB_4 0.11 ± 0.02 ng/min. Release was detected, however, with both 10^{-6}M (14.58±5.94 ng/min LTC_4/D_4; 1.46±0.38 ng/min LTB_4; 13.76±1.30 ng/min $6KPGF_{1\alpha}$) and 10^{-5}M A23187 (17.94±5.03 ng/min LTC_4/D_4; 0.87±0.20 ng/min LTB_4; 13.76±1.30 ng/min 6KPGF1α). Values quoted are mean ± S.E.M, n=3-6. Results for ischaemia-reperfusion are presented in the Table 1 (values are ng/min ± S.E.M).

Table 1. EFFECT OF ISCHAEMIA-REPERFUSION ON EICOSANOID RELEASE

	1 HR ISCHAEMIA (n=3-7)		4 HR ISCHAEMIA (n=4)	
	LTC_4/D_4	$6KPGF_{1\alpha}$	LTC_4/D_4	$6KPGF_{1\alpha}$
PRE ISCHAEMIA (BASAL)	0.13±0.04	0.85±0.22	0.46±0.35	0.79±0.29
ISCHAEMIA	0.01±0.00	0.52±0.14	0.00	0.39±0.15
REPERFUSION	0.26±0.08	1.46±0.44*	0.05±0.01	3.88±1.32*
10^{-6}M A23187	14.40±5.70*	22.27±2.22*	2.22±0.35*	4.94±1.33*

* $p<0.05$ increase over basal by paired T test

Levels of LTB_4 were undetectable during ischaemia-reperfusion. Low levels of release were evident with 10^{-6}M A23187: 1.16±0.24 ng/min (1hr) and 0.27±0.05 ng/min (4hr). Both values are significantly different from basal.

Thus, the rat isolated perfused heart synthesises LTs (particularly LTC_4/D_4) and $6KPGF_{1\alpha}$, when stimulated with A23187. Ischaemia-reperfusion, however, selectively stimulated the release of $6KPGF_{1\alpha}$. At present, neither the cellular source(s) of LT release in the perfused heart nor the pathophysiological stimulus capable of initiating their production is known.

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DIHYDROTESTOSTERONE POTENTIATES OESTRADIOL-INDUCED THYMUS ATROPHY IN MALE RATS

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Oestradiol (E2) and testosterone (T) both cause severe thymus atrophy in rats (Grossman, 1985; Fitzpatrick & Greenstein 1987). Dihydrotestosterone (DHT), a potent androgenic metabolite of T, does inhibit the post-orchidectomy rise in thymus weight (Pearce et al 1981), but is not atrophic to the organ (Fitzpatrick & Greenstein 1987). Androgens and oestrogens are simultaneously present and active in target tissues, and we therefore decided to test the effects of these steroids alone or in combination on the thymus. Adult male Wistar rats, weighing 300g-350g, were orchidectomized (OCX) or sham-orchidectomised via the scrotal route under ether anaesthesia; 3 days later, groups (4-6 per group) of OCX rats were anaesthetised with ether and given s.c. SILASTIC implants containing one of the following treatments: empty implant, 5mg T, 5mg DHT, 5mg E2, 5mg E2 + 5mg DHT. After 28 days the rats were anaesthetized with ether and blood taken by cardiac puncture for white cell counts. The rats were killed by cervical dislocation and the thymus removed, weighed and processed for histology. At autopsy, sham-operated thymuses weighed 443±11 mg(n=5); OCX 793±62 mg(5); T 431±31 mg (4); DHT 475±33 mg (4); E2 282±24 mg(6); E2 + DHT 175+18mg(6). The thymus was significantly heavier in OCX rats than in sham-OCX rats ($P<0.001$; Student's t-test), but not in OCX rats treated with T or DHT. Histologically, there were signs of atrophy in T- but not DHT-treated thymuses. Thymus weights in E2-treated rats were significantly lower than those from OCX animals ($P<0.001$), and weights from DHT + E2- treated rats were significantly lower than those from E2-treated rats. In these last two groups the thymus was grossly atrophied and the total white cell count reduced by about 50%. The results suggest that androgens and oestrogens may act in concert to cause thymus atrophy, and that the mechanisms underlying prevention of thymus growth on one hand, and thymus atrophy on the other, are different.

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MOTOR TRANSMISSION IN URINARY BLADDER OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

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A high incidence of bladder dysfunction amongst diabetics is well documented (Faerman et al, 1971, Buck et al, 1976, Frimodt-Moller, 1980). The motor transmission in mammalian bladder is believed to be partly cholinergic and partly non-cholinergic (Ambache & Zar, 1970; Burnstock et al, 1972; Krell et al, 1981). In the present study we have, therefore, examined the effect of streptozotocin-induced diabetes on the cholinergic and non-cholinergic motor transmission in rat bladder. Male Wistar rats (200-300g) were made diabetic by a single i.p. injection of buffered streptozotocin (75mg/kg); successful induction of diabetes was confirmed by high blood glucose levels (>300mg/100ml) and loss of body weight (weight of diabetic rats = 57% of controls). Four weeks after induction of diabetes, rats were sacrificed by decapitation. The volume of retained urine was measured and the bladder excised and weighed. Strips of detrusor muscle, 1-1.5cm x 0.2cm were prepared and suspended between parallel platinum electrodes in a 1 ml organ bath containing Krebs-Henseleit solution bubbled with a 95% O_2 + 5% CO_2 mixture at 37°C. The detrusor was contracted either through a direct action on the muscle by acetylcholine or indirectly by field stimulation of autonomic motor nerves (pulse duration: 0.1 ms; frequency: 0.5-10 Hz). Contractions were recorded isometrically. Atropine, 3μM was used to estimate the cholinergic and non-cholinergic contributions to the motor transmission.

There was a marked increase in bladder weight in diabetic rats (495% of control bladders). The volume of retained urine in bladder was significantly higher in diabetic rats (volume ± s.e. mean: diabetic 1.15 ml ± 0.3. Control 0.2 ml ± 0.05). There was no significant difference between the control and diabetic rats in the tension developed by the detrusor in response to acetylcholine. The atropine sensitive component of the response to electrical field stimulation remained undiminished in diabetic rats (atropine-sensitive response to field stimulation with a 10 Hz train in diabetic rats = 115% that of control rats). There was a marked reduction of the atropine-resistant component of the response to electrical field stimulation (atropine-resistant response to field stimulation with a 10 Hz train in diabetic rats = 45% that of control rats).

The results indicate that significant changes occur in rat bladder as early as 4 weeks following the induction of diabetes by streptozotocin. Although the cholinergic motor transmission and the response to acetylcholine remain unimpaired, the non-cholinergic motor transmission is considerably inhibited.

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INTRAGASTRIC CAPSAICIN ENHANCES GASTRIC ACID ELIMINATION AND MUCOSAL BLOOD FLOW BY ACTIVATION OF AFFERENT NEURONES

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Functional ablation of afferent neurones by systemic treatment of newborn rats with a high dose of capsaicin (160 µmol kg^{-1}) aggravates gastric lesion formation in various experimental ulcer models (Szolcsányi & Barthó, 1981; Holzer & Sametz, 1986a; Evangelista et al., 1986). On the other hand, intragastric administration of low doses of capsaicin (0.5-2.0 µmol kg^{-1}) has been shown to protect the gastric mucosa against ulceration (Szolcsányi & Barthó, 1981; Holzer & Sametz, 1986b). The aim of the present study was to investigate the effect of intragastric capsaicin on gastric acid secretion and mucosal blood flow in order to elucidate the mechanism of the antiulcer effect of capsaicin.

Gastric acid secretion and mucosal blood flow were measured in urethane-anaesthetized rats. Blood pressure was recorded from a carotid artery. The stomachs were continuously perfused with saline, and gastric acid secretion was determined by titrating the samples of gastric perfusate to pH 7. The clearance of ^{14}C-aniline into the gastric perfusion solution, adjusted to pH 3, was used to estimate changes in the mucosal blood flow (Main & Whittle, 1973).

Addition of capsaicin to the perfusion medium (160-640 nmol ml^{-1}) had no effect on basal acid secretion but significantly inhibited the secretory response to intravenous pentagastrin (8 nmol h^{-1}). When the stomachs were perfused with acid saline (pH 3), the acidity of the perfusate was also significantly reduced by capsaicin. This effect of intragastric capsaicin was absent in rats which had been treated with a high a dose of capsaicin as neonates.

Addition of capsaicin to the perfusion medium (160 nmol ml^{-1}) caused a 1.5-1.7 fold increase in mucosal blood flow, an effect which was not altered by combined pretreatment of the animals with atropine (0.35 µmol kg^{-1}), phentolamine (3.50 µmol kh^{-1}), and propranolol (1.90 µmol kg^{-1}). However, intragastric capsaicin failed to enhance mucosal blood flow in rats which had been treated with a high dose of capsaicin as neonates.

The data on gastric acid secretion indicate that, since basal secretion is not altered, intragastric administration of capsaicin enhances acid elimination from the stomach, possibly by increasing acid back diffusion. This effect is probably related to the increase in mucosal blood flow observed with intragastric capsaicin. The effects of capsaicin on both acid secretion and mucosal blood flow are due to activation of afferent neurones, because they were absent after ablation of afferent neurones by systemic pretreatment of rats with a high dose of capsaicin. The antiulcer effect of intragastric capsaicin may, at least in part, be accounted for by the increase in mucosal blood flow; as this effect was not altered by blockade of the autonomic nervous system it would appear that it is due to a local release of vasodilator mediators from sensory nerve endings in the gastric mucosa.

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IN VITRO RELEASE OF ACETYLCHOLINESTERASE FROM AUERBACH'S PLEXUS OF GUINEA-PIG ILEUM

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A soluble isoenzyme of acetylcholinesterase (AChE) is secreted from neurones in the brain into cerebrospinal fluid (CSF)(Greenfield, 1984). The presence of AChE in human CSF and its absence from plasma has led to a diagnostic test for neural tube defects by screening for AChE in amniotic fluid (Smith et al., 1979). However, AChE can also be detected in the amniotic fluid in certain cases of exomphalos in the absence of neural tube defects (Wald et al., 1984), indicating that AChE may also be released from the gut.

Most of the AChE present in the intestinal wall of the guinea-pig is present in the neurons, whereas the butyrylcholinesterase (BuChE) is confined to the muscle (Ambache et al., 1971). We have used this tissue to study whether there is both spontaneous and drug-evoked secretion of AChE and BuChE from the gut.

Strips of longitudinal muscle, retaining Auerbach's plexus, were prepared from the ileum of guinea-pigs (Paton & Aboo Zar, 1968). 150 mg segments were superfused with oxygenated Krebs' solution at 37°C in a 0.4 ml chamber at a rate of 0.25 ml/min. After an initial wash out phase of ten minutes, two-minute fractions were collected into vials containing bovine serum albumen. Ten minutes after the commencement of fraction collection, various drugs were added to the superfusing medium for a period of four minutes. All conditions were repeated using Ca^{++}-free medium throughout the whole procedure. Perfusates were assayed for AChE, BuChE (Chubb & Smith, 1975) and the soluble cytoplasm marker lactate dehydrogenase (LDH).

There was a rapid washout of AChE and BuChE activities over the first six minutes so that by ten minutes the levels of both were low and relatively constant (basal activity AChE = 0.3 mU/ml; BuChE = 1 mU/ml perfusion fluid).

Addition of 50 mM KCl led to a Ca^{++}-dependent 100 % increase in the activities of both AChE and BuChE in the perfusate. There was no accompanying increase in LDH activity indicating specific release, rather than an artefact due to tissue damage. When plexus-free muscle was used neither basal nor evoked release of AChE could be detected, although BuChE was still released.

Addition of 0.01 mM carbachol (n = 8) led to a 100% increase in the release of AChE with no accompanying increase in BuChE or LDH. This Ca^{++}-dependent increase in AChE release was blocked by hexamethonium (n = 4) but not by atropine (n = 4), indicating an involvement of nicotinic receptors.

It is concluded that spontaneous and evoked secretion of AChE and of BuChE occurs from the myenteric plexus and from its associated smooth muscle, respectively. This could account for the presence of both these enzymes in the amniotic fluids of cases of exomphalos, and could explain the lower ratio of AChE/BuChE in these cases compared to those with neural tube defects.

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ANTAGONISM BY BRL 43694 OF THE PSEUDOAFFECTIVE REFLEX INDUCED BY DUODENAL DISTENSION

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There is evidence that 5-HT may facilitate nociception within the peripheral nervous system. For example, when 5-HT is applied to a skin blister base in humans it causes pain, which can be reduced by topical application of the 5-HT_3 receptor antagonist ICS 205-930 (Donatsch et al 1984). In man, abnormal distension of the gut causes abdominal pain, particularly in patients with irritable bowel syndrome (Swarbrick et al, 1980). Distension of the jejunum or ileum in anaesthetised animals will induce a fall in blood pressure which can be blocked by morphine or capsaicin indicating that it may be part of a pain reflex (Lembeck & Skofitsch, 1982; Clark & Smith, 1985). In the present report, the effects of BRL 43694, a potent and selective 5-HT_3 receptor antagonist (Fake et al, 1987), have been studied on the response to duodenal distension and compared to ICS 205-930.

Male albino rats (220-350g) were anaesthetised with 25% urethane (6ml/kg ip). A 3-4cm portion of the duodenum was isolated by ligature immediately below the stomach. This segment was connected proximally to a saline (0.9%)-filled reservoir, and drained distally. The duodenum was distended with saline at a pressure of 75 cmH_2O for 20s every 5 min. Blood pressure and the intraluminal pressure of the duodenum were also monitored. The following regimen was used: 4 distensions (T1-T4); iv BRL 43694, ICS 205-930 or saline; 4 distensions (T5-T8). Drugs or saline were administered iv immediately after distension T4. Results were calculated as percentage (mean ± SEM) of the pre-drug response.

Effects of BRL 43694 and ICS 205-930 on the response to duodenal distension

	NaCl	BRL 43694 1µg/kg	BRL 43694 10µg/kg	BRL 43694 100µg/kg	ICS 205-930 10µg/kg	ICS 205-930 100µg/kg
	(6)	(7)	(7)	(9)	(4)	(4)
T4	100	100	100	100	100	100
T5	111±20	66±16	98±17	98±15	36±13*	92±27
T6	122±33	63±14*	63±8**	70±10*	70±14	67±7**
T7	131±30	68±14	53±14*	63+13*	65±14	51±18*
T8	113±23	65±16	47±9***	67±12*	80±5*	55±16*

*P 0.05; **P 0.01; ***P 0.005 Student's paired 't'-test; (n) = number of animals

Distension of the duodenum induced a rapid, reproducible fall in blood pressure of 16 ± 4mmHg (n=6). The response was blocked by morphine (20mg/kg sc; 92% block after 25 min; n=6) and this inhibition was reversed by naloxone (5mg/kg iv; 100% reversal after 20 min; n=6). BRL 43694 (10-100µg/kg iv) or ICS 205-930 (10-100µg/kg) significantly reduced the depressor response (see table) 20 min after administration; BRL 43694 (1µg/kg) was less effective. The intraluminal pressure of the duodenum was unaffected by either drug; similarly BRL 43694 does not increase rat intragastric pressure (Fake et al, 1987).

These results suggest that duodenal distension can evoke a visceral pain reflex. They also indicate a partial role for 5-HT acting through 5-HT_3 receptors in visceral nociception. Thus BRL 43694 antagonises this action of 5-HT as well as preventing emesis evoked by anti-cancer treatment (Boyle et al, 1987).

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SEVERAL REPUTED AGONISTS AT 5-HT_{1B} RECEPTORS BEHAVE AS PARTIAL AGONISTS ON MOUSE BLADDER PREPARATION

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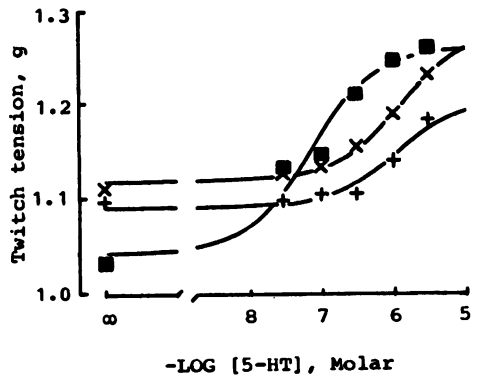
Holt et al. (1986) showed that a new pharmacological preparation, the isolated superfused mouse bladder strip (Wyllie et al., 1983) behaves as if it contains 5-hydroxytryptamine (5-HT) receptors of type 5-HT_{1B}. It may thus serve as a relatiely simple test object to study 5-HT receptors which are thought to be important in the brain. Some possible agonists at 5-HT_{1B} receptors have now been examined on this tissue - RU24969 (5-methoxy-3-[1,2,3,6-tetrahydropyridon-4-yl]1H-indole neutral succinate, 0.03 nM - 3.0 uM), BEA1654 (N-(3-acetylaminophenyl)-piperazine hydrochloride, 0.3 uM - 30 uM), TFMPP (1-(3-trifluoromethylphenyl)piperazine, 30 nM - 3.0 uM), PAPP (1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)piperazine, 100 nM - 10 uM), MCPP (1-(3-chlorophenyl)piperazine hydrochloride, 30 nM - 3.0 uM), 2-MPP (1-(2-methoxyphenyl)piperazine hydrochloride, 30 nM - 3.0 uM), MK-212 (6-chloro-2-(1-piperazinyl)pyrazine, 3 - 300 uM).

Two types on experiment were made. (1) We compared the concentration-response curve for each compound with that of 5-HT (4 expts. each): none of the compounds was a full agonist, and PAPP showed only feeble effects. (2) To test whether the compounds acted on 5-HT receptors we studied in 3 - 5 expts. the effects of mixtures of each compound with 5-HT. RU24969, BEA1654, TFMPP (Figure 1) & 2MPP augmented the effect of a low concentration of 5-HT (less than 0.1 uM) but depressed the effect of a high concentration of 5-HT (more than 1 uM). No interaction occurred with MK-212. The partial agonists showed efficacies in the range 50 - 70%. Their agonist effects, but not those of MK-212, could be suppressed by 50 uM pindolol, a beta adrenoceptor antagonist which also competes with 5-HT for 5-HT_{1B} receptors.



These data are consistent with the idea theat mouse bladder contains 5-HT_{1B} receptors. They also indicate a need for caution in interpreting the results of experiments <u>in vivo</u> because the putative agonists we tested all seem to be partial agonists and their effects <u>in vivo</u> wwould depend on 5-HT concentration at the receptors.

We are grateful to the MRC for support & to manufacturers for gifts of compounds.

Figure 1: Twitch tension developed by mouse bladder stimulated by trains of 16 pulses of 8 V, 10 Hz, 2-ms, as influenced by 5-HT (0 - 3 uM). [] = control curve, X = curve in presence of 0.3 uM TFMPP, + = curve in presence of 3.0 uM TFMPP. (Result of a single, typical experiment.)

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BRADYKININ RECEPTOR-INDUCED PHOSPHATIDYL INOSITOL TURNOVER IN HUMAN EMBRYONIC PITUITARY TUMOUR CELLS

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The nonapeptide,bradykinin (BK), is found in the mammalian brain, pituitary and other peripheral tissues (Regoli and Barabe,1980). Although bradykinin is known to evoke several biological actions including hypertension,nociception, inflammation and smooth muscle contraction the precise mechanism of action of this peptide is not known.Similarly, although two BK receptors ,namely B_1 and B_2,have been proposed and identified in mammalian tissues (Regoli and Barabe,1980) the signal transduction mechanism associated with these receptors is not understood.

The aim of the present study was to explore the possible existence of BK receptors on the human embryonic pituitary cell-line ,Flow 9000, using a bioassay involving the measurement of [^{3}H]inositol-1-phosphate ([^{3}H]IP_1) by ion exchange chromatography and receptor binding techniques. Flow 9000 cells were cultured as monolayers in supplemented Ham's F10 medium at 37°C in a humidified 95% O_2/CO_2 atmosphere. The cells (1-2 x10^6) were labelled with 2 µCi of myo-[2-^{3}H]inositol for 24-48 hours,washed with 2x1 ml of Krebs-bicarbonate buffer and then challenged with 1 ml solution of the test compound in Krebs buffer containing 10 mM LiCl for 30 min at 37°C. The assays were terminated by the addition of 1 ml of ice-cold chloroform/methanol (1:2) and the [^{3}H]IP_1 extracted and quantified according to Berridge et al.(1982).

Bradykinin and related analogues produced concentration-dependent accumulation of [^{3}H]IP_1 in Flow 9000 cells yielding half-maximal stimulations at the concentrations shown in parentheses (nM; mean ± SEM from 3-5 experiments) : BK (1.6 ± 0.24); Lys-Bk (2.1 ± 0.6); Met-Lys-BK (18.1 ± 2.7); Des-Arg^9-BK (436 ± 102); BK (1-6) (> 10^{-5}M); BK (2-7) (>10^{-5}M) and BK (2-9) (>10^{-5}M). In contrast, several other peptides unrelated to bradykinin,including substance P, neurotensin, somatostatin, vasoactive intestinal polypeptide, neuropeptide Y, calcitonin gene-related peptide and D-Ala^2-D-Leu^5-enkephalin were inactive in this system at 10 µM. Bradykinin and Des-Arg^9-BK produced half-maximal inhibitions (IC_{50}s) of specific [^{3}H]BK receptor binding on Flow 9000 cell homogenates at 13.9 ± 5.5 nM (n = 3) and 10 ± 1 µM (n = 3) respectively.

These studies suggest that bradykinin receptors of the B_2-type are present on Flow 9000 cells and that they appear to be coupled to the phosphatidyl inositol signal transduction mechanism. Flow 9000 cells may therefore represent a useful in vitro model for studying the peripheral actions of bradykinin at the cellular level.

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RU 31719, A NEW BENZODIAZEPINE RECEPTOR LIGAND WITH BENZODIAZEPINE-LIKE TRANQUILISING EFFECTS

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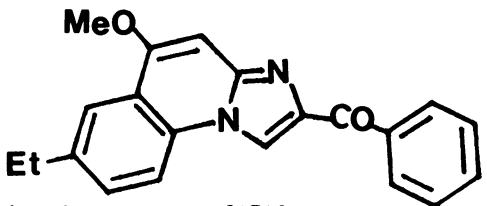
RU 31719, (7-ethyl-5-methoxy imidazo[1,2-a]quinolin-2-yl)phenyl methanone, is a ligand for benzodiazepine binding sites with an IC_{50} of 16.2 ± 3.5nM, n = 4, for displacing 0.6nM [^{3}H]-flunitrazepam from rat forebrain membranes (Squires and Braestrup, 1977). This interaction appears to be specific in as much as standard binding assays showed no interaction with dopamine receptors in the striatum or $5HT_2$, $\alpha 1$, $\alpha 2$, β or muscarinic receptors in the cerebral cortex up to 100uM. There was a weak interaction with [^{3}H]-naloxone-labelled opiate receptors in rat forebrain membranes ($IC_{50} \sim 100\mu M$).



RU 31719

In rat models of anxiety RU 31719 was active with minimal effective doses (MED) of <10mg/kg p.o. (Table 1), thus in a similar dose range and also with similar efficacy to chlordiazepoxide or diazepam. RU 31719 was also a potent anticonvulsant against both tonic leptazol seizures in rats or mice and tonic isoniazid seizures in mice (Table 1).

Table 1

Conflict		Social	Seizures			Rotating	Locomotor	Pull
Food[a]	Water[b]	Interaction[c]	Leptazol		INH	Drum	Activity	up
MED mg/kg p.o.			Rat	Mouse		ED_{50} mg/kg p.o.		
5-10	2.5	2.5	7.0	5.4	7.6	60	55	>640

ED_{50} values were calculated where dose-response curves were linear. All doses were 1h pre-test except food conflict when drugs were given 25 min prior to the 35 min test.

However, tests for sedative/myorelaxant activity showed only weak effects (Table 1) and in particular the pull up test which is specific for muscle relaxant activity (Deacon and Gardner, 1984) detected weak effects from 20mg/kg p.o. which did not reach 50% inhibition, even at doses up to 640mg/kg p.o.

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BENZODIAZEPINES ENHANCE THE EFFECT OF GABA AUTORECEPTOR AGONISTS

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It is well established that the release of GABA from central nerve terminals is modulated by presynaptic autoreceptors (Mitchell & Martin, 1978a). GABA receptors have been subdivided into GABA-A and GABA-B subtypes and the autoreceptor appears to resemble a GABA-A receptor in that it is sensitive to classical GABA-A agonists and is antagonised by bicuculline (Bowery et al, 1984). However, Brennan (1982) reported that unlike postsynaptic GABA-A receptors the autoreceptor may not be coupled to a benzodiazepine receptor since the IC_{50} value for muscimol-induced inhibition of (^3H)-GABA release was unchanged in the presence of flurazepam (10^{-6}M) although Mitchell & Martin (1978b) demonstrated that flurazepam and diazepam at 10^{-6}M enhanced the release of (^3H)-GABA from rat cortical slices. We have re-examined the effects of benzodiazepines both on the K^+-evoked release of (^3H)-GABA and on muscimol induced inhibition of (^3H)-GABA release in rat cortical slices.

Briefly, slices of rat cerebral cortex (0.2 x 0.2 x 2.0mm) were preloaded with (^3H)-GABA (10^{-7}M, specific activity 72 Ci $mmol^{-1}$) and superfused at 37°C with Krebs-Henseleit solution containing amino-oxyacetic acid (10^{-5}M) at a rate of 0.4 ml min^{-1}. Two 4 min pulses of Krebs solution containing 25mM K^+ were administered 68 (S_1) and 92 (S_2) min after the start of the superfusion. Drugs were added to the superfusing medium immediately after S_1. Superfusate fractions were collected every 4 min. The results were calculated as the S_2/S_1 ratio and statistical analysis performed using the Mann-Whitney U test, 2 tailed.

Muscimol (10^{-9} to 10^{-6}M) produced a concentration-dependent inhibition of K^+-evoked (^3H)-GABA release which was antagonised by bicuculline (10^{-6}M). The maximum inhibition produced by muscimol was 55± 6%; concentrations of muscimol above 10^{-6}M increased the basal release of tritium. Clonazepam 10^{-6} and 10^{-5}M produced an increase in K^+-evoked (^3H)-GABA release to 124 ± 5 and 144 ± 4% of controls respectively. Concentrations below 10^{-6}M had no significant effect. In the presence of clonazepam (10^{-7} and 10^{-6}M) the maximum inhibition produced by muscimol with no change in basal release was increased significantly from 55 ± 6 to 72 ± 4% and 77 ± 3% respectively ($p<0.05$). The concentration of muscimol required to inhibit K^+-evoked (^3H)-GABA release by 40% (IC_{40}) was decreased significantly from 178 ± 17nM to 22.9 ± 1.3nM and 12.6 ± 1.5nM in the presence of clonazepam 10^{-7} and 10^{-6}M respectively. There was no change in the threshold concentration of muscimol. The benzodiazepine antagonist flumazenil (Ro 15-1788) (10^{-7}M) had no significant effect on the concentration effect curve to muscimol but antagonised the increase in inhibition of (^3H)-GABA release produced by muscimol in the presence of clonazepam (10^{-7}M).

These results suggest that the presynaptic GABA autoreceptor is coupled to a benzodiazepine-sensitive site and that this site can modulate the response of the autoreceptor to agonists.

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BENZODIAZEPINE WITHDRAWAL: ENHANCED ANXIETY IN THE RAT REVERSED BY FLUMAZENIL (RO 15-1788)

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In the elevated plus-maze test of anxiety (Pellow et al, 1985) benzodiazepines increase the % time that rats spend on the open arms. We have previously shown that after 20 days of treatment with chlordiazepoxide, tolerance can be demonstrated to its anxiolytic effects in this test, and that the day after the last dose, the rats in spontaneous withdrawal spent significantly less % time on the open arms than did control animals (Baldwin et al, 1987). This indicates enhanced anxiety in benzodiazepine withdrawal.

The purpose of the present experiment was to explore the underlying mechanism of benzodiazepine withdrawal by investigating the effects of the benzodiazepine receptor antagonist, flumazenil (Ro 15-1788), and the benzodiazepine partial inverse agonist, FG 7142, in rats undergoing spontaneous withdrawal from chlordiazepoxide. Male hooded Lister rats were injected with chlordiazepoxide (CDP 10 mg/kg i.p.) or water control for 21 days. All injections were given in the morning and 24-30 hrs after the last injection of the chronic treatment the groups shown in the Table were tested in the plus-maze.

In both experiments, the rats in the withdrawal group spent significantly less % time on the open arms, compared with the controls (see Table), indicating enhanced anxiety during withdrawal. Ro 15-1788 (4 mg/kg) completely reversed this anxiogenic effect, whereas it was without effect in control animals (see Table). FG 7142 (5 mg/kg) was without significant effect in control animals and partially reversed the withdrawal anxiety, since the withdrawal + FG 7142 group was not significantly different from controls.

We suggest that tolerance to the anxiolytic effects of benzodiazepines arises from the production of an anxiogenic ligand. Ro 15-1788 antagonises this ligand and hence reverses its effects in animals undergoing withdrawal. This suggests that flumazenil might be a useful treatment in patients suffering increased anxiety in benzodiazepine withdrawal. The partial reversal of withdrawal by FG 7142 could be explained by its partial inverse agonist properties; it would act as an antagonist in the presence of a full agonist. This implies that the endogenous ligand has greater efficacy than FG 7142.

Chronic Treatment (21 days)	Acute treatment (20 min before test)	% time on open arms	N
Control	Control	21.2 ± 2.37	29
Control	Ro 15-1788	17.1 ± 4.14	14
CDP	Control	11.2 ± 1.83**	22
CDP	Ro 15-1788	22.0 ± 3.42~	17
	(30 min before test)		
Control	Control	23.6 ± 2.66	21
Control	FG 7142	18.2 ± 4.31	7
CDP	Control	12.1 ± 2.21*	14
CDP	FG 7142	19.6 ± 6.17	8

**p<.01 * p<.05, compared with controls ~p<.05 compared with withdrawal group

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RU 32698, A NEW BENZODIAZEPINE RECEPTOR LIGAND WITH ANXIOLYTIC BUT LITTLE SEDATIVE/MYORELAXANT PROPERTIES

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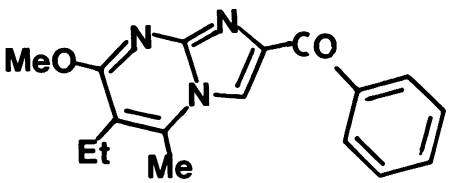
RU 32698 (2-benzoyl-6-ethyl-7-methoxy-5-methyl-imidazo[1,2-a]pyrimidine) is a member of a series of 2-benzoyl pyridines or pyrimidines which have selective affinity for benzodiazepine binding sites (RU 32698 - IC_{50} 56nM for displacement of ^{3}H-flunitrazepam binding to cortical membranes at 4°C).



RU 32698

In models in rats thought to detect anxiolytic drug effects such as food- or water-motivated conflicts in trained rats, social interaction or a leptazol cue, RU 32698 had similar potency to chlordiazepoxide (CDZP) (Table 1). However, RU 32698 was less active than CDZP in antagonising tonic seizures induced in mice by leptazol (ED_{50}s = 21 and 1.9mg/kg p.o. respectively) or electroshock (ED_{50}s = 16 and 5.1mg/kg p.o.). Furthermore, in contrast to CDZP, RU 32698 showed little or no effect in models detecting sedative and/or muscle relaxant effects such as rotating drum in rat or mouse, potentiation of chloral hydrate loss of righting reflex (Chloral Loss RR) and the pull up test in rats (Deacon and Gardner, 1984) (Table 1).

Table 1

Conflict		Social	Leptazol	Rotating Drum		Chloral	Pull up
Food[b]	Water[a]	Interaction[c]	Cue	Mouse	Rat	Loss RR	
MED	MED	MED	ED_{50}	ED_{50}	ED_{50}	ED_{50}	ED_{50}
2-5	2-5	5	4	200	>200	>200	>200
5	2-5	2	3	6.8	12	22	16

Data for RU 32698 are shown above those for CDZP in the same tests. MED = minimal effective dose tested. ED_{50} values were calculated where dose response curves were linear. All doses are mg/kg p.o. (1h pre-test) except food conflict when drugs were given 25 min prior to the 35 min test session.

The effect of RU 32698 in food-motivated conflict was fully antagonised by FG 7142 at 20mg/kg p.o. which itself only induced a small decrease in responding, suggesting the involvement of benzodiazepine receptors. RU 32698 is a candidate for clinical development as a selective anxiolytic therapy.

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SPECIFIC BINDING OF THE ANXIOLYTIC [^{3}H]-ALPIDEM TO CENTRAL RECEPTORS: DIFFERENCES WITH [^{3}H]-DIAZEPAM

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Alpidem (ALP) is an imidazopyridine (6-chloro(4chlorophenyl)2N,N di-n-propyl imidazo[1,2-a]pyridine-3-acetamide) which possesses anxiolytic and anticonvulsant properties in animal models, and weak myorelaxant and sedative effects in both animals and human volunteers (Saletu et al., 1986). ^{3}H-ALP was used as a ligand to explore its binding characteristics in various regions of the rat central nervous system. Suspensions of crude membrane preparations (20 µg of prot/ml) from cerebral cortex, cerebellum or spinal cord in chloride ion free buffer (K^+/Na^+, 50 mM, pH 7.5) were incubated for 30 min at 0°C with 0.1 - 7 nM ^{3}H-ALP (51.8 Ci/mmol, LERS Chemistry Department) or when indicated 1 nM ^{3}H-diazepam (72.5 Ci/mmol, N.E.N.). Binding assays were terminated by filtration in GF/B filters pretreated with 0.05% polyethylenimine. Non specific binding of ^{3}H-ALP or ^{3}H-DIAZ was determined in the presence of Ro 15-1788 2 µM or DIAZ 1 µM respectively.

In membranes preincubated with the peripheral benzodiazepine ligand Ro 5-4864 1 µM for 10 min, Scatchard analysis of saturation data indicate that ^{3}H-ALP binds with high affinity to a single class of recognition sites in the cerebral cortex (Kd = 1.50 ± 0.14 nM, Bmax = 1438 ± 129 (3) fmol/mg prot) as well as in cerebellum (Kd = 1.06 ± 0.26 nM, Bmax = 900 ± 163 fmol/mg prot). Under these conditions, recognition sites for ^{3}H-ALP were not detectable in the spinal cord which does not possess central receptors of BZ_1 type (Watanabe et al., 1985).

The binding of ^{3}H-ALP to rat cortex is displaced by benzodiazepines (Ki (nM) : triazolam 1.3; clonazepam 1.4; flunitrazepam 2.6; diazepam 6.9) as well as by non-benzodiazepines (Ki (nM) : CGS 9896 0.3; β-CCE 1.4; zolpidem 6.9; CL 218872 24.0).

Table 1 Changes in ^{3}H-alpidem or ^{3}H-diazepam binding in the cerebral cortex

	% of change (a)		
	GABA (10 µM)	Cl^- (100 mM)	Pentobarbital (1 mM)
^{3}H-ALPIDEM 1 nM	+ 82 ± 11.8 (3)	+ 11 ± 4.8 (3)	+ 1.8 ± 4.4 (4)
^{3}H-DIAZEPAM 0.5 nM	+105 ± 7.0 (4)	+ 48 ± 2.8* (3)	+32.0 ± 8.0* (3)

(a) percent of change in the specific binding relative to control values. Values are mean ± S.E.M. from ( ) experiments in duplicate. *$p<0.05$ when compared with ^{3}H-ALP.

Specific ^{3}H-ALP and ^{3}H-DIAZ binding to washed cerebral cortex membranes was enhanced by GABA (0.01 - 100 µM) (Table 1). On the other hand, chloride ions (1 - 30 mM) enhanced significantly ^{3}H-DIAZ but not ^{3}H-ALP binding (Table 1) suggesting the association of ^{3}H-ALP binding to BZ_1 receptors (Lo and Snyder, 1983). Pentobarbital (0.01 - 1 mM), in the presence of Cl^- ions 100 mM) enhanced ^{3}H-DIAZ binding but failed to modify ^{3}H-ALP binding (Table 1).

The lack of influence of chloride ions and pentobarbital on ^{3}H-ALP binding as well as the selectivity of ^{3}H-ALP for BZ_1 receptors are characteristics that differentiate this ligand from ^{3}H-DIAZ. The atypical characteristics of ^{3}H-ALP binding when compared with ^{3}H-DIAZ may explain its selective association with anxiolytic activity.

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FLURAZEPAM ENHANCES THE ABILITY OF DELTA-9-TETRAHYDROCANNABINOL TO INDUCE CATALEPSY IN MICE

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Previous experiments with mice have shown that flurazepam (FZ) and delta-9-tetrahydrocannabinol (THC) can abolish the righting reflex when given consecutively to the same animals at doses which, given singly, have no effect on this reflex (Morrison and Pertwee, 1985). It was also shown in this earlier study that FZ could enhance THC-induced hypothermia. The present experiments were carried out to test whether FZ increases the intensity of THC-induced catalepsy.

Experiments were performed with groups of 6 unrestrained, adult, male, MF1 mice. THC was mixed with 2 parts of Tween 80 by weight, dispersed in saline and given intraperitoneally at time zero. FZ was dissolved in saline and injected subcutaneously, 30 min before THC. The injection volume for both drugs was 0.25 ml/25 g body weight. The ambient temperature was kept at 34°C to avoid hypothermia. The intensity of catalepsy was determined by noting the length of time a mouse remained immobile with both forelegs over a 4 cm high bar and both hindlegs on the bench (bar test). This test was based on the method of Costall and Olley (1971). Testing commenced at +30 min after which mice were observed for periods of up to 30 min (maximum score = 1800 s).

Table 1 Effect of FZ on THC-induced immobility in the bar test

Treatment at -30 min		Treatment at time zero		Time spent immobile	
Drug	Dose (mg/kg)	Drug	Dose (mg/kg)	Range (s)	Mean (s±s.e.)
FZ	3	Tween	40	0-7	3±1
Saline	-	Tween	40	1-7	3±1
FZ	3	THC	2.5	1-48	23±8
Saline	-	THC	2.5	1-26	8±4
FZ	3	THC	5	68-215	160±30*
Saline	-	THC	5	1-7	4±1
FZ	3	THC	10	55-1800	798±253*
Saline	-	THC	10	2-41	18±6
FZ	3	THC	20	1009-1800	1544±162*
Saline	-	THC	20	29-661	189±104

*Increases significant to $P<0.001$ (Mann-Whitney U-test).

FZ markedly extended the time that THC-treated mice remained immobile in the bar test (Table 1). The immobility was not accompanied by any detectable loss of skeletal muscle tone. It is concluded that FZ can enhance the ability of THC to induce catalepsy and that it does so by an as yet unknown mechanism.

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2-CHLOROADENOSINE PREVENTS KAINIC ACID-INDUCED LESIONS OF RAT STRIATUM

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Injection of kainic acid (< 3 nmol) into the rat striatum results in an extensive perikaryal-specific lesion (Coyle et al., 1978). The mechanism underlying this toxicity is still unclear, although it appears that pre- as well as postsynaptic components may be involved. Kainic acid evokes the release of endogenous glutamate from striatal slices (Ferkany and Coyle, 1983) and related to this, is the observation that destruction of the cortico-striatal glutamatergic input confers protection against kainic acid (Biziere and Coyle, 1978).

Adenosine inhibits the release of many transmitter substances including glutamate, in the nervous system (Dolphin and Archer, 1983). In this study, we have investigated whether local injection of the stable adenosine analogue 2-chloroadenosine, might afford protection against kainic acid. Bilateral intrastriatal injections of kainic acid (2.5 nmol) were made in 200g male Wistar rats. 2-chloroadenosine (3-100 nmol) was co-injected into one striatum. Animals were allowed to recover for 14 days, anaesthetised and then transcardially perfused with fixative. Forty micron sections were stained for NADPH-diaphorase, AChE or thionin. As expected, kainic acid alone, elicited complete disruption of neuronal perikarya throughout the entire striatum. In contrast, co-injection of 2-chloroadenosine resulted in a dose-related protection. At the highest dose, neuronal loss was minimal except in the area close to the injection site.

To investigate whether the protection against kainic acid lesions by 2-chloroadenosine was related to a presynaptic mechanism, we have investigated its ability to influence the release of endogenous glutamate from a rat striatal P2 preparation. The basal release was approximately 0.4 nmol mg protein per min. Following depolarisation with 30 mM K^+ or addition of 1 mM kainic acid, glutamate release increased to 1.2 and 0.9 nmol mg protein per min, respectively. Inclusion of 100 µM 2-chloroadenosine did not affect the basal release, but produced a significant inhibition of the K^+-stimulated release. Similar results were found using purified guinea pig cortical synaptosomes, where 2-chloroadenosine abolished the K^+-evoked release in a dose-dependent manner. Although an inhibition by 2-chloroadenosine of kainic acid-induced release was observed in some experiments, it was not a consistent phenomenon.

These results demonstrate that 2-chloroadenosine prevents the direct striatal lesioning action of kainic acid. It seems possible that this acion is mediated presynaptically.

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STRYCHNINE-RESISTANT ACTION OF GLYCINE AT MAMMALIAN MOTONEURONES

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Hyperpolarizing or depolarizing actions of glycine associated with inhibitory conductance changes are characterized by sensitivity to strychnine. However, a strychnine-resistant depolarizing action of glycine (Evans et al 1976) associated with increased excitability of motoneurones (Nicoll et al 1976) has been reported previously to occur at motoneurones in the amphibian spinal cord. The present results, from mature rat preparations, suggest that this effect may not be peculiar to amphibian motoneurones.

Recordings of d.c. polarity were made from ventral roots (SIII to CoI) of isolated hemisected spinal cords obtained from rats of 180 to 250g body weight. Depressant actions of neutral amino acids were assessed from inhibition of N-methyl-D-aspartate (NMDA) receptor-mediated spontaneous synaptic activity which developed in the absence of added Mg^{2+} Allan et al (1980). γ-Aminobutyrate (GABA), glycine and taurine, all (1-5 mM), had depressant actions. The depressant actions of GABA and glycine were associated with depolarization. Both features of the GABA, but not the glycine- or taurine-induced responses were sensitive to picrotoxin (25μM). Depressant actions of glycine and taurine, but not GABA were antagonised in the presence of strychnine (1μM). In three preparations the depolarizing action of 5mM glycine (mean=0.21mV) was increased by 0.35mV±0.08 S.D. in the presence of strychnine, presumably due to blockade of the inhibitory conductance. This strychnine-resistant depolarizing action of glycine was still present following blockade of regenerative activity with tetrodotoxin. Kynurenic acid (1mM), which in three preparations depressed 1mM L-glutamate-induced depolarizations (mean=0.55mV) by 0.33mV±0.21 S.D., had no effect on the strychnine-resistant depolarizing action of glycine. The strychnine-sensitive and strychnine-insensitive depolarizing actions of glycine could be distinguished also by absence of the latter from isolated ventral root fibres.

Therefore it would appear that mature rat motoneurones possess strychnine-insensitive receptors for glycine in addition to those which mediate strychnine-sensitive inhibitory effects. It is unlikely that this strychnine-insensitive effect corresponds to the glycine-induced closure of potassium channels reported by Biscoe et al (1987) since the latter action was sensitive to strychnine. Glycine has been shown to potentiate the effects of NMDA on cultured neurones from embryos (Johnson & Ascher 1987). Resistance of the present phenomenon to kynurenate suggests that it is unlikely to be explained through such action at NMDA receptors.

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INTERACTION OF MK-801 WITH NMDA RECEPTORS AND SYNAPTIC TRANSMISSION IN THE RAT VENTROBASAL THALAMUS

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The anticonvulsant, MK-801, has been shown to be a non-competitive antagonist of N-methyl-D-aspartate (NMDA) excitatory amino acid receptors (Wong et.al., 1986). NMDA receptors are involved in the mediation of responses of ventrobasal thalamus (VB) neurones to maintained stimulation of non-nociceptive somatosensory afferents (Salt, 1986). This study investigates the possibility that MK-801 might interact with VB synaptic NMDA receptors, and that such an interaction might contribute to the effects of systemically administered MK-801.

Extracellular single neurone recordings were made with multi-barrel iontophoretic electrodes in VB of urethane anaesthetised (1.2g/kg, i.p.) rats. Iontophoretically ejected MK-801 (0-20nA, 5mM in 150mM NaCl), was found to selectively antagonise the responses of twenty VB neurones to iontophoretically applied NMA, whilst having little effect on responses to iontophoretically applied kainate or quisqualate. Under such conditions of selective antagonism, VB neurone responses to sensory stimulation (2-second air jet directed at the peripheral receptive field) were also reduced, on average, by 62%. Recovery from the effects of MK-801, seen in the majority of cases, took between 30 and 60 minutes. Five VB neurones were also tested with intravenous administration of MK-801 (0.1-0.4 mg/kg). This produced similar effects to those seen with iontophoretically applied antagonist. However, the duration of action of i.v. MK-801 was much longer, with no recovery from its effects even when neurones were recorded for three hours or more following administration of the antagonist.

These results are consistent with previous findings which showed MK-801 to be an NMDA antagonist in various brain regions (Aram et.al., 1986; Wong et.al., 1986), and with previous data obtained with other NMDA antagonists in VB (Salt, 1986; Salt & Wilson, 1987). Furthermore, it is evident that systemically applied MK-801 is able to block, at least partially, NMDA-receptor mediated sensory synaptic input to VB.

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COMPARISON OF THE EXCITATORY AMINO ACIDS QUISQUALATE AND AMPA IN SLICES OF RAT CEREBRAL CORTEX

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The classification of excitatory amino acid receptor sub-types is primarily based on the actions of selective agonists (Watkins & Evans, 1981). One of these sub-types, the "quisqualate receptor", is reported to be preferentially activated also by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (Krogsgaard-Larsen et al., 1980) and both quisqualate (QUIS) and AMPA share a common membrane binding site (Honoré et al 1982). We have recently found some differences in the dose-response relationships of AMPA and QUIS that have led us to compare the pharmacology of these two agonists in more detail.

Slices of rat cerebral cortex were prepared as previously described (Harrison & Simmonds, 1985) and were perfused with Krebs medium containing 1mM Mg^{2+}. Depolarising responses to QUIS and AMPA were normalised with respect to the response obtained with 5μM AMPA in each slice. The antagonist kynurenate caused parallel shifts of the log. dose-response lines allowing Schild plots to be constructed. The slopes of the Schild plots and pA_2 values were as follows:

		agonist	slope	pA_2	n
KYNURENATE	0.1-10.0 mM	QUIS	0.77±0.06	3.96	26
	0.1-3.0 mM	AMPA	1.01±0.04	3.92	16

The values are very similar to those previously reported (Kemp et al 1987), and suggest that the antagonism is competitive. The lower slope of the Schild plot with QUIS may be due to the influence of an uptake process for which QUIS is a substrate (see Lodge et al 1980). AMPA was also antagonised by pentobarbitone and phenobarbitone, to a similar extent as QUIS (Horne & Simmonds 1986) and in a non-competitive manner.

Typical QUIS dose-response curves extended over approximately two log units (2.5μM -320μM). AMPA was approximately equipotent with QUIS at the bottom of the dose-response curves but the AMPA dose-response curve was appreciably steeper such that, near the top, AMPA was about 8 times more potent than QUIS. The possibility was tested that QUIS might be a partial agonist. Near maximal concentrations of 400μM QUIS and 40μM AMPA were applied first separately and then in combination. The mean response to 400μM QUIS (± SEM) was 2.00 ± 0.15 (n=4), and that to 40μM AMPA was 2.64 ± 0.40 (n=4). When these two doses were added in combination the mean response was 2.40 ± 0.40 (n=4). In each experiment the combination response was smaller than that to 40μM AMPA alone and this difference was statistically significant overall ($p<0.05$, Students paired t-test). These results suggest that QUIS may, indeed, be a partial agonist acting at the same receptor site as AMPA.

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INHIBITION BY CARBACHOL OF THE SYNAPTICALLY-EVOKED EXCITATION OF HIPPOCAMPAL CA1 NEURONES: MEDIATION BY M_1 MUSCARINIC RECEPTORS?

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The ability of exogenously applied acetylcholine to reduce the amplitude of the CA1 population spike in the hippocampus in vitro has been attributed to a presynaptic muscarinic action of the amine, resulting in a decreased amount of excitatory amino acid release in response to the afferent volley in the Schaffer collateral/commissural pathway (Hounsgaard, 1978; Valentino & Dingledine, 1981). The differentiation of muscarinic receptors into M_1 and M_2 subclasses (Hammer et al., 1980) has prompted us to re-examine the muscarinic inhibition of the hippocampal population spike with particular reference to the subtype of muscarinic receptor involved. Carbachol was chosen as the agonist because of its resistance to hydrolysis by acetylcholinesterase. The selective M_1 receptor ligand, pirenzepine (Hammer et al., 1980), was used as the antagonist.

Transverse hippocampal slices (330 µm), prepared from adult male Wistar rats, were submerged in continuously flowing artificial CSF in a perspex recording chamber. Slices were maintained at 21-22 °C. Pyramidal neurones in area CA1 were synaptically activated by means of electrical stimulation of the Schaffer collateral/commissural pathway in stratum radiatum. Conventional intracellular and extracellular recording techniques were used to monitor the activity of single CA1 pyramidal neurones or the population spike, respectively. The effect of carbachol on the population spike was investigated by constructing cumulative dose-response curves to the agonist (15 min exposure to each concentration). The slice was then superfused with artificial CSF containing pirenzepine, allowing 45 min for equilibration before repeating the dose-response curve to carbachol. The apparent pA_2 for pirenzepine was estimated from the following relationship (Arunlakshana & Schild, 1959):

$$pA_2 = - \log ([\text{pirenzepine}] / (\text{dose ratio} - 1))$$

Data are expressed as mean ± s.e. mean.

Intracellular records were made from six CA1 pyramidal neurones having membrane potentials ranging from -60 to -72mV. One cell exhibited spontaneous firing (<0.5Hz). Schaffer collateral/commissural stimulation reliably evoked single constant latency spikes in each of the cells studied (amplitude 82-100 mV, latency 5.6-11.2 ms). Superfusion of the slices for 15 min with carbachol (3-10 µM) consistently led to failure of the synaptically-evoked spike together with a variable degree of depolarization (4.2 ± 1.6 mV, range 0-10 mV). One neurone developed slow (<0.5Hz) spontaneous firing during the application of carbachol.

Carbachol (1-10 µM) produced a dose-dependent reduction of the population spike amplitude (EC_{50} =1.9 ± 0.2 µM; n=5). Pirenzepine (100 nM) shifted the dose-response curve to carbachol to the right (dose ratio = 10.9 ± 1.3). The apparent pA_2 for this antagonism was 8.0 ± 0.1, corresponding to the high affinity M_1 muscarinic receptor (Hammer et al., 1980).

Taken together, these data provide evidence that the inhibition by carbachol of the synaptically-evoked excitation of hippocampal CA1 neurones may be mediated by muscarinic receptors of the M_1 subtype.

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COMPARISON OF THE POSITIVE INOTROPIC AND PHOSPHODIESTERASE INHIBITORY EFFECTS OF IBMX AND MILRINONE IN THE RABBIT MYOCARDIUM

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A variety of new cardiotonic drugs that produce their effects by selective inhibition of a high affinity cAMP Specific-phosphodiesterase have recently been described in the literature (Weishaar et al., 1986). However, the exact relationship between inhibition of various phosphodiesterase isoenzymes, elevation of intracellular cyclic nucleotides and positive inotropism remains unclear. In an attempt to analyse these aspects the effects of a specific (milrinone) and non-specific (IBMX) inhibitors of phosphodiesterases on the rabbit myocardium were examined.

Tension and cyclic nucleotide measurements, in rabbit papillary muscles, were made as described by Rodger and Shahid (1984). Phosphodiesterase isoenzymes, from rabbit heart ventricle, were partially purified using DEAE-Sepharose anion exchange chromatography. Enzyme activity was assayed according to the procedure described by Methven et al., (1980).

Both IBMX and milrinone produced concentration-dependent positive inotropic effects with mean EC_{50} values of 130 μM ± 20 and 518 μM ± 67 respectively. Threshold concentrations of IBMX (4.5 μM) and milrinone (32 μM) produced a 5-6 fold potentiation of isoprenaline responses. The effects on tissue cyclic nucleotide levels are summarized in Table 1:

Table 1 Effects of IBMX and milrinone on cyclic nucleotide levels

Compound	Conc. (μM)	Tension (mg)	cAMP (pmol/mg wet wt.)	cGMP
Control	-	463±82	0.763±0.079	0.0250±0.0065
IBMX	450	1067±152	2.36 ±0.20**	0.1350±0.021**
Milrinone	800	981±113	1.298±0.225*	0.029 ±0.0061

Although both compounds elevate cAMP, it is clear that IBMX produced a much larger increase than milrinone. Phosphodiesterases from rabbit cardiac ventricle were resolved into three main fractions which were labelled PDE I, II and III representing the order of elution from DEAE-sepharose. PDE I showed almost equal affinity for cAMP and cGMP and, unlike fractions II and III, was activated by Ca/calmodulin (5-10 fold). PDE III displayed high affinity and high activity for cAMP. In contrast, PDE II had low affinity and low activity for cAMP and cGMP. IBMX inhibited cAMP hydrolysis by all three fractions in an almost equal but potent manner (IC_{50} values (μM): 15.7, 10.5 and 5.6 for I, II and III respectively). Furthermore IBMX (IC_{50}: 4.3 μM) also inhibited the Ca/calmodulin-induced activation of PDE I. In contrast, milrinone showed high selectivity for inhibiting cAMP hydrolysis by PDE III (IC_{50}: 2.24 μM) compared to PDE I (IC_{50} > 200 μM) and PDE II (IC_{50}: 65.7 μM).

The results indicate that IBMX is a potent but non-selective inhibitor of phosphodiesterases from rabbit heart, whereas milrinone shows selectivity for inhibiting PDE III. These data may explain the differing effects of the drugs on cAMP levels as well as the lower inotropic potency of milrinone in rabbit isolated papillary muscles.

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FAILURE OF (-)-N^6-PHENYLISOPROPYLADENOSINE TO AFFECT THE CAMP-DEPENDENT PROTEIN KINASE

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In ventricular cardiac muscle adenosine exerts negative inotropic effects in the presence of cAMP-increasing agents although the drug has no inotropic or even a weak positive inotropic effect when given alone (Schrader et al., 1977). The mechanism of the negative inotropic effect is still a matter of debate. A decrease in cellular cAMP level via inhibition of the adenylate cyclase has been reported (Schrader et al., 1977) while others found no change in the cellular cAMP content (Böhm et al., 1985). In this context it has been suggested that a direct effect on cAMP-dependent protein kinase (cAdPK) could attribute to the negative inotropic effect of adenosine. Therefore we investigated the effects of the A_1-adenosine receptor agonist (-)-N^6-phenylisopropyladenosine (PIA) on isolated bovine heart cAdPK and protein kinase and force of contraction (FC) in intact guinea-pig papillary muscles.

Protein kinase activity was determined by the method of Witt and Roskowski (1975). PIA (0.001- 100 µM) failed to inhibit the activity of the bovine heart cAdPK in the presence of 0.1, 0.3 and 1.0 µM cAMP. In electrically driven guinea-pig papillary muscles (1 Hz) the protein kinase activity ratio as defined as enzyme activity ratio in the absence and in the presence of 1 µM cAMP was determined according to Keely et al. (1975). Isoprenaline (ISO; 0.01 µM) increased FC to 305%. Additionally applied PIA (1 µM) reduced FC by 38%. In the same preparations ISO increased the protein kinase activity ratio from 0.39 $\pm$ 0.05 (n=7) to 0.58 $\pm$ 0.05 (n=11, $p < 0.05$) while additionally applied PIA did not change the ratio (0.55 $\pm$ 0.05, n=10).

The failure of PIA to affect isolated cAdPK and the protein kinase activity ratio in intact papillary muscles indicates that direct inhibitory effects on the cAdPK are unlikely to contribute to the adenosine receptor-mediated negative inotropic effects in ventricular cardiac muscle.

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β1-ADRENOCEPTOR RESERVE FOR THE POSITIVE INOTROPIC EFFECTS OF DENOPAMINE (TA 064) IN KITTEN HEART TISSUES

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Catecholamines and other agonists can elicit maximum inotropic responses in kitten ventricle at concentrations considerably smaller than their equilibrium constants K for β-adrenoceptors (Kaumann, 1978 & 1981). The nature of the amplification of β-adrenoceptor activation leading to inotropic responses is unknown. Do both β_1- and β_2- adrenoceptors, known to coexist in kitten heart (Kaumann & Lemoine, 1985), contribute to amplification? To study the role of β_1-adrenoceptors we chose the β_1-selective agonist denopamine (Nagao et al.,1984). Experiments were carried out at 32.5°C on isolated heart tissues (5μM phenoxybensamine 2h) from kittens (pretreated with reserpine or syrosingopine) and on ventricular membranes for binding of ^{3}H-(-)-bisoprolol to β_1-adrenoceptors and for adenylate cyclase assays as described by Kaumann & Lemoine (1985). The affinity of denopamine for β_2-adrenoceptors was estimated on membranes (labelled with ^{3}H-ICI 118,551) from smooth muscle cells of calf trachea and guinea-pig lung as described by Lemoine et al.(1985). The intrinsic activity of denopamine with respect to catecholamines was 1.0 on isolated heart tissues and 0.3 for ventricular adenylate cyclase. The affinity of denopamine, estimated from binding inhibition and from the antagonism of agonist-induced stimulation of cyclase, was only 3-5 times higher for β_1- than for β_2-adrenoceptors (Table 1). 1μM of the β_1-specific antagonist CGP 20712 A (Kaumann, 1986; $pK\beta_1$=9.5) caused a 3½ log unit shift of the inotropic concentration-effect curves for denopamine without revealing β_2-components. The affinity of denopamine for β_1-adrenoceptors was lower than its inotropic potency (Table 1) suggesting the existence of a β_1-adrenoceptor reserve, which was confirmed by desensitising the tissues with (-)-isoprenaline as described by Kaumann & Birnbaumer (1976). Desensitisation caused a rightward shift of the concentration-effect curves for denopamine without reduction of maximum responses.

Table 1

Affinity of denopamine for β_1- and β_2-adrenoceptors (SD<0.2 log)

Binding assays (0.2 mM GTP)				Adenylate cyclase		
Tissue	Competing ligand	$pK\beta_1$	$pK\beta_2$	Agonist	$pK\beta_1$	$pK\beta_2$
Kitten ventricle	^{3}H-(-)-bisoprolol	5.8		(-)-Adrenaline	5.6	4.9
Guinea-pig lung	^{3}H-ICI 118,551		5.4	(-)-Noradrenaline	5.6	5.0
Calf trachea	^{3}H-ICI 118,551		5.2			

Positive inotropic effects of denopamine in kitten tissues (x±SD)

	Controls			Desensitised		
Tissue	n	-logEC50	%of isoprenaline max	n	-logEC50	%of isoprenaline max
Left atrium	8	7.5±0.2	98±2	8	6.2±0.2	98±2
Papillary muscle	8	7.2±0.2	99±3	7	5.8±0.1	112±13

Conclusions: 1) The small β_1-selectivity of denopamine estimated from affinity measurements does not account for its inotropic β_1-specificity. 2) Small increases of cyclic AMP mediated through activation of β_1-adrenoceptors by denopamine appear sufficient to generate maximum increases in contractile force. 3) Although denopamine recognizes ventricular β_2-adrenoceptors, their occupancy does not contribute to positive inotropic effects.

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INFLUENCE OF MEMBRANE POTENTIAL ON THE NEGATIVE INOTROPIC EFFECTS OF HALOTHANE ON CELLS ISOLATED FROM GUINEA-PIG VENTRICULAR MUSCLE

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In voltage-clamped guinea-pig ventricular cells halothane reduces the amplitude of calcium currents and may reduce the calcium transient that activates contraction produced in response to a step depolarization from -40 mV to 0 mV (Terrar & Victory, 1987). The mechanisms which generate a calcium transient and contraction in response to a depolarisation to +60 mV may be different. In this study the effect of halothane upon contractions of isolated guinea-pig ventricular cells evoked by 200 ms step depolarisations to +60 mV was investigated. Contraction was measured by an optical method. The effect of 2% halothane applied in solution flowing over the cells upon contraction at this potential was variable. In 10 cells the contraction increased, in 7 contraction decreased and in 2 no change occurred. The time to peak of the contraction was, however, consistently increased by 51±10%. This effect of halothane differred from that observed at 0 mV ($p < 0.001$), where a profound depression of contraction (54±2%; Figure 1) and no significant change in time to peak contraction were consistently observed. When a prepulse to 0 mV preceded the depolarisation to +60 mV, the time to peak contraction was significantly increased by 46±9%. The administration of 2% halothane caused no further increase in the time to peak contraction when a prepulse was present. In another series of experiment, pairs of step depolarizations were applied. When the first pulse was a 200 ms depolarization to 0 mV, the amplitude of the calcium current and contraction in response to a second depolarization to 0 mV were initially greatly reduced and recovered as the interpulse interval was increased over the range 10 ms to 1s. Halothane caused a marked slowing of the recovery of contraction, but had little or no detectable effect on the recovery of calcium current. However, when the first of a pair of depolarizations was to +60 mV for 200 ms, there was little reduction in the amplitude of contraction in response to a second depolarization to +60 mV, even at short intervals such as 10 ms; there was little change in the amplitude of these contractions over the range 10 ms to 1s, and little or no influence of halothane on contraction under these conditions, in contrast to the effects at 0 mV described above .

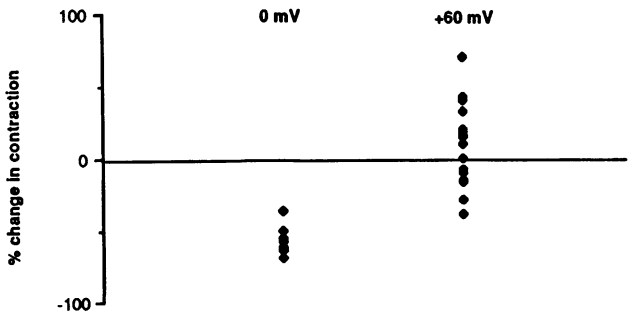

Figure 1. Modification of contraction by application of 2% halothane (1.16 mM). 12 cells at 0 mV, 19 cells at +60 mV. 36 C, pH 7.4, 0.3 Hz.

These observations provide support for the suggestion that mechanisms for control of contraction at 0 and +60 mV may differ. The observed variation of the actions of halothane on contractions at these two potentials may result from these possible differences in mechanisms of contraction, although a more direct influence of membrane potential on halothane action cannot be excluded.

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NIFEDIPINE-RESISTANT CONTRACTIONS AT POSITIVE POTENTIALS IN CELLS ISOLATED FROM GUINEA-PIG VENTRICULAR MUSCLE

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Mechanisms of calcium entry to control contraction in guinea-pig myocytes may differ at different potentials (Mitchell. Powell, Terrar & Twist, 1987). This possibility was investigated by comparing the actions of nifedipine and dodecylamine on contractions and currents in response to step depolarizations to various potentials for 200 ms; the potential preceding the depolarization was always -40 mV, but in some cells a complex protocol was employed where the cell was held at a more negative level (such as -70 mV), and a slow ramp of depolarization was applied to bring the membrane potential to -40 mV to inactivate fast sodium current without significantly activating it (duration of ramp, 0.5 s; duration of plateau at -40 mV, 0.5 s; protocol repetition rate, 0.3 Hz). Contractions in response to a step depolarization to +60 mV, unlike those at 0 mV, were not abolished by a preceding depolarization to 0 mV to inactivate calcium current (10 ms interval between pulses); in the same cells this contraction at +60 mV was resistant to block by 5 μM nifedipine, in contrast to the marked reduction by nifedipine of the current and contraction in response to the prepulse to 0 mV. One possible mechanism for calcium entry at positive potentials is via Na:Ca exchange, and dodecylamine has been reported to inhibit this (e.g. Bielfield, Hadley, Vassilev & Hume, 1986). When cells were exposed to 20 μM dodecylamine, the contractions at +60 mV were greatly reduced, while the calcium current was reduced to a lesser degree (Figure 1). Qualitatively similar observations were made at less positive potentials (e.g. +20 mV).

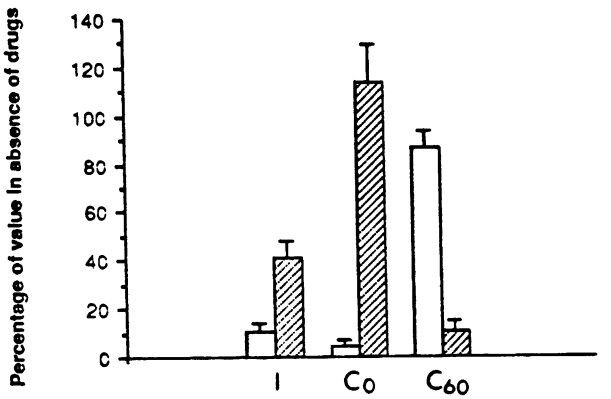

Figure 1: Effects of nifedipine (5 μM; open columns) and dodecylamine (20 μM; shaded columns) on peak calcium current at 0 mV (I), contraction at 0 mV (C0) and contraction at +60 mV (C60). 36°C. Bars show S.E. (9 cells in each case).

The resistance of the contractions at +60 mV to nifedipine, and their relative sensitivity to dodecylamine, are consistent with the suggestion that calcium may enter at +60 mV via mechanisms other than nifedipine-sensitive calcium channels. In view of the lack of specificity of dodecylamine (Bielfield et al, 1986), further work (perhaps with more specific inhibitors) will be necessary to establish the role of Na:Ca exchange, and of other mechanisms which might permit calcium entry (e.g. calcium-activated non-selective channels) for contraction at positive potentials.

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EFFECTS OF BRL 34915 ON MEMBRANE CURRENTS RECORDED FROM SINGLE SMOOTH MUSCLE CELLS FROM THE RABBIT PORTAL VEIN

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BRL 34915 is a novel vasodilatory agent which has been suggested to have a mechanism of action primarily involving an increase in membrane K^+ conductance (Weir & Weston, 1986). We have studied the effects of BRL 34915 on single, enzymatically dispersed, smooth muscle cells from the rabbit portal vein using standard patch clamp techniques (Hamill et al., 1981) at room temperature.

In physiological salt solution, cells had resting membrane potentials between -30 and -40mV when in current clamp. Bath application of BRL 34915 (1μM) induced small hyperpolarizations of 2 to 5mV, and BRL 34915 (10μM) larger hyperpolarizations of 30 to 40mV. In cells clamped at -40mV, BRL 34915 (10μM) evoked a sustained outward current of 43.7 ± 18.3 pA (mean ± s.d., n=12), which was associated with an increase in membrane conductance. The reversal potential of the current induced by BRL 34915 (10μM) (I_B) was close to the calculated K^+ equilibrium potential (E_k) in all cells studied; with E_k at -78mV it was -72.5 ± 3.5mV (n=5), and with E_k at -58 mV it was -55 ± 0.5mV (n=3). Instantaneous current evoked by hyperpolarizing pulses from a positive holding potential (e.g. +30mV) showed a slight curvilinear relationship with respect to clamp potential, but no marked voltage dependence of I_B has been observed.

The pharmacological characteristics of I_B were studied by bath application of TEA, 4 -aminopyridine or quinidine during the sustained outward current evoked by BRL 34915 (10μM) at a holding potential of -40mV. TEA (0.5mM) had little or no effect on I_B but at higher concentrations blocked the current dose - dependently (IC_{50}≈5mM). 4-AP also reduced I_B (IC_{50}≈0.2mM) although cell-to-cell variations did occur and complete block was not always observed. Quinidine was more potent, blocking I_B dose-dependently with an IC_{50} of about 10μM. The insensitivity of I_B to TEA suggests the majority of the current is not carried by large conductance Ca^{2+}-activated K^+ channels (see Beech & Bolton, 1987). BRL 34915 (10μM) had little effect on the time- and voltage-dependent outward current evoked by depolarization.

The results suggest that BRL 34915 (1-10 μM) induces an increase in membrane K^+ conductance which shows little voltage sensitivity and which is not associated with the opening of large conductance Ca^{2+}-activated K^+ channels, but with other K^+ channels whose identity is at present unknown.

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