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Release of endothelium-derived relaxing factor from pig cultured aortic endothelial cells, as assessed by changes in endothelial cell cyclic GMP content, is inhibited by a phorbol ester

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1 Cultured aortic endothelial cells of the pig respond to the endothelium-derived relaxing factor (EDRF) they release with an increase in cyclic GMP content. This response is inhibited by haemoglobin or by L-N^G-monomethyl-arginine (L-NMMA), and has been used to investigate the effects of phorbol esters on EDRF release.

2 Pretreatment with phorbol-12,13-dibutyrate (PDB) but not the inactive 4α-phorbol-12,13,-didecanoate (PDD), inhibited increases in cyclic GMP induced by substance P (10^{-8} M) in a time and concentration-dependent manner. PDB did not affect basal cyclic GMP levels.

3 PDB (3×10^{-7} M), but not PDD (3×10^{-7} M), also inhibited ATP (10^{-5} M)-induced increases in cyclic GMP, but did not affect those induced by bradykinin (10^{-7} M).

4 Increases in cyclic GMP induced by low (10^{-7} M) but not high (10^{-6} M) concentrations of the calcium ionophore A23187 were inhibited by PDB (3×10^{-7} M). This inhibitory effect was due to enhanced destruction of EDRF by superoxide anions rather than inhibition of EDRF release, as the inhibition was abolished in the presence of superoxide dismutase (SOD, 30 u ml^{-1}) and catalase (CAT, 100 u ml^{-1}).

5 SOD and CAT did not affect the inhibitory action of PDB on substance P or ATP-induced increases in cyclic GMP.

6 Increases in endothelial cell cyclic GMP content induced by sodium nitroprusside (10^{-5} M) were unaffected by PDB pretreatment.

7 The inhibitory effects of PDB are probably a result of an action of protein kinase C on the steps between receptor occupation and phospholipase C activation.

Introduction

Endothelium-derived relaxing factor (EDRF) has been the subject of intense investigation since it was first described by Furchgott & Zawadzki (1980). Recently attention has focussed on the factors controlling the release of EDRF. Extracellular calcium and ATP have been shown to be necessary for EDRF release (Singer & Peach, 1982; Edwards *et al.*, 1985), and an inhibitory role has been suggested for the intracellular messenger guanosine 3′:5′-cyclic monophosphate (cyclic GMP). Busse *et al.* (1988) have shown that atrial natriuretic peptide (ANP) reduces EDRF release from cultured cells, and this has been confirmed in intact blood vessels by Hogan *et al.* (1989). ANP, like EDRF and the nitrovasodilators, increases cyclic GMP in cultured endothelial cells (Leitman & Murad, 1986; Martin *et al.*, 1988), and it has been suggested that the elevated cyclic GMP levels are responsible for the inhibition of EDRF release (Busse *et al.*, 1988; Hogan *et al.*, 1989). Support for this view comes from the observation that 8-bromo-cyclic GMP inhibits EDRF release from rabbit aorta, and this led to the suggestion that EDRF, by elevating endothelial cell cyclic GMP levels, would inhibit its own release through a negative feedback mechanism (Evans *et al.*, 1988). It has been shown that cyclic GMP inhibits phosphatidyl inositol hydrolysis in vascular smooth muscle (Lewis *et al.*, 1988), and cyclic GMP may inhibit EDRF release by a similar mechanism in endothelium.

Another intracellular messenger has been implicated in the control of EDRF release by the demonstration that phorbol esters can inhibit EDRF release from intact rings of guinea-pig pulmonary artery (Weinheimer *et al.*, 1986) or rabbit aorta (Lewis & Henderson, 1987) and from bovine cultured aortic endothelial cells in a bioassay system (de Nucci *et al.*, 1988). Each of these studies used smooth muscle relaxation as a measure of EDRF release. Tumour promoting phorbol esters mimic the stimulating action of diacylglycerol (DAG) on protein kinase C (Castagna *et al.*, 1982). The study of Lewis & Henderson (1987) was necessarily limited to short exposure times and lower concentrations of phorbol esters, because of their complicating smooth muscle constrictor effects. The study of de Nucci *et al.* (1988) used only a single concentration of phorbol ester, and did not examine the time course of the effects of the phorbol ester. The study of Weinheimer *et al.* (1986) used a range of phorbol ester concentrations, but again did not examine the time course of the effects of the phorbol ester.

Martin *et al.* (1988) demonstrated that cultured aortic endothelial cells of the pig respond to the EDRF they produce with an increase in cyclic GMP levels. This technique has been used in this study to investigate the time course and concentration-dependence of the effects of phorbol esters on agonist-stimulated EDRF release from pig cultured aortic endothelium. The specific inhibitor of EDRF production L-N^G-monomethyl arginine (L-NMMA) has also been used to confirm that the elevation of cyclic GMP in the endothelial cells is due to release of EDRF.

A preliminary account of some of these findings has been published (Smith, 1989a,b).

Methods

Endothelial cell culture

Pig aortae, obtained from a local abattoir, were flushed immediately after removal from the animal (approx 10–15 min after death) with sterile saline containing 200 u ml^{-1} benzylpenicillin and 200 μg ml^{-1} streptomycin. One end of the aorta

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was tied off, and the other end cannulated with a 60 ml syringe containing the same saline. The lumen of the aorta was then filled with saline from the syringe, and the vessel transported to the laboratory, where the endothelial cells were isolated essentially as described by Gordon & Martin (1983). Briefly, the intercostal arteries were ligated, the lumen filled with 0.2% collagenase (type II, Sigma) and the vessel incubated at 37°C for 20 min. The endothelial cells were then harvested into 40 ml of Medium E199 supplemented with 10% foetal bovine serum, 10% newborn bovine serum, glutamine (6 mM), benzyl penicillin (200 u ml^{-1}), streptomycin (200 $\mu g\,ml^{-1}$) and kanamycin (100 $\mu g\,ml^{-1}$). The cells were then seeded into three plates each containing 6 wells (9.6 cm^2). Culture medium was replaced the following day and subsequently every other day, and the cells used once they became confluent, usually within 5–7 days of isolation.

Experimental protocol

The tissue culture medium was aspirated and the endothelial cells washed with 2 × 2 ml of Krebs solution of the following composition (mM): NaCl 94.8, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The cells were then incubated in 2 ml of Krebs solution at 37°C under an atmosphere of 5% CO_2 in air for at least 90 min. Drug concentrations and exposure times were selected on the basis of previous work by other authors (Martin *et al.*, 1988), and from previous experience with the drugs in intact ring preparations from various species. Drugs were added at the concentrations and times indicated in Results, and at the appropriate time the Krebs solution was rapidly aspirated and the cells immediately extracted with 0.5 ml of ice cold 6% trichloroacetic acid (TCA). The cells were scraped from the well and together with the TCA, aspirated into plastic tubes. Each well was extracted with a second 0.5 ml of ice-cold 6% TCA. The combined extracts were centrifuged at 13,000 r.p.m. for 2 min and the pellet and supernatant separated. The DNA content of the cell pellet was measured by the fluorimetric method of Kissane & Robins (1958). The supernatant was neutralised to approx pH 6 by vortexing for 90 s with 2 ml of 0.5 M tri-n-octylamine in freon (1,1,2 trichlorotrifluoroethane). The cyclic GMP content of the aqueous upper layer was measured by radioimmunoassay with a commercially available kit (New England Nuclear). The cyclic GMP content of each well was expressed as fmol μg^{-1} DNA.

Drugs

Adenosine triphosphate (ATP), calcium ionophore A23187, catalase (bovine liver), haemoglobin (bovine type I), phorbol-12,13-dibutyrate (PDB), 4α-phorbol-12,13-didecanoate (PDD), sodium nitroprusside (SNP), substance P and superoxide dismutase (horseradish) were obtained from Sigma Chemical Co. L-NMMA (L-N^G-monomethyl arginine) was obtained from Ultrafine Chemicals (Manchester). All drugs were dissolved in distilled water except for A23187, which was dissolved in dimethyl sulphoxide (DMSO), and PDB and PDD which were dissolved in ethanol. The final concentrations of these solvents in the medium bathing the cells were 0.002% and 0.02% DMSO for 10^{-7} M and 10^{-6} M A23187 respectively, and 0.003%, 0.03% or 0.3% ethanol for 3×10^{-8} M, 3×10^{-7} M or 3×10^{-6} M PDB or PDD respectively.

Haemoglobin solutions were reduced to the ferrous form with dithionite as described by Martin *et al.* (1986). Sera, culture medium, glutamine and kanamycin were obtained from Flow Laboratories, benzyl penicillin (crystapen) from Glaxo and streptomycin sulphate from Evans Medical Ltd.

Statistical analysis

The resting endothelial cell content of cyclic GMP was found to vary from batch to batch, hence in all experiments each 6 well plate served as its own internal control. For the time course experiments a large number of batches of cells were needed, and it was necessary to normalise the cyclic GMP levels by expressing the cyclic GMP content of each experimental well on a plate as a percentage of the cyclic GMP content of the control well on the same plate. Mean cyclic GMP levels were compared by two way analysis of variance between time points and drug pretreatment. Significant differences between means and the fixed value of 100% were detected by paired comparison, by use of the residual mean square from the analysis of variance and the *t* statistic. In all other experiments, absolute values of cyclic GMP were used, and results are expressed as arithmetic mean ± s.e.mean. Data were analysed by one-way analysis of variance followed by Student Newman-Keuls (SNK) multiple range test to identify significant differences at the $P < 0.05$ level.

Results

Phorbol esters and substance P

Substance P (10^{-8} M, 1.5 min) induced a 17.5 fold increase in the cyclic GMP content of the cultured endothelial cells, which was significantly reduced to a 10.7 fold increase by pretreating the cells for 2 min with phorbol-12,13-dibutyrate (PDB, 10^{-7} M), but was unaffected (21.6 fold increase) by 2 min pretreatment with the inactive 4α-phorbol-12,13-didecanoate (PDD, 10^{-7} M) (Figure 1a). Pretreatment of the endothelial cells

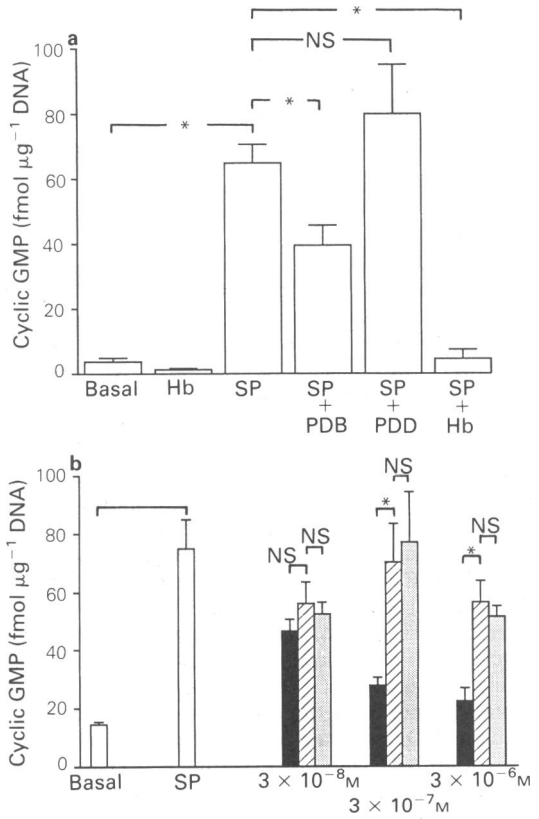

Figure 1 (a) Effects of 2 min pretreatment with phorbol-12,13-dibutyrate (PDB, 10^{-7} M) or 4α-phorbol-12,13-didecanoate (PDD, 10^{-7} M), or 20 min pretreatment with haemoglobin (Hb, 10^{-5} M) on substance P (SP, 10^{-8} M for 1.5 min)-induced increases in pig aortic endothelial cell cyclic GMP content. Results are expressed as the mean of 5 or 6 observations. (b) Effects of 5 min pretreatment with various concentrations of PDB (solid columns), PDD (hatched columns) or the appropriate solvent control (ethanol, EtOH; stippled columns) on SP (10^{-8} M for 1.5 min)-induced increases in pig aortic endothelial cell cyclic GMP content. Concentrations of phorbol esters are indicated under each group of columns. The concentrations of EtOH are 0.003%, 0.03% abd 0.3% for 3×10^{-8} M, 3×10^{-7} M and 3×10^{-6} M phorbols respectively. Results are expressed as the mean of 4 to 8 observations. Vertical bars indicate s.e.mean, NS = not significant, $*P < 0.05$.

with the specific EDRF inhibitor haemoglobin (10^{-5} M) for 20 min before the addition of substance P significantly inhibited the substance P-induced increase in endothelial cell cyclic GMP content (Figure 1a).

To provide confirmation that the increases in cell cyclic GMP content were due to EDRF release, cells were incubated with the competitive inhibitor of EDRF synthesis, L-N^G-monomethyl arginine (L-NMMA, 5×10^{-5} M) (Palmer *et al.*, 1988), for 15 min before the addition of substance P. This concentration of L-NMMA inhibited the substance P-induced increase in cyclic GMP by 55% (Table 1).

As 10^{-7} M PDB for 2 min only produced 41% inhibition of the response, the higher concentration of 3×10^{-7} M was used for all subsequent experiments with the phorbol esters. The time course of the effects was investigated in a series of experiments using different pretreatment times (Figure 2). Pretreatment with PDD (3×10^{-7} M) produced a statistically significant ($P < 0.05$) initial inhibition of the substance P (10^{-8} M)-induced increases in cyclic GMP at the 30 s time point, but no inhibition at later time points. The reason for this effect of PDD at 30 s is not known. Increasing pretreatment time with the active PDB (3×10^{-7} M) produced a progressive inhibition of the response to substance P (10^{-8} M) which became significant at 1 min pretreatment ($P < 0.01$ at 1 and 2 min, $P < 0.001$ at 3, 5 and 10 min cf. 100%), and after 3 min prevented significant elevation of cyclic GMP above basal levels by substance P (10^{-8} M). Five minutes was chosen as the pretreatment time for all subsequent experiments.

Preatreatment for 5 min with 3×10^{-7} M PDB or PDD, or the equivalent concentration of the solvent, ethanol (0.03%), had no effect on basal levels of cyclic GMP in the cells (Table 2).

The effects of 5 min pretreatment with PDB on substance P-induced increases in cyclic GMP levels were concentration-dependent (Figure 1b). When the response to substance P in the presence of PDB was compared with the response in the presence of the same concentration of PDD (after subtraction of basal levels), there was no inhibition of the substance P-induced increase with 3×10^{-8} M PDB, 76.4% inhibition with 3×10^{-7} M PDB ($P < 0.05$) and 81.3% inhibition with 3×10^{-6} M PDB ($P < 0.01$). The response in the presence of PDD is the most appropriate control, as both PDD and ethanol are present. At each concentration of phorbol ester used, the effect of the solvent (ethanol) alone was tested. There were no significant differences between the substance P-induced increases in cyclic GMP in the presence of any of the concentrations of ethanol or PDD in ethanol, hence only solvent plus PDD was examined in all subsequent experiments.

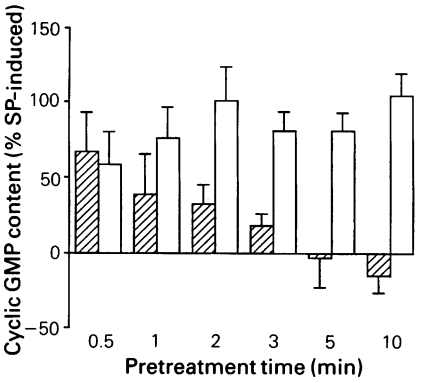

Figure 2 Time course of the effects of phorbol ester pretreatment on substance P (SP)-induced increases in pig aortic endothelial cell cyclic GMP content. The SP (10^{-8} M, 1.5 min)-induced increases in the cyclic GMP content of the cells are normalised to 100%. Zero cyclic GMP content is equivalent to basal levels. The pairs of columns show the effects on the SP-induced increase of pretreatment with phorbol-12, 13-dibutyrate (PDB, 3×10^{-7} M, hatched columns) or 4α-phorbol-12, 13-didecanoate (PDD, 3×10^{-7} M, open columns) for the times indicated under each pair of columns. Results are expressed as the mean of 7 or 8 observations, vertical bars indicate s.e.mean.

The inhibitory effect of PDB on the substance P-induced increases in endothelial cell cyclic GMP levels was also examined in the presence of superoxide dismutase (SOD, 30 u ml^{-1}) and catalase (CAT, 100 u ml^{-1}). Substance P (10^{-8} M, 1.5 min) produced a 5 fold increase in cyclic GMP content of the cells (from 13.1 ± 1.1 to 66.1 ± 12.5 fmol cyclic GMP μg^{-1} DNA, $n = 9$) in the presence of SOD and CAT, which was significantly ($P < 0.001$) reduced to 2.7 fold by 5 min PDB (3×10^{-7} M) pretreatment (to 35.8 ± 3.7 fmol cyclic GMP μg^{-1} DNA, $n = 9$), but unaffected (5.4 fold) by 5 min PDD (3×10^{-7} M) pretreatment (70.1 ± 6.1 fmol cyclic GMP μg^{-1} DNA, $n = 9$). The effect of substance P (with SOD and CAT) was completely inhibited by 20 min haemoglobin (10^{-5} M) pretreatment (4.4 ± 1.2 fmol cyclic GMP μg^{-1} DNA, $n = 9$). The 5 fold increase in cyclic GMP produced by substance P (10^{-8} M) in this experiment appears at first to be considerably less than the 17.5 fold increase produced by substance P (10^{-8} M) in the absence of SOD and CAT (Figure 1). However, this is almost entirely due to the higher resting levels of cyclic GMP in this experiment in the presence of SOD and CAT (13.1 ± 1.1 vs 3.7 ± 1.1 in Figure 1), as the stimulated values in the presence and absence of SOD and CAT are similar (66.1 ± 12.5 and 64.7 ± 5.9 respectively). It is not clear whether these higher resting levels in the presence of SOD and

Table 1 Effect of L-N^G-monomethyl arginine pretreatment on substance P-induced increases in pig aortic endothelial cell cyclic GMP content

Group	*Stimulus*	*Preatreatment*	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (control)	None	18 ± 1.8	8	
2	SP, 10^{-8} M	None	40.6 ± 2.6	8	$P < 0.001$ cf group 1
3	SP, 10^{-8} M	L-NMMA, 5×10^{-5} M	28.1 ± 3.1	9	$P < 0.05$ cf group 2

Group 1 (control) shows mean ± s.e.mean resting levels of cyclic GMP in the endothelial cells. The other two groups were exposed to substance P (SP, 10^{-8} M) for 1.5 min, with or without 15 min pretreatment with 5×10^{-5} M L-N^G-mono methyl arginine (L-NMMA).

Table 2 Effect of phorbol esters on the basal levels of cyclic GMP in pig aortic endothelial cells

Group	*Treatment*	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None	18 ± 1.7	8	No significant
2	PDB, 3×10^{-7} M	20 ± 6.2	9	differences between
3	PDD, 3×10^{-7} M	26.5 ± 5.5	9	any of the groups
4	Ethanol, 0.03%	15.9 ± 2.7	9	

Group 1 shows mean ± s.e.mean resting levels of cyclic GMP in the endothelial cells. Groups 2, 3 and 4 were respectively exposed to phorbol-12,13-dibutyrate (PDB), 4α-phorbol-12,13-didecanoate (PDD) or the equivalent concentration of the solvent ethanol (0.03% final concentration) for 5 min.

CAT are due to enhanced effects of basally released EDRF (because its half-life is prolonged), or variation between batches of cells, as resting levels were found to vary between batches.

Phorbol esters and ATP

PDB produced a similar inhibition of the responses of the endothelial cells to adenosine 5'-triphosphate (ATP; Figure 3). ATP (10^{-5} M, 3 min) induced a 3.2 fold increase in endothelial cell cyclic GMP content that was prevented by PDB pretreatment (3×10^{-7} M, 5 min, $P < 0.05$), but was unaffected by PDD pretreatment (2.2 fold increase; 3×10^{-7} M, 5 min, $P > 0.1$). Haemoglobin pretreatment (10^{-5} M, 20 min) also prevented the ATP-induced increase in cyclic GMP ($P < 0.05$). Similar results were seen in the presence of SOD (30 u ml^{-2}) and CAT (100 u ml^{-1}) where ATP (10^{-5} M, 3 min) induced a 2.8 fold increase in cyclic GMP (from 19.4 ± 2.3 to 55.1 ± 3.4 fmol μg^{-1} DNA, $n = 6$) which was significantly ($P < 0.05$) reduced to a 1.5 fold increase (28.3 ± 5.8 fmol μg^{-1} DNA, $n = 6$) by PDB pretreatment (3×10^{-7} M, 5 min), but unaffected (3 fold increase, 58.5 ± 10.1 fmol μg^{-1} DNA, $n = 5$, $P > 0.7$) by PDD pretreatment (3×10^{-7} M, 5 min). The ATP-induced increase in cyclic GMP (with SOD and CAT) was prevented by haemoglobin pretreatment (10^{-5} M, 20 min, 4.5 ± 0.8 fmol μg^{-1} DNA, $n = 5$, $P < 0.001$).

Phorbol esters and bradykinin

The experiments with bradykinin were only done in the presence of SOD and CAT. Bradykinin (BK, 10^{-7} M, 1.5 min) induced a significant ($P < 0.001$) 3.4 fold increase in endothelial cell cyclic GMP content that was not affected by pretreatment with PDB (3.1 fold increase) or PDD (3 fold increase) (Table 3). However, the BK-induced rise in cyclic GMP was significantly ($P < 0.001$) reduced to a 1.5 fold increase by 20 min pretreatment with 10^{-6} M haemoglobin.

Phorbol esters and sodium nitroprusside

Sodium nitroprusside (10^{-5} M, 3 min) induced a 6.4 fold increase in endothelial cell cyclic GMP content, that was prevented by 20 min haemoglobin (10^{-5} M) pretreatment (Table 4). The response to sodium nitroprusside was not inhibited by 5 min pretreatment with 3×10^{-7} M PDB or PDD (Table 4).

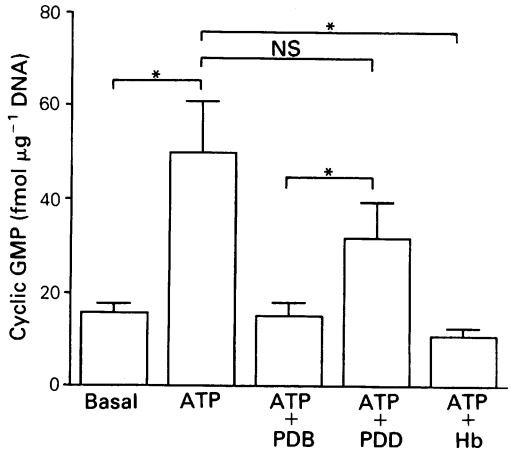

Figure 3 Effects of 5 min pretreatment with phorbol-12,13-dibutyrate (PDB, 3×10^{-7} M) or 4α-phorbol-12,13-didecanoate (PDD, 3×10^{-7} M), or 20 min pretreatment with Hb (10^{-5} M) on adenosine trisphosphate (ATP, 10^{-5} M for 3 min)-induced increases in pig aortic endothelial cell cyclic GMP content. Results are expressed as the mean of 6 observations, vertical bars indicate s.e.mean, NS = not significant, $^*P < 0.05$.

Phorbol esters and calcium ionophore

A23187 (calcium ionophore, 10^{-7} M, 3 min) induced a 6.3 fold increase in endothelial cell cyclic GMP content that was significantly ($P < 0.05$) reduced to 4.2 fold by PDB pretreatment (3×10^{-7} M, 5 min), but unaffected (6.3 fold increase) by PDD pretreatment (3×10^{-7} M, 5 min; Figure 4). The response to 10^{-7} M A23187 was completely inhibited by 20 min haemoglobin pretreatment (10^{-5} M, $P < 0.001$). The response to the higher concentration of 10^{-6} M A23187 (3 min) was much greater, producing a 13.1 fold increase in endothelial cell cyclic GMP content that was unaffected by 5 min PDB (3×10^{-7} M,

Table 3 Effect of phorbol esters or haneoglobin pretreatment on bradykinin-induced increases in pig aortic endothelial cell cyclic GMP content in the presence of superoxide dismutase and catalase

Group	*Stimulus*	*Pretreatment* (30 u ml^{-1} SOD + 100 u ml^{-1} CAT in all groups)	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (control)	None	41.3 ± 3.2	5	
2	BK, 10^{-7} M	None	141.7 ± 6.6	5	$P < 0.001$ cf group 1
3	BK, 10^{-7} M	PDB, 3×10^{-7} M	129.5 ± 6.6	5	NS cf group 4
4	BK, 10^{-7} M	PDD, 3×10^{-7} M	123.1 ± 9.5	5	NS cf group 2
5	BK, 10^{-7} M	Hb, 10^{-6} M	61.3 ± 4.8	5	$P < 0.001$ cf group 2

Group 1 (control) show mean ± s.e.mean resting levels of cyclic GMP in endothelial cells treated with SOD (30 u ml^{-1}) and CAT (100 u ml^{-1}) for 6.5 min. All other groups received SOD and CAT 5 min before the addition of bradykinin (BK). Hb was added 20 min before BK, and PDB or PDD added 5 min before BK. Groups 2 to 5 were exposed to BK (10^{-7} M) for 1.5 min following the various pretreatments indicated. NS = not significant.

Table 4 Effects of phorbol esters or haemoglobin pretreatment on sodium nitroprusside-induced increases in pig aortic endothelial cell cyclic GMP content

Group	*Stimulus*	*Pretreatment*	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (Controls)	None	15.6 ± 1.6	12	
2	SNP, 10^{-5} M	None	100.4 ± 9.2	6	$P < 0.001$ cf group 1
3	SNP, 10^{-5} M	PDB, 3×10^{-7} M	86 ± 10.7	6	NS cf group 4
4	SNP, 10^{-5} M	PDD, 3×10^{-7} M	78.9 ± 3.2	6	NS cf group 2
5	SNP, 10^{-5} M	Hb, 10^{-5} M	10.1 ± 1.1	6	$P < 0.001$ cf group 2

Group 1 (control) shows mean ± s.e.mean resting levels of cyclic GMP in the endothelial cells. The other groups were exposed to sodium nitroprusside (SNP, 10^{-5} M) for 3 min, with or without 5 min pretreatment with PDB or PDD, or 20 min pretreatment with Hb. Abbreviations as in Table 3.

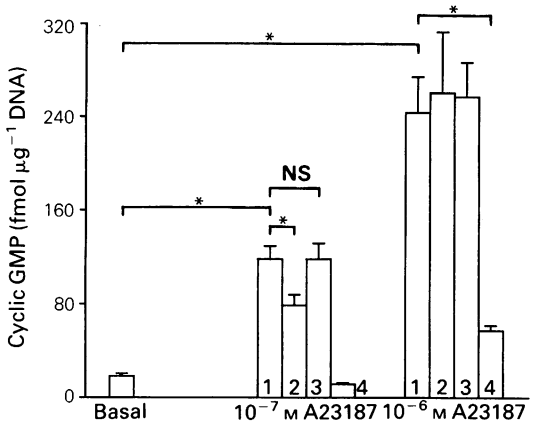

Figure 4 Effects of 5 min pretreatment with phorbol-12,13-dibutyrate (PDB, 3×10^{-7} M) or 4α-phorbol-12,13-didecanoate (PDD, 3×10^{-7} M), or 20 min pretreatment with Hb (10^{-5} M) on calcium ionophore (A23187, 10^{-7} or 10^{-6} M for 3 min)-induced increases in cyclic GMP content. In each group of 4 columns, (1) represents A23187 treated cells, (2) A23187 + PDB, (3) A23187 + PDD, (4) A23187 + Hb. Concentrations of A23187 are shown under each group of columns. Results are expressed as the mean of 6 or 12 observations, vertical bars indicate s.e.mean, NS = not significant, $*P < 0.05$.

14 fold increase) or PDD (3×10^{-7} M, 13.8 fold increase) pretreatment. The response to 10^{-6} M A23187 was significantly reduced ($P < 0.01$) to a 3 fold increase by 20 min pretreatment with haemoglobin (10^{-5} M) (Figure 4).

In the presence of SOD (30 u ml^{-1}), 5 min pretreatment with 3×10^{-7} M PDB no longer inhibited the response to 10^{-7} M A23187 (3 min; 1.7 fold increase in cyclic GMP) but tended to increase the response (2.2 fold increase in cyclic GMP), although the effect just failed to reach significance (Table 5). Pretreatment with PDD (3×10^{-7} M, 5 min) was without effect (1.9 fold increase in cyclic GMP), and 20 min pretreatment with haemoglobin (10^{-5} M) completely inhibited ($P < 0.01$) the response to 10^{-7} M A23187 (Table 5).

In the presence of SOD (30 u ml^{-1}) plus CAT (100 u ml^{-1}), A23187 (10^{-7} M, 3 min) produced a 1.7 fold increase in endothelial cell cyclic GMP that was unaffected by 5 min pretreatment with 3×10^{-7} M PDB (1.8 fold increase) or PDD (1.7 fold increase). In the presence of SOD and CAT the response to 10^{-7} M A23187 was completely inhibited ($P < 0.01$) by 20 min haemoglobin (10^{-5} M) pretreatment. Thus the addition of CAT had no significant additional effect to that of SOD alone.

Discussion

Agonist-induced elevation of porcine cultured endothelial cell cyclic GMP levels has previously been shown to be due to EDRF released from those cells stimulating soluble guanylate cyclase (Martin *et al.*, 1988). The inhibition by L-NMMA of substance P-induced increases in endothelial cell cyclic GMP content obtained in this study provides further evidence that the increase in cyclic GMP is due to EDRF release. This technique has been used in the present study to investigate the effects of phorbol esters on EDRF release.

Figure 1 shows that 10^{-7} M PDB but not the inactive 4α-PDD inhibited the elevation of cyclic GMP levels in pig cultured aortic endothelial cell, induced by substance P. These results in cultured endothelial cells from a different species (pig rather than rabbit) are in agreement with the observations of Lewis & Henderson (1987) that PDB inhibits substance P-stimulated EDRF release from intact rings of rabbit aorta. The present study extends these observations, showing that the inhibitory effects of PDB are both time and concentration-dependent. These results also agree with the observations of de Nucci *et al.* (1988) in bovine cultured aortic endothelial cells.

The mechanism by which phorbol esters, through stimulation of protein kinase C, inhibit receptor-mediated release of EDRF has yet to be established. Ryan *et al.* (1988) have shown that phorbol myristate acetate inhibits agonist-induced increases in endothelial cell free intracellular calcium. Brock & Capasso (1988) have further shown that a number of activators of protein kinase C inhibited agonist-induced inositol 1,4, 5-triphosphate (IP_3) formation and increases in free intracellular calcium in endothelial cells. The inhibitory effects of phorbol esters probably result from an action of protein kinase C on the steps linking receptor occupation to phospholipase C activation. However, other possible actions of phorbol esters need to be excluded.

Endothelial cells have recently been shown to generate and secrete superoxide anions (Rosen & Freeman, 1984), which are known to inactivate EDRF (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). Matsubara & Ziff (1986) have demonstrated that the phorbol ester, phorbol myristate acetate, increases superoxide anion production by endothelial cells. Hence increased superoxide anion production and destruction of EDRF may be a potential mechanism for the inhibitory effects of PDB shown in Figures 1 and 2. However, PDB was still able to exert its inhibitory effects in the presence of SOD and CAT, and PDB did not inhibit bradykinin-induced EDRF release, hence it is unlikely that PDB inhibits substance P-induced EDRF release by enhancing superoxide anion production. The concentration of SOD used (30 u ml^{-1}) would be sufficient to remove phorbol ester-induced superoxide production, as the same concentration of SOD was able to reverse the synergistic production of superoxide by phorbol and ionophore (Figure 4). The inhibitory effects of PDB on substance P-induced EDRF release are also unlikely to be due to antagonism of substance P at its receptors, as PDB similarly inhibited ATP-induced EDRF release in the presence or absence of SOD and CAT.

SOD, CAT and indeed haemoglobin, are all large molecules which are unable to enter the cell, and can only interact with superoxide anions, hydrogen peroxide and nitric oxide respectively, when they are in the extracellular space. However, this

Table 5 Effect of phorbol esters or haemoglobin pretreatment on A23187-induced increases in pig aortic endothelial cell cyclic GMP content in the presence of superoxide dismutase (SOD)

Group	*Stimulus*	*Pretreatment* (30 u ml^{-1} SOD in all groups)	*Cyclic GMP* (fmol μg^{-1} DNA)	n	*Statistical significance*
1	None (Control)	None	27 ± 6.7	6	
2	A23187, 10^{-7} M	None	46.7 ± 4.6	6	$P < 0.05$ cf group 1
3	A23187, 10^{-7} M	PDB, 3×10^{-7} M	60.4 ± 6.8	6	NS cf group 4
4	A23187, 10^{-7} M	PDD, 3×10^{-7} M	51.1 ± 5.7	6	NS cf group 2
5	A23187, 10^{-7} M	Hb, 10^{-5} M	10.9 ± 2.2	6	$P < 0.001$ cf group 2

Group 1 (control) shows mean ± s.e.mean resting levels of cyclic GMP in endothelial cells treated with SOD (30 u ml^{-1}) for 8 min. All other groups received SOD 5 min before the addition of A23187. Hb (10^{-5} M) was added 20 min before A23187, and PDB or PDD added 5 min before A23187. Groups 2 to 5 were exposed to A23187 (10^{-7} M) for 3 min following the various pretreatments indicated. Abbreviations as in Table 3.

does not mean that they are unable to affect the concentrations of these agents inside the cell. Haemoglobin for example will avidly bind extracellular nitric oxide, producing a steep concentration gradient out of the cell, increasing the net rate of diffusion of nitric oxide out of the cell, and hence effectively lowering the intracellular nitric oxide concentration. Whether or not superoxide anions cross endothelial cell membranes, and if so how, is at present unclear. However, it is clear from bioassay experiments that addition of extracellular SOD prolongs the half-life of released EDRF (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986).

Protein kinase C has been shown to phosphorylate rat brain soluble guanylate cyclase *in vitro* (Zwiller *et al.*, 1985). However, the inhibitory effects of PDB in this study cannot be explained by an action on soluble guanylate cyclase (which is stimulated by EDRF), as the elevation of endothelial cell cyclic GMP levels by sodium nitroprusside was unaffected by PDB pretreatment. Sodium nitroprusside generates nitric oxide spontaneously in solution (Feelisch & Noack, 1987) and thus stimulates soluble guanylate cyclase directly (Braughler *et al.*, 1979). EDRF and sodium nitroprusside will stimulate the same soluble guanylate cyclase, as the active principle in both cases is nitric oxide, a freely lipid permeable gas capable of reaching all parts of the cell.

The calcium ionophore, A23187, is thought to act by directly translocating calcium ions across the cell membrane. Its effects are not therefore subject to the same intracellular regulatory mechanisms as receptor-mediated agonists. However, in the absence of SOD or CAT, PDB pretreatment inhibited the increase in cyclic GMP levels produced by 10^{-7} M A23187, but not that produced by 10^{-6} M A23187. Phorbol esters and calcium ionophore have been shown to act synergistically to increase superoxide anion production by endothelial cells (Matsubara & Ziff, 1986). It is possible therefore that at the lower concentration (10^{-7} M) of calcium ionophore the enhanced superoxide anion production results in more rapid EDRF destruction, less elevation of cyclic GMP and an apparent inhibition of release. With 10^{-6} M A23187, very large increases in cyclic GMP were seen (Figure 4), indicating a large release of EDRF, which was not affected by PDB pretreatment. This suggests that the amount of EDRF released is more than sufficient to saturate the soluble guanylate cyclase, and any enhanced destruction of EDRF by increased superoxide anion production is not apparent.

Evidence to support this interpretation comes from the experiments with A23187 in the presence of SOD and SOD plus CAT. In the presence of SOD only, PDB pretreatment no longer inhibited 10^{-7} M A23187-induced elevation of cyclic GMP levels, but if anything tended to increase the response, although this was not significant when compared to the response in the presence of PDD. Care must be exercised in interpreting these results as a lack of inhibitory effect of PDB on A23187-induced EDRF release, as the end product of SOD-mediated superoxide anion dismutation is hydrogen peroxide, which can stimulate soluble guanylate cyclase either directly (White *et al.*, 1986) or indirectly by reacting with superoxide anions to produce hydroxyl radicals which in turn stimulate guanylate cyclase (Murad *et al.*, 1978). The apparent reversal of PDB-induced inhibition by SOD alone could therefore be due to elevation of cyclic GMP levels by hydrogen peroxide and/or hydroxyl radical. Similar results were obtained when the experiment was repeated in the presence of SOD and CAT so that any hydrogen peroxide formed by SOD would be converted to water by CAT. Pretreatment with PDB in the presence of SOD and CAT had no effect on 10^{-7} M A23187-induced elevation of cyclic GMP levels. Failure of PDB to inhibit A23187-induced EDRF release in rabbit aorta was similarly shown by Lewis & Henderson (1987). It is apparent that A23187-induced cyclic GMP production is less in the presence of SOD or SOD and CAT than in their absence (cf Table 5 and Figure 4). This may well reflect protein binding of the ionophore, as A23187 is known to bind to albumin: indeed binding of A23187 to albumin has been used as a technique for rapid removal and reversal of the effects of A23187 (Sarkadi *et al.*, 1976).

The lack of an inhibitory effect of PDB on BK-induced EDRF release was an unexpected result, and implies that BK has a different pathway of cellular activation from substance P and ATP. These experiments were only performed in the presence of SOD and CAT, but there is no obvious reason why a different result would be obtained in the absence of SOD and CAT. Not all agonists release EDRF through the same mechanism. For example pertussis toxin, which inactivates a G_i-protein involved in receptor-response coupling (Murayama & Ui, 1983), inhibits endothelium-dependent relaxation of pig coronary arteries produced by the α_2-agonist UK 14304 (Pfizer, Sandwich, Kent), 5-hydroxytryptamine and thrombin, but not those produced by bradykinin and ADP (Flavahan *et al.*, 1989).

Evidence that phospholipase C can participate in the pathways leading to EDRF release has been provided by a number of studies. For example, de Nucci *et al.* (1988) found that addition of purified phospholipase C to bovine cultured aortic endothelial cells released both EDRF and prostacyclin. Bradykinin is known to stimulate phosphatidyl inositol hydrolysis in cultures of both bovine (Derian & Moskowitz, 1986) and porcine (Hong & Deykin, 1982; Lambert *et al.*, 1986) aortic endothelial cells, and ATP has been shown to increase the formation of inositol-1,4,5-trisphosphate in bovine cultured aortic endothelial cells (Pirotton *et al.*, 1987). Substance P stimulates phosphatidyl inositol hydrolysis in smooth muscle (Watson & Downes, 1983; Yousufzai *et al.*, 1986) and paratoid acinar cells (Aub & Putney, 1984), and is likely to act in a similar manner in endothelial cells. Thus, if all three agonists act by increasing phosphatidyl inositol hydrolysis (proven for bradykinin and ATP, assumed for substance P), but PDB only inhibits EDRF release induced by substance P and ATP, one must question whether the mechanism of action of PDB acting via protein kinase C, is through inhibition of the phospholipase C enzyme *per se*.

It has been demonstrated that phorbol esters induce the phosphorylation of a number of receptor types, e.g. insulin (Jacobs *et al.*, 1983; Takayama *et al.*, 1984), α_1-adrenoceptor (Leeb-Lundberg *et al.*, 1985) and β-adrenoceptor (Sibley *et al.*, 1984; Kelleher *et al.*, 1984), and that in some of these systems this is associated with a reduction in agonist binding affinity and in response (see for example Leeb-Lundberg *et al.*, 1985). Protein kinase C has also been shown to phosphorylate a G_i protein *in vitro* (Katada *et al.*, 1985). It is possible that different agonists utilise different G proteins to couple receptors to phospholipase C, and that protein kinase C only affects some G proteins. There is therefore a number of possible sites where phorbol ester-stimulated protein kinase C could act to inhibit agonist-induced EDRF release. Brock & Capasso (1988) suggested a similar mechanism to explain inhibition of thrombin- and histamine-induced increases in IP_3 formation and intracellular calcium by various activators of protein kinase C. The exact mechanism by which protein kinase C acts requires further investigation.

In conclusion, PDB but not the inactive 4α-PDD inhibit increases in pig aortic endothelial cell cyclic GMP content induced by substance P and ATP, but not by bradykinin, sodium nitroprusside and, in the presence of SOD and CAT, the calcium ionophore A23187. The inhibitory effects of PDB probably result from an action of protein kinase C on the steps leading from receptor occupation to phospholipase C activation.

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